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Dietary supplementation with sodium butyrate protected by medium-chain fatty acid salts to promote gut health in broiler chickens and piglets

MERITXELL SADURNÍ PÉREZ

Universitat Autònoma de Barcelona

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Tesi doctoral presentada per

MERITXELL SADURNÍ PÉREZ

Dirigida per

ANA CRISTINA BARROETA LAJUSTICIA

LORENA CASTILLEJOS VELÁZQUEZ

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Lorena Castillejos Velázquez i **Ana Cristina Barroeta Lajusticia**, investigadores del Departament de Ciència Animal i dels Aliments de la Facultat de Veterinària de la Universitat Autònoma de Barcelona,

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Dra. Ana Cristina Barroeta Lajusticia

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A la meva família

Resum

La suplementació dietètica amb àcid butíric i àcids grassos de cadena mitjana (**AGCM**) és una de les estratègies nutricionals més prometedores per a promoure la salut intestinal en pollastres de carn i garrins. De fet, existeix una àmplia gamma de formes de presentació que es poden utilitzar en les dietes dels monogàstrics: àcids grassos lliures, sals o glicèrids, i formes protegides. Entre aquestes, es destaca el butirat sòdic protegit, ja que pot presentar una alliberació lenta de l'àcid butíric a l'intestí i promoure els seus efectes beneficiosos sobre la salut intestinal. No obstant això, existeix poca informació sobre els additius protegits amb greix, en concret amb AGCM, en relació amb la protecció i alliberació intestinal de l'àcid butíric i els efectes sobre la barrera intestinal. Per aquest motiu, l'objectiu global de la present tesi va ser investigar els efectes del butirat sòdic protegit amb sals d'AGCM sobre la salut intestinal i els paràmetres productius en pollastres de carn i garrins. Es van avaluar dos additius a base de butirat sòdic protegit amb sals d'AGCM, procedents dels àcids grassos destil·lats del coco, que es diferenciaven per la quantitat de butirat sòdic: l'additiu avaluat en pollastres de carn incloïa un 70% de butirat sòdic, mentre que l'additiu per a garrins contenia un 50% de butirat sòdic.

Al primer experiment (Capítol 3), es va realitzar un estudi *in vivo* per a determinar l'alliberació intestinal de l'àcid butíric provinent del butirat sòdic protegit amb sals d'AGCM. Els pollastres de carn van ser alimentats amb una dieta suplementada amb l'additiu protegit que contenia un colorant blau (marcador inert), i la seva alliberació al llarg del tracte intestinal va ser determinada per espectrofotometria. Al duodè i al jejú anterior es va observar poca quantitat del colorant blau alliberat, fet que indica una mínima alliberació d'àcid butíric, mentre que la major part de marcador es va detectar a l'ili distal ($p < 0,05$), suggerint una alliberació gradual de l'àcid butíric.

Per altra banda, es van realitzar dos assaigs en pollastres de carn per avaluar els efectes del butirat sòdic (70%) protegit amb sals d'AGCM sobre la barrera intestinal i els paràmetres productius (Capítol 4). Al primer experiment, un total de 192 pollastres (Ross 308), allotjats en condicions òptimes, van ser alimentats amb un dels quatre tractaments experimentals, incloent-hi la no suplementació o la suplementació a 0,5, 1 o 2 kg/t durant 44 dies d'edat. No es van observar diferències en els paràmetres productius al llarg de l'estudi. L'ús de 0,5 i 1 kg/t de l'additiu no va afectar els recomptes de cèl·lules caliciformes a l'epiteli ileal dels pollastres de carn de 10 dies ($p = 0,023$). A més, la suplementació a 1 kg/t va disminuir els recomptes de limfòcits intraepitelials a l'ili en comparació amb l'ús de 2 kg/t, als 39 dies de vida ($p = 0,085$). Els nivells d'àcid làuric i mirístic al dipòsit de greix abdominal van augmentar gradualment amb la

dosi de suplementació de l'additiu, que contenia àcids grassos destil·lats de coco ($p < 0,001$). Al segon experiment, es va fer un desafiament de coccidiosi que alterava la barrera intestinal dels pollastres de carn. Un total de 360 pollastres (Ross 308) van ser assignats aleatòriament a tres tractaments experimentals: no desafiat, desafiat-control i desafiat-suplementat rebent l'additiu a 1 kg/t de pinso. El desafiament coccidial es va dur a terme als 7 dies d'edat i, una setmana després de la inoculació (**PI**), es va observar una reducció de la ingesta d'aliment i del guany de pes dels pollastres de carn desafiats. Pel que fa a la barrera intestinal, els resultats van mostrar que la coccidiosi alterava la histomorfometria ileal, reduint la relació entre l'altura de les vellositats i la profunditat de les criptes, i el nombre de cèl·lules caliciformes. Per altra banda, el butirat sòdic protegit amb AGCM va augmentar la profunditat de les criptes als 7 dies PI i va restablir el nombre d'aquestes cèl·lules secretores de mucina als 14 dies PI ($p < 0,05$). A més, l'ús de l'additiu va modificar l'alteració provocada per la coccidiosi a la població de *Lactobacillus* i *Enterobacteriaceae* ($p < 0,05$) de l'ili. No es van observar canvis en la digestibilitat dels nutrients als 4 dies PI. Tot i això, sembla que el butirat sòdic protegit amb AGCM afavoreix la recuperació dels paràmetres productius al llarg dels 21 dies d'estudi.

El quart assaig (Capítol 5) es va dur a terme amb 96 garrins deslletats (Landrace x Large White, 21 dies d'edat) seguint un disseny factorial amb 2 nivells de proteïna bruta (**PB**) a la dieta (18,8% vs. 22,2%) i 2 dosis de suplementació amb butirat sòdic (50%) protegit amb sals d'AGCM (0 vs. 1 kg/t). L'objectiu d'aquest estudi va ser avaluar les dues estratègies per millorar la salut intestinal de garrins deslletats. D'una banda, la reducció de la PB va comprometre els rendiments productius dels garrins deslletats ($p < 0,05$), va reduir la profunditat de les criptes ileals ($p = 0,057$) i va modificar la població de *Lactobacillus* a l'ili i al còlon ($p = 0,032$). A més, la reducció dels nivells de PB de la dieta va millorar la digestibilitat de la matèria orgànica ($p = 0,026$) i la consistència fecal ($p < 0,10$). D'altra banda, l'ús de l'additiu va augmentar el nombre de cèl·lules caliciformes ($p = 0,036$), va millorar la digestibilitat dels nutrients a l'ili i va modificar els recomptes de *Lactobacillus* i enterobacteries ($p < 0,05$), així com els metabòlits microbians al còlon.

En conjunt, els resultats suggereixen que el butirat sòdic protegit amb AGCM permet un alliberament gradual d'àcid butíric a l'intestí, un factor clau per promoure els efectes beneficiosos d'aquests àcids grassos sobre la barrera intestinal. En particular, aquesta estratègia nutricional és especialment efectiva quan la salut intestinal dels pollastres de carn es veu compromesa per la coccidiosi, afectant la histomorfometria de l'ili i la població microbiana. En la mateixa línia, l'ús de butirat sòdic protegit amb AGCM sembla reforçar la barrera intestinal durant el deslletament dels garrins, observant així una millor digestibilitat ileal. A més, atès que

la reducció de la PB a la dieta també promou efectes beneficiosos sobre la salut intestinal dels garrins, és important seguir investigant la combinació del butirat sòdic protegit amb AGCM conjuntament amb altres estratègies nutricionals. De fet, aquest enfocament ha rebut una gran atenció en els darrers anys, especialment en condicions desafiants, per tal de promoure la salut animal i reduir l'ús d'antibiòtics i l'impacte ambiental.

Resumen

La suplementación dietética con ácido butírico y ácidos grasos de cadena media (**AGCM**) es una de las estrategias nutricionales más prometedoras para promover la salud intestinal de los pollos de carne y los lechones. De hecho, encontramos una amplia gama de presentaciones disponibles para su incorporación en el pienso de los animales monogástricos: ácidos grasos libres, sales o glicéridos y formas protegidas. Entre todas ellas, destaca el butirato sódico protegido, que puede liberar lentamente el ácido butírico en el intestino, potenciando su efecto positivo sobre la salud intestinal. Sin embargo, existe poca información sobre los aditivos protegidos con grasa, y en concreto con AGCM, en relación con la protección y liberación intestinal de ácido butírico y sus efectos sobre la barrera intestinal. Por todo ello, el objetivo global de la presente tesis fue investigar los efectos del butirato sódico protegido con sales sódicas de AGCM sobre la salud intestinal y la eficiencia productiva en pollos de carne y lechones. Se evaluaron dos aditivos a base de butirato sódico protegido con sales de AGCM procedentes de ácidos grasos destilados de coco que diferían en la cantidad de butirato sódico: 70% en el pienso de los pollos de carne vs. 50% en el pienso de lechones.

En el primer estudio (Capítulo 3), se llevó a cabo un estudio *in vivo* para determinar la liberación intestinal de ácido butírico a partir de butirato sódico protegido con sales de AGCM. Los pollos de carne fueron alimentados con una dieta suplementada con el aditivo protegido que contenía un colorante azul (marcador inerte), y se determinó por espectrofotometría su liberación a lo largo del tracto intestinal. Tanto en el duodeno como en el yeyuno anterior, se observaron trazas del colorante azul liberado, lo que indica una mínima liberación de ácido butírico. Por el contrario, la mayor cantidad de marcador se detectó en el íleon distal ($p < 0,05$), sugiriendo una liberación gradual del ácido butírico.

Por otro lado, se realizaron dos ensayos con pollos de carne para evaluar los efectos del butirato sódico (70%) protegido con sales de AGCM sobre la barrera intestinal y los parámetros productivos (Capítulo 4). En el primer experimento, un total de 192 pollos (Ross 308), alojados en condiciones óptimas, fueron alimentados con uno de los cuatro tratamientos experimentales, incluyendo la no suplementación o la suplementación a 0,5, 1 o 2 kg/t durante 44 días de edad. No se observaron diferencias en los parámetros productivos a lo largo del estudio. El uso de 0,5 y 1 kg/t del aditivo no afectó los recuentos de células caliciformes en el epitelio ileal de los pollos de carne de 10 días ($p = 0,023$). Además, la suplementación a 1 kg/t disminuyó los recuentos de linfocitos intraepiteliales en el íleon en comparación con el uso de 2 kg/t a los 39 días ($p = 0,085$). Los niveles de ácido láurico y mirístico en el depósito de grasa

abdominal aumentaron gradualmente con la dosis de suplementación del aditivo ($p < 0,001$), que incluía ácidos grasos destilados de coco. En el segundo experimento, se realizó un desafío de coccidiosis que alteraba la barrera intestinal de los pollos de carne. Un total de 360 pollos (Ross 308) fueron asignados aleatoriamente a uno de los tres tratamientos experimentales: no desafiado, desafiado-control y desafiado-suplementado a 1 kg de aditivo/t de pienso. El desafío coccidial se llevó a cabo a los 7 días de edad y una semana después de la inoculación (**PI**) se observó una reducción de la ingesta de alimento y de la ganancia de peso de los pollos desafiados. En cuanto a la barrera intestinal, los resultados mostraron que la coccidiosis alteraba la histomorfometría ileal, reduciendo la relación entre la altura de vellosidades y la profundidad de criptas y el número de células caliciformes. Por otro lado, la suplementación del butirato sódico protegido con AGCM aumentó la profundidad de las criptas a los 7 días PI y restableció el número de estas células secretoras de mucina en el íleon a los 14 días PI ($p < 0,05$). Además, el uso del aditivo modificó la alteración provocada por la coccidiosis en la población de *Lactobacillus* y *Enterobacteriaceae* ($p < 0,05$) en el íleon. No se observaron cambios en la digestibilidad de los nutrientes a los 4 días PI. Sin embargo, parece que la incorporación de butirato sódico protegido con AGCM favoreció la recuperación de los parámetros productivos a lo largo de los 21 días de estudio.

El cuarto ensayo (Capítulo 5) se llevó a cabo con 96 lechones destetados (Landrace x Large White, 21 días de edad) siguiendo un diseño factorial con 2 niveles de proteína bruta (**PB**) en la dieta (18,8% vs. 22,2%) y 2 niveles de suplementación con butirato sódico (50%) protegido con sales de AGCM (0 vs. 1 kg/t). El objetivo de este estudio fue evaluar ambas estrategias para mejorar la salud intestinal de lechones destetados. Por un lado, la reducción de la PB comprometió los rendimientos productivos de los lechones destetados ($p < 0,05$), redujo la profundidad de las criptas a nivel de íleon ($p = 0,057$) y modificó la población de *Lactobacillus* en el íleon y el colon ($p = 0,032$). Además, la digestibilidad de la materia orgánica ($p = 0,026$) y la consistencia fecal ($p < 0,10$) se vieron mejoradas al reducir los niveles de PB de la dieta. Por el otro lado, el uso del aditivo aumentó el número de células caliciformes ($p = 0,036$), mejoró la digestibilidad de los nutrientes en el íleon y modificó los recuentos de *Lactobacillus* y enterobacterias ($p < 0,05$), así como los metabolitos microbianos en el colon.

En conjunto, los resultados sugieren que el butirato sódico protegido con AGCM permite una liberación gradual del ácido butírico en el intestino, un factor clave para promover los efectos beneficiosos de estos ácidos grasos sobre la barrera intestinal. En particular, esta estrategia nutricional es especialmente efectiva cuando la salud intestinal de los pollos de carne se ve comprometida por la coccidiosis, afectando a la histomorfometría del íleon y a población

microbiana. En la misma línea, el uso de butirato sódico protegido con AGCM parece reforzar también la barrera intestinal durante la fase de destete de los lechones, observándose asimismo una mejor digestibilidad ileal. Además, dado que la reducción de la PB en la dieta también promueve efectos beneficiosos sobre la salud intestinal de los lechones, es importante seguir investigando la combinación del butirato sódico protegido con AGCM con otras estrategias nutricionales. De hecho, en la actualidad se realizan nuevas investigaciones en esta línea, especialmente en condiciones de desafío, con la finalidad de promover la salud animal y reducir el uso de antibióticos y el impacto ambiental.

Abstract

The dietary supplementation with butyric acid and medium-chain fatty acids (**MCFA**) is a promising nutritional strategy under research to promote gut health in broiler chickens and piglets. In fact, there is a wide range of presentation forms that can be used in monogastric diets including free fatty acids, salts or glycerides and protected forms. Among them, protected sodium butyrate highlights, since it can obtain a slow release of butyric acid in the intestine and promote intestinal health. There is, however, limited information about the intestinal release of butyric acid from fat-protected additives and the potential of MCFA to protect butyric acid and promote beneficial effects on the intestinal barrier. Therefore, the global aim of the present thesis was to investigate the potential effects of sodium butyrate protected by sodium salts of MCFA on gut health and, consequently, growth performance in broiler chickens and piglets. Two additives based on sodium butyrate protected by MCFA salts from coconut fatty acid distillates differing in the amount of sodium butyrate were evaluated: the additive tested for broilers included 70% sodium butyrate, while for piglets the additive contained 50% of sodium butyrate.

In the first study (Chapter 3), an *in vivo* trial was carried out to test the intestinal release of butyric acid from sodium butyrate protected by MCFA salts. Broilers fed a diet supplemented with the protected feed additive. It contained a blue dye, which was used as an inert marker, and its release throughout the intestinal tract was determined by spectrophotometry. Few traces of delivered blue dye, and thus butyric acid, were observed in the duodenum or anterior jejunum, whereas the greatest amount was detected in the distal ileum ($p < 0.05$), suggesting a gradual release of butyric acid.

Furthermore, two trials were performed to evaluate the effects of the sodium butyrate (70%) protected by MCFA salts on the gut barrier and performance parameters in broiler chickens (Chapter 4). In the first experiment, a total of 192 chicks (Ross 308), housed under optimal conditions, were fed one of the four experimental treatments including non-supplementation or supplementation at 0.5, 1 or 2 kg/t for 44 days of age. No differences were observed on performance parameters throughout the study. Using 0.5 and 1 kg/t of the feed additive did not affect goblet cell counts in the ileum epithelium of 10 days broilers ($p = 0.023$). In addition, supplementation at 1 kg/t decreased intraepithelial lymphocyte counts in the ileum compared to 2 kg/t in the ileum at 39 days ($p = 0.085$). Lauric and myristic acid levels in the abdominal fat pad gradually increased with the supplementation dose due to coconut fatty acid distillates included in the additive ($p < 0.001$). In the second experiment, a coccidiosis challenge disrupting the gut barrier of broilers was performed. A total of 360 chicks (Ross 308) were randomly

assigned to three experimental treatments: non-challenged, control-challenged and supplemented-challenged treatment receiving the feed additive at 1 kg/t. The coccidiosis-challenge was carried out at 7 days of age, and it reduced feed intake and growth gain one week post-inoculation (**PI**) in challenged broilers. Concerning the gut barrier, the results showed that coccidiosis disrupted ileal histomorphometry reducing the villus height:crypt depth ratio ($p < 0.10$) and the number of goblet cells, but sodium butyrate protected by MCFA increased crypt depth at 7 days PI and restored the number of these mucin-secreting cells at 14 days PI ($p < 0.05$). Furthermore, the use of the feed additive interacted with the modulation led by coccidiosis in the *Lactobacillus* and *Enterobacteriaceae* population in the ileum ($p < 0.05$), although nutrient digestibility was not affected at 4 days PI. However, it seems that sodium butyrate protected by MCFA promoted the recovery of growth performance parameters for the 21 days of study.

The fourth trial (Chapter 5) was conducted in 96 weaned piglets (Landrace x Large White, 21 days of age) following a factorial design with 2 dietary crude protein (**CP**) levels (18.8% vs. 22.2%) and 2 supplementation doses with sodium butyrate (50%) protected by MCFA salts (0 vs. 1 kg/t). The aim of this study was to evaluate both strategies to improve the gut health of weaned piglets. On one hand, reducing CP compromised growth performances ($p < 0.05$), decreased ileal crypt depth ($p = 0.057$) and modified the *Lactobacillus* population in the ileum and colon ($p = 0.032$) of weaned piglets. In addition, organic matter digestibility ($p = 0.026$) and fecal consistency ($p < 0.10$) were improved by reducing dietary CP levels. On the other hand, the use of the feed additive increased the number of goblet cells ($p = 0.036$), enhanced the nutrient digestibility in the ileum and modified the *Lactobacillus* and enterobacteria counts ($p < 0.05$) as well as microbial metabolites in the colon.

Overall, the results suggest that MCFA-fat protected sodium butyrate enables a gradual release of butyric acid in the intestine, a key factor in promoting the beneficial effects of both fatty acids on the gut barrier. Particularly, the potential to reinforce the gut health impacting ileum histomorphometry and microbial populations by this nutritional strategy has been exacerbated when broiler intestinal health was compromised by coccidiosis. In the same line, the use of protected sodium butyrate protected by MCFA also appears to reinforce the intestinal barrier during the weaning phase of piglets, observing improved ileal digestibility as well. Furthermore, as reducing dietary CP also promotes beneficial effects on piglet intestinal health, the potential of combining sodium butyrate protected by MCFA with other nutritional strategies appears to be a promising research direction in animal production. In fact, these approaches have received

a great deal of attention in recent years, especially in challenging conditions, in order to promote animal health and reduce antibiotic use and environmental impact.

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Abbreviations

AA	amino acids
ADG	average daily gain
ADFI	average daily feed intake
AFP	abdominal fat pad
AID	apparent ileal digestibility
AME	apparent metabolizable energy
ATTD	apparent total tract digestibility
BW	body weight
CD	crypt depth
CEEAH	ethical committee on human and animal experimentation
CFU	colony-forming unit
CP	crude protein
DM	dry matter
ETEC	enterotoxigenic *Escherichia coli*
FA	fatty acid (s)
FCR	feed conversion ratio
GALT	gut-associated lymphoid tissue
GE	gross energy
G:F	gain-to-feed ratio
GIT	gastrointestinal tract
IEL	intraepithelial lymphocytes
LCF	long-chain fatty acid (s)
L:E	*Lactobacillus*:Enterobacteria ratio
MCFA	medium-chain fatty acid (s)
MUFA	monounsaturated fatty acid (s)
NH_3	ammoniac
OM	organic matter
PI	post-inoculation
PUFA	polyunsaturated fatty acid (s)

PW	post-weaning
SCFA	short-chain fatty acid (s)
SFA	saturated fatty acid (s)
TiO_2	titanium dioxide
TNF-α	tumor necrosis factor-α
UFA	unsaturated fatty acid (s)
VFA	volatile fatty acids
VH	villus height
VH:CD	villus height:crypt depth ratio

CHAPTER 1

General introduction

For many years, antibiotics have been worldwide used in poultry and swine production (Piva et al., 2002; Naeem et al., 2018). However, the development of antimicrobial resistances and environmental contamination (Long et al., 2018; Polianciuc et al., 2020) promoted doubts on their use and increased human health concerns. As a result, the European Union banned the use of antibiotics as growth promoters in January 2006 (European Parliament and European Council, 2003). Furthermore, the European Union made public the antimicrobial resistance related with zoonosis, and since 2022 has also imposed new restrictions on the use of antibiotics in animal production (EFSA, 2018; European Parliament and European Council, 2018).

The antibacterial action favors animal performance through different ways, such as reducing the incidence and severity of subclinical infections and reducing the waste of nutrients by the intestinal microbiota (Huyghebaert et al., 2011). Therefore, reduced use of antibiotic led to limited growth performance and the rise of the incidence of certain diseases (Naeem et al., 2018; Liu et al., 2018). In swine production, the impact of antibiotic removal from diets is more marked at weaning than at the grower-finisher stage (Cromwell, 2013). Thus, the use of medicinal zinc oxide in the post-weaning diet has been used for years until the European Union banned its use in June 2022 (Lynegaard et al., 2021). Given this situation, promoting gut health has become a crucial tool in animal production (Celi et al., 2017; Kogut et al., 2017). In this sense, different nutritional strategies have been in research according to the objectives of the global action plan of the World Health Assembly (2015) to invest in new medicines and alternative interventions to antibiotics.

Therefore, dietary supplementation with additives is being considered. According to the European Parliament and European Council (Regulation (EC) No 1831/2003), feed additives are any substances, microorganisms, or preparations, other than feed material and premixtures, which are intentionally added to feed or water with the aim to perform one or more functions. The additives results beneficial for the feed properties or the animal health, performance, as well as improve the animal product quality or reduce the negative impacts of the animal production in the environment. It can be differentiated organic acids, pre- and probiotics, symbiotics, phytogenic actives, minerals, bacterial and yeast fermentation products, or enzymes, among others (EFSA, 2021).

Focusing on organic acids, long used as feed acidifiers and preservers, they also seem to be an effective way to limit the antibiotic use by improving the gut barrier and, consequently, to achieve a good health status (Piva et al., 2002; Mallo et al., 2010; Polycarpo et al., 2017). Among them, butyric acid is a short-chain fatty acid (**SCFA**) widely researched as a dietary supplement.

It is a natural product of intestinal fermentation and, in addition, synthetic forms are available. More recently, medium-chain fatty acids (**MCFA**) have received more attention for promoting gut health. In this case, natural sources rich in MCFA such as coconut and palm kernel have become increasingly important.

In both cases, the use of butyric acid and MCFA seems to have a significant role in the gut barrier, promoting the development of the intestinal epithelium and playing antibacterial properties. However, their effect may be limited due to their high efficiency to be absorbed and metabolized in the gastrointestinal tract. For this reason, the use of protected forms and their combination are highly in research. In addition, their ability to improve the development and function of the gut may be emphasized when the animals are under challenged conditions (Del Alamo et al., 2007).

Therefore, to understand the goal scenario, the concept of "gut health" will be described. Afterwards, generalities and sources of SCFA, butyric acid in particular, and MCFA will be presented, as well as their digestion and absorption process. Finally, the effects of butyric acid and MCFA on performance parameters, intestinal barrier considering the histomorphology of the epithelium and the microbial population, as well as digestibility capacity will be summarized. In this context, a compilation of the effects observed in previous studies that have evaluated products based on butyric acid and/or MCFA, differentiating their form of presentation, will be presented.

1.1 The concept of gut health

Optimal gut health is vitally important for poultry and swine performance as it has broad implications in regulating physiological homeostasis (equilibrium) that provides animal welfare (Celi et al., 2017; Ji et al., 2019). In this way, the interest in the concept of "gut health", "intestinal health", "enteric health" or similar terms has increased since the last two decades due to increasing demands for economic efficiency in the animal production, promoting the animal welfare and reducing the environmental impact as well as the use of antibiotics (Kogut et al., 2017).

This issue is repeatedly used in animal nutrition to describe animal health, but according to Celi et al. (2017) still lacks a clear definition. The simple definition would be "absence of clinical diseases". Nevertheless, it is well known that animal performance can be negatively affected without any clinical signs. A healthy animal is preferably defined as one that can perform its

physiological functions to reach its performance standards (Kogut and Arsenault, 2016; Kogut et al., 2017). Celi et al. (2017) proposed the definition of gut health as “steady state where the microbiome and the intestinal tract exist in symbiotic equilibrium and where the welfare and performance of the animal is not constrained by intestinal dysfunction”. Therefore, to clear the concept, it is required to deepen the knowledge on the gut barrier (Naeem et al., 2018; Pluske et al., 2018; Oviedo-Rondón, 2019).

1.1.1 The gut barrier

The gut is a vital organ of the digestive system which have a great complexity and dynamics (Celi et al., 2017; Moeser et al., 2017; Naeem et al., 2018). It is related with the digestion and absorption process, as well as representing a barrier against antigens and pathogens, and plays an important role in the immunity system (Vighi et al., 2008; Choct, 2009; Van der Flier and Claviers, 2009; Celi et al., 2017).

Anatomically, the gut is divided into the small intestine subdivided in duodenum, jejunum and the ileum, and the large intestine subdivided in cecum, colon and rectum. The endpoint of the intestinal tract in mammals is the anus, while birds have the cloaca (Van der Flier and Clevers, 2009; Nasrin et al., 2012). The wall throughout the intestine is divided into four layers. All of them are linked by connective tissue and by neural and vascular elements. Among them, mucosa is the first layer facing the intestinal lumen, and it is subdivided in three sublayers (from the intestinal lumen): epithelium, lamina propria and muscularis mucosae (Rao and Wang, 2010). Hence, the mucosa, and particularly its sublayer epithelium, is of great interest in the concept of “gut health”.

The intestinal epithelium has a unique architecture, organized into villi and crypts. Villi are protrusions that enlarge the surface area for the nutrient absorption. In pig case, villi’s description is restricted to small intestine whereas in chicken are also described in proximal cecum, although their function is until not clear at present (McLelland et al., 2000, Illanes et al., 2006; Nofrarias et al., 2006; Van der Flier and Clevers, 200; Nasrin et al., 2012). Crypts also called “crypts of Lieberkühn” are epithelial invasions through the amount intestine where stem cells reside (Mescher, 2009). These cells turn into specialized intestinal epithelial cells and transited through the villi, continuously and rapidly turnover in 3 to 5 days (Mescher, 2009; Van der Flier and Clevers, 2009; Xiong et al., 2019).

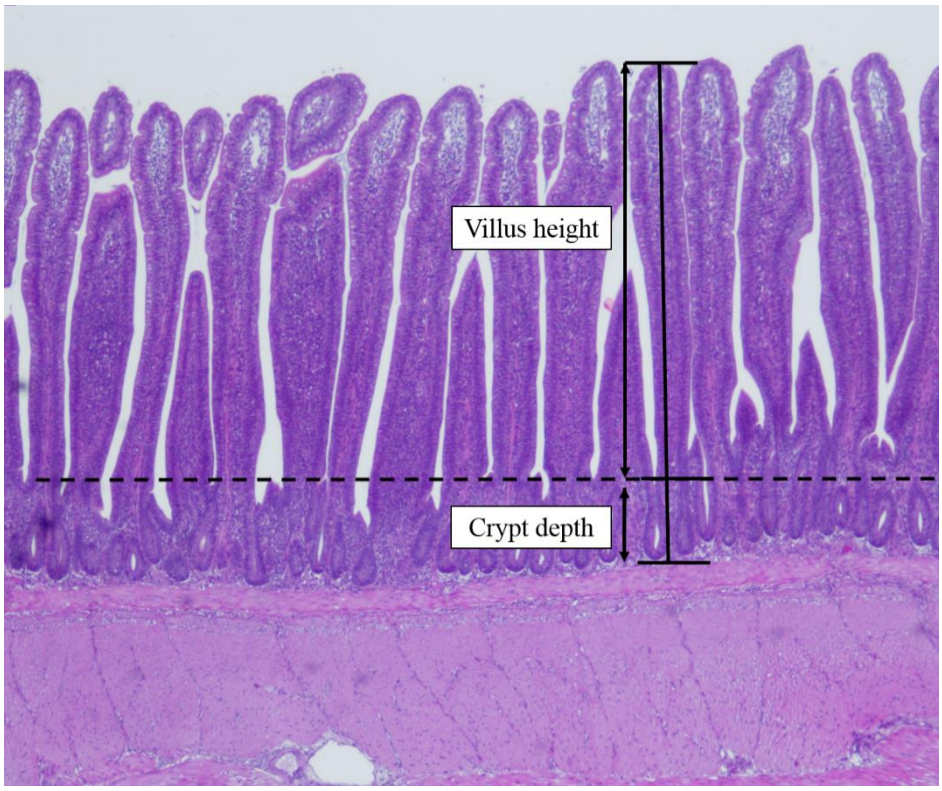

Figure 1.1 Histological picture of 39 d broiler's ileum at 40x magnification. Villus height and crypt depth measurements. Haematoxylin and eosin stain. Image by author using light microscope BHS, Olympus (Tokyo, Japan) in Centre de Recerca en Sanitat Animal (CReSA).

Differenced epithelial cells can be classified into the following broad categories: absorptive enterocytes, mucous-secreting goblet cells, and hormone-secreting enteroendocrine cells (Van der Flier and Clevers, 2009). The last ones are a minority, while the enterocytes are more than the 80% of all intestinal epithelial cells. The enterocytes are highly polarized columnar cells with an apical rush border, the microvilli, which is responsible for the absorption and transport of nutrients across the epithelium (Aman et al., 2005; Mescher, 2009). Between enterocytes, there are epithelial tight junction proteins such as occludin, claudin-1, and zonula occludens protein 1 that are the rate-limiting factor for paracellular permeability. The tight junctions are highly dynamic complexes that seal the paracellular pathway and conduct gate and fence functions: they allow the transport of nutrients and essential ions and restrict the entry of harmful substances as bacterial toxins or pathogens (Chelakkot et al., 2018; Xiong et al., 2019).

Goblet cells are specialized secretory cells (Jakobsson et al., 2015). Their proportion among all epithelial cell types increases throughout the intestine of chickens and pigs. According to Uni et al. (2003), goblet cells account for 23% of the total intestinal epithelial cells in the jejunum and 26% in the ileum of chickens. In the case of mammals, goblet cells comprised about 4% in the duodenum and 16% in the descending colon (Karam, 1999). Nevertheless, goblet cells secrete mucins which are glycoproteins with high molecular weight that form the intestinal mucus. In

fact, the oligosaccharide side chains of these glycoproteins contribute to the mucus gel-forming properties. Mucus layer facilitates the movement and effective passage of gut contents, as well as providing a physical and chemical protective barrier. Indeed, the absence of mucus has been described to increase the vulnerability to intestinal inflammation.

Furthermore, among the aforementioned cells in the villi, there are intraepithelial lymphocytes (**IEL**). These IEL are a heterogeneous population of cells with a diverse set of functions in epithelial surveillance as part of the diffused gut-associated lymphoid tissue (**GALT**). The GALT constitutes the most extensive and complex part of the immune system. It is structured in organized tissue that consists of Peyer's patches located along the small intestine, and lymph nodes, besides the diffused tissue (Ramiro-Puig et al., 2008; Rieger, 2015).

It is important to keep in mind that all components of the intestinal mucosa are co-influenced, and play together to achieve a healthy state. In this sense, several studies have described that physiology and function of the intestinal mucus are affected by "gut microbiota" (Castillo et al., 2006; Schroeder, 2019) referring to the tremendous number of microorganisms including bacteria, archaea, viruses, and unicellular eukaryotes that inhabits in the intestinal tract (Kogut, et al., 2007; Rieger, 2015).

For years, it has been described that the microbiota colonizes the intestine of animals after birth. Thus, the gut of newborn has been considered sterile (Mackie et al., 1999). However, recent studies have demonstrated earlier microbiota colonization in the gut. In broiler chickens, Ding et al. (2022) showed that the yolk microbiota contributes to the formation of chicken embryo intestinal microbiota. On the other hand, Leblois et al. (2017) observed microbial counts in umbilical cord blood indicating a maternal transfer of gut microbiota during the gestation period of sows. In any case, the microbiota colonization in the intestinal tract of both species is extremely fast. Apajalahti et al. (2004) described that 10^8 and 10^{10} colony-forming unit (**CFU**) of microbes per gram of digesta in the ileum and cecum, respectively, of one day post hatching broilers increased to 10^9 to 10^{11} in 3 days and remained stable for the following 30 days of age. Therefore, the source of microbiota colonizing the intestinal tract post-hatching in modern poultry practices may be the environment, diet, and animal handlers post-hatch (Maki et al., 2020). In pigs, establishment of the gut microbiota reaches counts of 10^9 CFU/g in colonic digesta at 12 h post-birth (Swords et al., 1993; Jensen-Waern et al., 1998). As piglets remain with the sows until weaning, the microbiota colonization for the first week of age is mainly determined by the sow's feces (Sansom and Gleed, 1981). However, microbiota patterns then change in a few days, and become characteristic for each piglet increasing its diversity with age and

achieving total bacterial counts of 10^{10} UFC/g in feces at d 120 of age (Swords et al., 1993; Katouli et al., 1997). The bacterial population colonizes the gut permanently living in a mutualistic relationship with its host (Mackie et al., 1999). The gut microbiota receives nutrients from host or the animal diet and, in turn, it assists in the degradation of complex non-fermentative carbohydrates and provides metabolites as SCFA for host nutrition and intestinal development. In addition, it has been described that some intestinal bacteria may confer health benefits to the host by promoting intestinal motility, regulating immune development and maturation, and providing competitive exclusion of pathogens for nutrients and mucosa adhesion (Castillo et al., 2006; Hooper et al., 2012; Kogut and Arsenault, 2016; Kogut et al., 2017).

Hence, there is clear evidence of the importance of the gut microbiota in maintaining health and normal intestinal function (Mulder et al., 2009). Hooper et al. (2012) described that germ-free animals have an underdeveloped mucosa layer and immune system, resulting in altered tolerance to some dietary proteins. In contrast, increased resistance to infections with pathogens as *Escherichia coli*, *Clostridium perfringens* or *Salmonella* spp. has been described in chicks that experimentally received gut contents of healthy adult chicks (Rychlik, 2020).

Therefore, it seems that microbiota population results beneficial for the host animal when it remains stable, in a homeostatic stage. However, changes in diet or environmental conditions, the use of antibiotics or infections are some of the external issues that can alter this intestinal homeostasis, leading to the proliferation of non-desirable or potentially pathogenic bacteria (dysbiosis). Hence, the relationship between lactic acid-producing bacteria (as *Lactobacillus*) and enterobacteria population has been traditionally considered as an index of desirable to non-desirable bacteria, relating a high ratio (lactic acid bacteria:enterobacteria) to a greater resistance to intestinal disorders (Ewing and Cole, 1994). In fact, it has been described that lactic acid bacteria acidify the host gut, creating a non-favorable environment for acid-sensitive members of family *Enterobacteriaceae* (Kim et al., 2019). Besides that, there are nutrition and site competition that limit bacteria overgrowth and pathogen colonization (Mulder et al., 2009).

To summarize, the dynamic balance between the mucus layer, the immune system and the gut microbiota determines the functionality of the intestinal barrier, which provides the ability to battle against challenging scenarios (Rodríguez-Lecompte et al., 2012; Naeem et al., 2018). In the next section, two of the most concurrent disrupting challenges on poultry and swine production, respectively, will be exposed.

1.1.2 Challenging scenarios for gut health

Numerous infectious and non-infectious stressors can damage gut barrier functions, as well as compromise digestion and absorption processes. Following, avian coccidiosis is presented as a worldwide infectious disease characterized by affecting the intestinal mucosa in broilers. On the other hand, weaning phase is next explained as a non-infectious scenario that disrupts the gastrointestinal tract in pigs.

1.1.2.1 Coccidiosis in poultry production

Avian coccidiosis is one of the most widely reported and economically important diseases that destabilize poultry production worldwide. The incidence of coccidiosis in commercial poultry can range from 5 to 70%, and it can affect any poultry reared in any type of facility (Czerwiński et al., 2012; Abdullahi et al., 2020).

Coccidiosis is an infectious disease caused by an intracellular protozoan that belongs to the Apicomplex phylum, *Eimeriidae* family and *Eimeria* genus (Gazoni et al., 2020; Cervantes et al., 2020). There are described 7 *Eimeria* species, although three of them have the most impact on broiler chickens production: *E. acervulina*, *E. maxima* and *E. tenella*. They have a common life cycle with different duration according to each species (from 4 to 7 days). In any case, *Eimeria* multiplication results in invasion and rupture of intestinal cells, while downregulating the expression of the tight junctions (Del Cacho, 2013; Adedokun and Adeola, 2016; López-Osorio et al., 2020). The parasitized intestinal region is particular to each *Eimeria* species as shown in **Figure 1.2**.

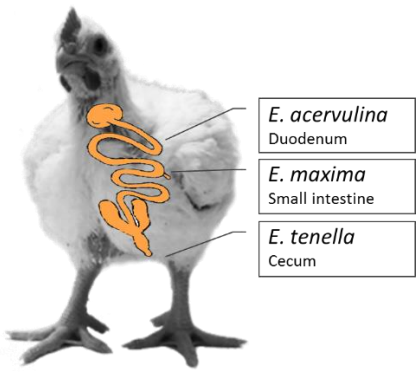

Figure 1.2 Intestinal regions parasitized by Eimeria species in broilers: *E. acerculina, E. maxima, E. tenella.*

Most infections are relatively mild or subclinical, resulting from the ingestion of a few sporulated oocysts, and can scape of notice. Indeed, coccidiosis has been described as the most common subclinical disease of commercial broilers (Cervantes et al., 2020). Nonetheless, coccidiosis can

be severe because of the ingestion of millions of sporulated oocysts. Therefore, impaired growth performance and lower skin coloration have been described because of the detrimental effect on intestinal histomorphometry and, consequently, nutrient and pigment absorption. In addition, it has been demonstrated that *Eimeria* infection impacts on the microbiota population that may result in dysbiosis increasing the susceptibility of the broilers against pathogenic bacteria as *Salmonella*, *Escherichia coli* or *Clostridium perfringens*¸ leading to necrotic enteritis (Takimoto et al., 1984; Ahari et al., 2020; Cervantes, 2020; Lin and Olukosi, 2021). Dehydration, blood loss and even increased mortality have also been described in coccidiosis cases (Su et al., 2015; Gazoni et al., 2020).

Many coccidiosis cases are concurrent infections with two or more *Eimeria* species. In this regard, it is important to know that there is not cross-immunity between species. For this reason, the anticoccidial vaccines used to control coccidiosis include a mixture of attenuated oocysts of the most common *Eimeria* species (Cervantes, 2020; Gazoni et al., 2020). Following with the aim of controlling coccidiosis, chemicals and ionophores have been used as effective anticoccidials for extended periods of time without the emergence of new products in the last years (Czerwiński et al., 2012; Adhikari et al., 2020). However, drug-resistance developed by coccidian involves the most serious limitation to their efficacy. As a consequence, different anticoccidial programs involving only one or a combination of different types of drugs have been proposed as single, dual or shuttle program, respectively. The single program consists of the continuous use of a single drug, while the dual program includes one drug in the starter feed and another one in the grower feed. Sometimes a third drug is used in the finisher feed. The *shuttle* program is characterized by the addition of a chemical anticoccidial to the starter feed and an ionophore anticoccidial to the grower feed. In addition, seasonal rotation of anticoccidial in feed and vaccines is another strategy currently used in poultry production (Ronsmans et al., 2015; Adhikari et al., 2020; Cervantes et al., 2020). Furthermore, nutritional strategies against coccidiosis are also under research in recent years. In fact, it has been described that feed additives may promote gut health and modulate the effect of coccidiosis on the gut barrier (Allen et al. 1998; Baltić et al., 2018; Adhikari et al., 2020; Abdullahi et al., 2020; Lin and Olukosi, 2021). Therefore, in the present thesis, coccidiosis has been considered interesting to evaluate the efficacy of dietary supplementation with sodium butyrate protected by MCFA salts on the gut health of broilers under a challenging scenario (Chapter 4).

1.1.2.2 Weaning in swine production

Weaning is the most challenging stage in swine production with many stress factors, such as nutritional, management, environmental and social issues (Lallès et al., 2004; Biagi et al., 2007). In nature, weaning in pigs occurs gradually around 10-12 weeks of age, which coincides with the near complete gastrointestinal development (Moeser et al., 2017). However, the high economic-competitive properties in the current swine production system results in early weaning between 3 and 4 weeks of age. Therefore, the period of 5 to 8 weeks after weaning to match nature systems continues to be a critical production stage (Pluske et al., 2001; Piva et al., 2002; Moeser et al., 2017).

It is well stablished that weaning is a multi-factorial process. In all cases, maternal separation is considered the major stressor for piglets , although there are many additional stressors including transport, the new housing environment with separation from littermates and exposure to non-familiar counterparts (new social hierarchy), vaccination and nutritional changes (Lallès et al., 2004; Moeser et al., 2017). Furthermore, the immune factors are supplied by maternal calostrum and milk but the development of the piglet immune system is not achieved before commercial weaning (Lallès et al., 2007). Transition from milk to solid feed also involves a rapid change in the intestine. Indeed, increased intestinal permeability has been described when piglets start eating feed consisting of more complex proteins and carbohydrates (Lallès et al., 2007). In addition, due to the multi stress factors, piglets usually reduce feed intake, resulting in structural damage in small intestine morphology, reducing the area for absorption and enzymatic activity (Pluske et al., 1997; Heo et al., 2013).

Moreover within a short period of time, the intestinal microbiota must develop from a simple non-stable community with low biodiversity to a complex and stable population (Lallès et al., 2007). So, in the immediate post-weaning period, opportunistic enteric pathogens such as pathogenic *Escherichia coli* or *Salmonella spp.* are highly likely to colonize the gut and further compromise the health of weaned pigs, causing diarrhea. In this situation, the post-weaning mortality ratio is around 6 to 10%, but can sometimes raise up to 20% (Fairbrother et al., 2005; Casanova-Higes et al., 2018; Xiong et al., 2019).

For decades, antibiotics have been commonly utilized in weaning but, nowadays, their use is more restricted for clinical cases. In addition, the use of pharmacological levels of zinc oxide in weaned pig diets has been banned in the European Union from June 2022 (European Parliament and European Council, 2018). Therefore, in this scenario a wide range of nutritional strategies are under research to promote gut health of weaned piglets (Molist et al., 2021). According to

Huting et al. (2021), several functional ingredients and feed additives are receiving the most attention. In the following sub-section, the reduction of crude protein (**CP**) level is provided to explain the role of a main nutrient in supporting gut health and functionality in weaned piglets.

1.1.2.2.1 Reducing dietary crude protein as a nutritional strategy to promote gut health in weaned piglets

Diets for weaned piglets have traditionally been rich in CP to compensate for the reduced and variable feed intake during immediate post-weaning phase (Pluske et al., 1997; Lallès et al., 2007). However, intestinal function is still developing, and the piglet does not produce enough endogenous enzymes to digest all the dietary CP. The non-digested CP remains available for microbial fermentation, which disrupts intestinal homeostasis and, consequently, weaning diarrhea appears (O'Doherty et al., 2017). Therefore, increasing precision in feed formulation by reducing CP level seems to be effective to reduce post-weaning diarrhea and promoting gut health in piglets. However, growth performance may also be reduced (Bikker et al., 2006; Nyachoti et al., 2006; Wellock et al., 2006; Heo et al., 2008; Yue and Qiao, 2008; Opapeju et al., 2009; Lynegaard et al., 2021). There is a wide range of dietary CP levels evaluated among recent studies focused on promote intestinal health in weaned piglets. Therefore, **Table 1.1** summarizes the effect of reducing the amount of CP intake by weaned piglets on growth performance and gut health.

Some authors evaluated the dietary CP reduction in weaned pig diets meeting nutrient requirements (FEDNA, 2013): 19.4% to 21.8% CP for 5 to 7 kg body weight (**BW**) weaned piglets; 18.9% to 20.8% CP for 7 to 12 kg BW weaned piglets. According to the reviewed literature, growth performance was not compromised in these cases. Furthermore, it seems to have beneficial effects on intestinal health: improved fecal consistency, increased villus height:crypt depth ratio (**VH:CD**), reduced microbial fermentation, as well as improved CP digestibility were observed.

Nonetheless, further reductions of dietary CP levels below nutrient requirements have been evaluated in several studies. In these cases, there are discrepancies about the effect of this nutritional strategy on performance parameters. Indeed, some authors observed that piglets receiving reduced-CP diets with 19 ± 0.1% % CP levels had improved intestinal morphometry with lower fermentation and enhanced fecal consistency, without compromised growth performance. Lee et al. (2017) showed no impact on growth gain, although the number of *Lactobacillus* and nutrient digestibility decreased, due to the increased intake.

On contrary, limited growth gain and feed efficiency were observed in other cases. In this context, intestinal fermentation was reduced, but the impact on intestinal morphometry was according to the amount of CP reduced for feeding piglets with 19% CP diets. Reducing the CP from 23% and 22% to 19% decreased intestinal villus height, but the reduction from 21% to 19% increased the VH:CD ratio (Limbach et al., 2021; Nyachoti et al., 2006). Since Wu et al. (2015) evaluated 4%-reduction from 23% to 19% in weaned piglets and observed higher VH:CD ratio, it seems that the time period during which the animal consumes the diets is a factor to be considered.

Pierce et al. (2007), in turn, showed that the reduction from 21% to 18.5% CP impaired piglet growth performance, but also had beneficial effects on the intestinal barrier, increasing the number of *Lactobacillus*, *Bifidobacteria* and reducing the number of *Escherichia coli*. In addition, intestinal fermentation was also modified, although nutrient digestibility was not compromised.

In the context of reducing (1.5% to 3%) dietary CP levels to 18%, different authors did not observe negative effects on growth performance during 14 to 35 days post-weaning. Furthermore, Rattingan et al. (2020) also observed better gut health with lower incidence of diarrhea and lower numbers of *Enterobacteriaceae* in piglets when reducing 3 points to obtain 18% CP in the diet. On the other hand, Manzanilla et al. (2009) described changes in morphometric parameters (higher VH:CD and reduced numbers of IEL and mitoses) and lower concentration of volatile fatty acids (**VFA**), but without affecting productive parameters or digestibility by reducing 2 points of CP (from 20 to 18%).

Other authors observed no differences in performance parameters or in the intestinal tract (Tang et al., 2019), while other studies showed worse G:F ratio and nutrient digestibility in piglets fed approximately 18 ± 0.1% CP (instead of 20, 21 or 23% CP). In the last case, although performance was compromised, 5%-reduction from 23% CP improved fecal score and increased *Lactobacillus*:*Enterobacteriaceae* ratio, as well as decreased coliform numbers in 14 d post-weaned piglets. On the other hand, the reduction from 20% CP to 17.9% decreased the number of *Lactobacillus* and the intestinal fermentation of 42 days post-weaning piglets.

Further research evaluated the effect of reducing the level of CP in the diet for feeding piglets with diets of 17 ± 0.6% CP. According to the literature reviewed, it is possible to reduce a wide range (from 2 to 8.1% points) of CP content weaned piglet diets to reach approximately 17% CP

Table 1.1 Published studies evaluating the effect of different dietary crude protein (CP) levels in performance and gut health of weaned piglets.

						Effects of crude protein (CP) reduction					
	High CP, %	**Low CP, %**	**Diff. CP, %**	**Weaning age, d (BW kg)**	**Experimental period, d PW**	**Performance**	**Fecal consistency**	**Histomorphometry**	**Microbiota**	**Fermentation**	**Digestibility**
Fang et al (2019)	23.7	21.7	2.0	8.7 kg	0-21 d	NS	-	-	-	-	↑ CP
Yue and Qiao (2008)	23.1	21.2	1.9	18 d (6.8 kg)	0-14 d	NS	↓	NS	-	-	-
Nyachoti et al. (2006)	23	21	2.0	18 d (6.2 kg)	0-21 d	NS	NS	↑ VH:CD	NS	↓ NH_3, VFA	-
Htoo et al. (2007)	24	20	4.0	19 d (8.2 kg)	6-27 d	-	-	-	-	NS	↓ AA
Htoo et al. (2007)	24	20	4.0	20 d (8.2 kg)	6-27 d	-	-	-	-	↓ NH_3, VFA	-
Htoo et al. (2007)	24	20	4.0	21 d (8.2 kg)	6-27 d	NS	NS	-	-	-	-
Júnior et al. (2021)	24	20	4.0	21 d (7.9 kg)	0-14 d	↓ ADFI	-	↑ VH:CD	-	-	-
Fang et al (2019)	21.7	19.7	2.0	8.7 kg	0-21 d	↑ G:F	-	-	-	-	↑ CP
Fang et al (2019)	23.7	19.7	4.0	8.7 kg	0-21 d	NS	-	-	-	-	↑ CP
Toledo et al. (2014)	21	19.5	1.5	21 d (6.0 kg)	0-30 d	NS	-	-	-	-	-
Nyachoti et al. (2006)	21	19	2.0	18 d (6.2 kg)	0-21 d	↓ G:F	NS	↑ VH:CD	NS	↓ NH_3, VFA	-
Lee et al. (2017)	21.7	19	2.7	6.8 kg	0-14 d	↑ ADFI	-	-	↓ *Lactobacillus*	↓ NH_3, VFA	↓ DM, GE
Limbach et al. (2021)	22	19	3.0	20 d (5.5 kg)	0-28 d	↓ ADG	NS	↓ VH	-	NS	-
Nyachoti et al. (2006)	23	19	4.0	18 d (6.2 kg)	0-21 d	↓ G:F	NS	↓ VH:CD	NS	↓ NH_3, VFA	-
Wu et al. (2015)	23	19	4.0	21 d (6.0 kg)	0-14 d	NS	↓	↑ VH:CD	-	-	-
Yue and Qiao (2008)	21.2	18.9	2.3	21 d (6.8 kg)	0-14 d	NS	↓	NS	-	-	-
Yue and Qiao (2008)	23.1	18.9	4.2	19 d (6.8 kg)	0-14 d	NS	↓	NS	-	-	-
Pierce et al. (2007)	21	18.5	2.5	21 d (7.6 kg)	0-28 d	↓ G:F	-	-	↑ *Lactob.*, *Bifid.*, ↓ *E. coli*	↓ isovaleric, ↑ propionic	NS
Toledo et al. (2014)	19.5	18	1.5	24 d (6.0 kg)	0-30 d	NS	-	-	-	-	-
Manzanilla et al. (2009)	20	18	2.0	20 d (5.4 kg)	0-19 d	NS	-	↑ VH:CD, ↓ IEL, mitosis	NS	↓ acetic acid, ↑ butyric acid	NS
Tang et al. (2019)	20.5	18	2.5	25 d (7.6 kg)	0-14 d	NS	-	NS	NS	NS	-
Rattingan et al. (2020)	21	18	3.0	26 d (6.5 kg)	0-35 d[1]	NS	↓	NS	↓ *Enterobacteria*		
Rattingan et al. (2020)	21	18	3.0	26 d (7.4 kg)	0-35 d	NS	↓	-	NS	-	-
Toledo et al. (2014)	21	18	3.0	22 d (6.0 kg)	0-30 d	NS	-	-	-	-	-
Wellock et al. (2006)	23	18	5.0	28 d (10.7 kg)	0-14 d	↓ G:F	↓	-	↓ coliform, ↑ L:E	-	↓ CP
Lee et al. (2017)	20	17.9	2.1	(6.8 kg)	15-42 d	↓ G:F	-	-	↓ *Lactobacillus*	↓ NH_3, VFA	↓ DM, GE
Opapeju et al. (2009)	22.5	17.6	4.9	17 d (5.3 kg)	0-14 d[2]	NS	↓	↑ VH:CD	↓ richness, diversity	↓ NH_3	-
Heo et al. (2009)	25.6	17.5	8.1	21 d (5.9 kg)	0-14 d[2]	NS	↓	-	-	↓ NH_3, VFA	-
Opapeju et al. (2015)	22.2	17.3	4.9	19 d (7.0 kg)	0-9 d[2]	-	-	↑ goblet cells	↓ *E. coli*	-	-
Heo et al. (2008)	24.3	17.3	7.0	21 d (6.0 kg)	0-14 d	NS	↓	-	↓ *E. coli*	↓ pH	NS
Yue and Qiao (2008)	18.9	17.2	1.7	23 d (6.8 kg)	0-14 d	↓ G:F	NS	↓ VH	-	-	-

Yue and Qiao (2008)	21.2	17.2	4.0	22 d (6.8 kg)	0-14 d	↓ G:F	↓	↓ VH	-	-	-
Yue and Qiao (2008)	23.1	17.2	5.9	20 d (6.8 kg)	0-14 d	↓ G:F	↓	↓ VH	-	-	-
Nyachoti et al. (2006)	19	17	2.0	18 d (6.2 kg)	0-21 d	NS	NS	↑ VH:CD	NS	↓ NH_3, VFA	-
Wu et al. (2015)	19	17	2.0	23 d (6.0 kg)	0-14 d	NS	↓	↑ VH:CD	-	-	-
Tang et al. (2019)	19.5	17	2.5	26 d (7.6 kg)	15-42 d	NS	-	NS	NS	NS	-
Yu et al. (2019)	20	17	3.0	28 d (9.6 kg)	3-48 d	NS	-	↓ VH:CD	NS	↓ NH_3	NS
Nyachoti et al. (2006)	21	17	4.0	18 d (6.2 kg)	0-21 d	↓ G:F	NS	↑ VH, ↓ VH:CD	NS	↓ NH_3, VFA	-
Bhandari et al. (2010)	22	17	5.0	21 d (6.7 kg)	0-12 d[2]	↓ ADG	↓	-	-	-	-
Nyachoti et al. (2006)	23	17	6.0	18 d (6.2 kg)	0-21 d	↓ ADG, G:F	NS	↑ VH:CD	NS	↓ NH_3, VFA	-
Wu et al. (2015)	23	17	6.0	22 d (6.0 kg)	0-14 d	NS	↓	↑ VH:CD	-	-	-
Fang et al (2019)	20.9	16.9	4.0	(8.7 kg)	22-42 d	NS	-	-	-	-	-
Lynegaard et al. (2021)	19.1	16.6	2.5	28 d (7.0 kg)	(6-9 kg)	↓ G:F	-	-	-	-	-
Toledo et al. (2014)	18	16.5	1.5	26 d (6.0 kg)	0-30 d	NS	-	-	-	-	-
Toledo et al. (2014)	19.5	16.5	3.0	25 d (6.0 kg)	0-30 d	NS	-	-	-	-	-
Toledo et al. (2014)	21	16.5	4.5	23 d (6.0 kg)	0-30 d	NS	-	-	-	-	-
Pierce et al. (2007)	18.5	16	2.5	23 d (7.6 kg)	12-40 d	↓ G:F	-	-	↑ *Lactob.*, *Bifidob.*, ↓ *E. coli*	↓ isovaleric, ↑ propionic	NS
Limbach et al. (2021)	19	16	3.0	21 d (5.5 kg)	0-28 d	↓ G:F	NS	NS	-	NS	-
Pierce et al. (2007)	21	16	5.0	22 d (7.6 kg)	12-40 d	↓ G:F	-	-	↑ *Lactob.*, *Bifidob.*, ↓ *E. coli*	↓ isovaleric, ↑ propionic	NS
Limbach et al. (2021)	22	16	6.0	22 d (5.5 kg)	0-28 d	↓G:F	↓	NS	-	↓ pH, butyric	-
Hermes et al. (2009)	19.4	15.4	4.0	21 d (9.1 kg)	14-35 d	NS	NS	↓ goblet cells, ↑ IEL	NS	↓ VFA	-
Toledo et al. (2014)	18	15.0	3.0	26 d (6.0 kg)	0-30 d	NS	-	-	-	-	-
Toledo et al. (2014)	19.5	15.0	4.5	25 d (6.0 kg)	0-30 d	NS	-	-	-	-	-
Toledo et al. (2014)	21	15	6.0	23 d (6.0 kg)	0-30 d	NS	-	-	-	-	-
Yu et al. (2019)	17	14	3.0	30 d (9.6 kg)	3-48 d	↓ G:F	-	-	NS	NS	NS
Lynegaard et al. (2021)	19,1	14	5.1	29 d (7.0 kg)	(6-9 kg)	↓ G:F	-	-	-	-	-
Yu et al. (2019)	20	14	6.0	29 d (9.6 kg)	3-48 d	↓ G:F	-	↓ VH:CD	NS	NS	↑ CP, AA
Wellock et al. (2006)	23	13	10	29 d (10.7 kg)	0-14 d	↓ G:F	↓	-	↓ coliform, ↑ L:E	-	↓ CP
Wellock et al. (2008)	23	13	10	28 d (8 kg), 40 d (13 kg)	0-42 d[2]	↓ G:F	-	-	-	-	-
Wellock et al. (2008)	23	13	10	28 d (8 kg), 40 d (13 kg)	0-28 d[2]	-	NS	NS	↓ *Lactobacillus*	↓ pH	-

Diff. CP, % = difference between the high and low CP diets; PW = post-weaning; NS = non-significance; ADG = average daily gain; ADFI = average daily feed intake; G:F = gain-to-feed ratio; ↓ fecal consistency = better fecal consistency or lower diarrhea; VH = villus height; CD = crypt depth; IEL = intraepithelial lymphocytes; L:E = *Lactobacillus*:*Enterobacteria* ratio; NH_3 = ammoniac; VFA = volatile fatty acids; AA = amino acids; DM = dry matter; GE = gross energy. [1]Unsanitary conditions. [2]Enterotoxigenic *Escherichia coli* infection.

without affecting performance parameters or digestibility and reducing diarrhea as well as microbial fermentation. These results were also observed in piglets challenged by enterotoxigenic *Escherichia coli* (**ETEC**). In addition, reducing by 2, 4.9 or 6% points to feed piglets with 17% CP diets for 14 or 21 days increased the intestinal VH:CD ratio. However, it has been described that reduction of 2.5 or 3 points did not affect or even decreased the VH:CD ratio in 48 days post-weaned piglets. As for antibacterial effects, Heo et al. (2008) showed a reduction in the number of *Escherichia coli* in feces, positively affecting fecal consistency, without impacting performance parameters.

Conversely, some authors observed worse productive performances in piglets fed reduced-CP diets with 17 ± 0.2% CP (diets reduced between 1.7 and 6% CP points). The protein reduction for two weeks post-weaning improved fecal consistency, even in piglets infected with ETEC (Bhandari et al., 2010), although it reduced intestinal villus height (Yue and Qiao, 2008). However, if this nutritional strategy is used for 3 weeks post-weaning, Nyachoti et al. (2006) showed that a 6% reduction in dietary CP levels increased the VH:CD ratio throughout the intestine, whereas a 4% reduction particularly improved it in the jejunum. In any case, microbiota fermentation was reduced in piglets fed 17% CP diets.

Moreover, it was described that reduction of 2.5 to 6 points to obtain diets with 16% CP can impair growth performance but improve the intestinal environment (Lynegaard et al., 2021). The number of *Escherichia coli* and fermentation products decreased, as well as the number of *Lactobacillus* and *Bifidobacterium* increased. In addition, Pierce et al. (2007) observed that nutrient digestibility was not compromised in the piglet fed this amount of CP in the diet.

Controversially, others authors evaluated the effect of further reducing dietary CP to feed piglets with around 15% CP diets, and no differences in productive parameters were observed (Toledo et al., 2014). However, reduced numbers of goblet cells, increased number of IEL and higher serum concentration of acute-phase proteins, indicating tissue disruption, was observed in 9.1 kg BW piglets. On the other hand, no antibacterial effects on the microbial population were observed, but piglets that fed less CP had lower bacterial fermentation in the intestinal tract (Hermes et al., 2009; Piñeiro et al., 2009).

Further reductions to 13 and 14% CP was studied in weaned piglet diets. To the best of our knowledge, performance parameters were compromised in all of these cases, including piglets challenged by ETEC (Wellock et al., 2008). In fact, Yu et al. (2019) observed lower VH:CD, although CP digestibility increased. It should be noted that these authors observed that reduction of CP by 3 or 6 points from 20% led to higher ileal digestibility. However, they observed

that growth performance and feed efficiency were only compromised by the 6% reduction to obtain diets with 14% CP, even when ileal digestibility of amino acids was improved. Therefore, the results suggested that compromised growth performance may be the result of reduced feed intake. However, animals feeding diets with 13% CP had worse CP digestibility.

In contrast, few results showed improvements in performance parameters by reducing dietary CP. Fang et al. (2019) reported that reduction from 21.7% to 19.7% CP increased weight gain and feed efficiency, although feed intake decreased for 21 d in heavy piglets (8.7 kg). These results supports the nutrient requirements standard for 7 to 12 kg BW piglets (FEDNA, 2013).

Therefore, according to the literature consulted, it appears that reducing CP level may be a nutritional strategy to consider promoting the functionality of the intestinal barrier of weaning piglets during the most challenging phase of pig production. However, reducing CP levels below the nutrient recommendations may compromise the growth of weaned piglets. Thus, further research is needed to assess this nutritional strategy as a promising candidate to improve piglet intestinal health or even combine dietary CP reduction with other nutritional strategies to achieve the objective. Thus, in the present thesis, this nutritional strategy reducing CP has been evaluated in combination with the dietary supplementation with sodium butyrate protected by MCFA salts on piglet intestinal health in Chapter 5.

1.2 Organic acids in poultry and swine nutrition: butyric acid and medium-chain fatty acids

Dietary supplementation with SCFA and MCFA to promote the gut health in monogastrics is increasing. Therefore, the most relevant generalities and sources of these organic acids are described in this section. In addition, the processes of fat digestion and absorption are presented to finally show different forms of presentation of these fatty acids (SCFA, in particular butyric acid, and MCFA) that are already use and/or under research in poultry and swine nutrition.

1.2.1 Generalities of organic acids

A wide range of organic acids with different physical and chemical properties are used as feed additives after being considered safety by the European Union (Adil et al., 2010; Kaczmarek et al., 2016; Naeem et al., 2018). An organic acid is any substance that contains the R-COOH group in its structure and has acidic properties as fatty acids (**FA**) (Dibner and Buttin, 2002).

The FA are monocarboxylic acids that contain a hydrocarbon chain as the hydrophobic part and the R-COOH group as the hydrophilic part. They can be classified according to the presence of double bonds between their carbon atoms: saturated FA have no double bonds and unsaturated FA have at least one double bond (usually in *cis* conformation, although some FA have *trans* configuration). There are monounsaturated fatty acids (**MUFA**) with a single bond in the chain or polyunsaturated (**PUFA**) if they have two or more double bonds. Another way to classify FA is according to the length of the aliphatic carbon chain. They can be differenced into SCFA, MCFA and long-chain fatty acids (**LCFA**). There is controversy about the maximum length of SCFA. While some authors define the SCFA as organic FA with 1 to 4 carbons (butyric acid), others include the FA with 6 carbons (caproic acid). In the present thesis, caproic acid is considered as MCFA. About LCFA, they are clearly defined as consisting FA with a chain length of more than 12 carbons (Hanczakowska et al., 2016; Baltić et al., 2017; Khatibjoo et al., 2018).

Table 1.2 Short- and medium-chain fatty acids trivial names and chemical formulas.

Cx	Trivial name	Chemical formula
C1:0	Formic acid	HCOOH
C2:0	Acetic acid	CH3COOH
C3:0	Propionic acid	CH3CH2COOH
C4:0	Butyric acid	CH3CH2CH2COOH
C5:0	Valeric acid	CH3CH2CH2CH2COOH
C6:0	Caproic acid	CH3CH2CH2CH2CH2COOH
C7:0	Enanthic acid	CH3CH2CH2CH2CH2CH2COOH
C8:0	Caprylic acid	CH3CH2CH2CH2CH2CH2CH2COOH
C9:0	Pelargonic acid	CH3CH2CH2CH2CH2CH2CH2CH2COOH
C10:0	Capric acid	CH3CH2CH2CH2CH2CH2CH2CH2CH2COOH
C11:0	Undecyclic acid	CH3CH2CH2CH2CH2CH2CH2CH2CH2CH2COOH
C12:0	Lauric acid	CH3CH2CH2CH2CH2CH2CH2CH2CH2CH2CH2COOH

Regarding on physical properties, SCFA and MCFA have unpleasant odor and are potentially volatiles (Ahsan et al., 2016; Wu et al., 2018). They have relatively high solubility in water due to the hydrophilic -COOH group, and their melting points increase with chain length being at -5.3°C with butyric acid (C4:0) and 44.8°C with lauric acid (C12:0). Therefore, at room temperature, SCFA are normally liquid meanwhile MCFA have a buttery presentation (Gunstone, 2008).

About chemical properties, it has been highlight the pK_a value, a logarithmic measure of the acid dissociation constant. Knowing that $HA \rightleftharpoons A^- + H^+$, the acid dissociation constant is:

$Ka = \frac{[A^-]\times[H^+]}{[AH]}$, that it is usually presented in logarithmic units $pKa = -log_{10}Ka$, where A^- is the dissociated form of the acid as an ion, and H^+ is the proton delivered from the non-dissociated acid (AH).

Hence, according to the Henderson-Hasselbach equation, $pH = pKa + log_{10}\left(\frac{A^-}{AH}\right)$, each FA has a specific pK_a value that results in the pH level of the environment where there is an equilibrium in the reaction between the non-dissociated and dissociated form of the FA (50:50):

$$pH = pKa + log_{10}\left(\frac{A^-}{AH}\right) \rightarrow pH = pKa + log_{10}\left(\frac{50}{50}\right) \rightarrow pH = pKa + log_{10}(1) \rightarrow$$

$$pH = pKa + 0 \rightarrow pH = pKa$$

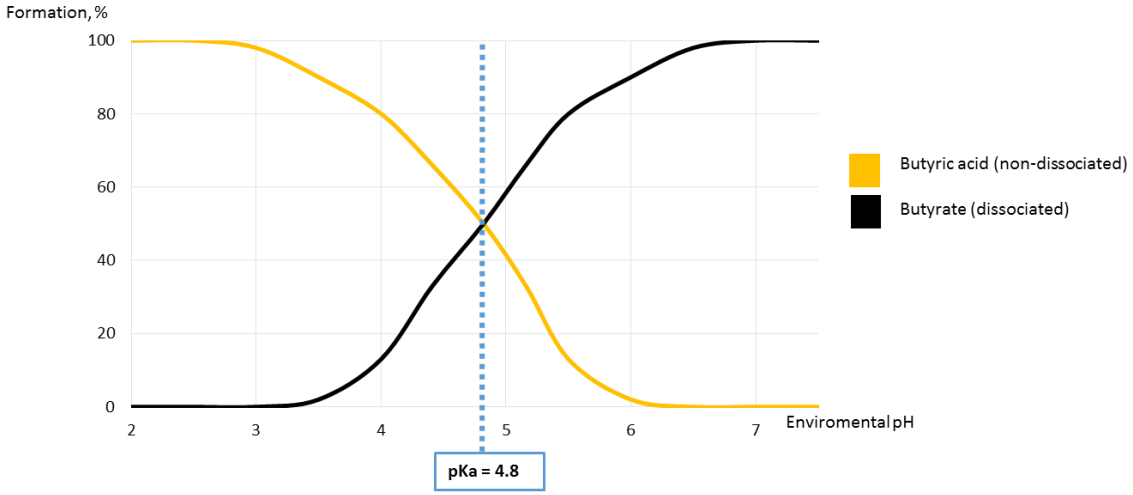

Figure 1.3 Effect of environmental pH on dissociation of butyric acid (pKa = 4.82). Adapted from Ahsan et al. (2016).

Therefore, a change in the environment pH level shifts this equilibrium reaction between the dissociated and non-dissociated form of the acid as it is represented in the **Figure 1.3** with the butyric acid as example: a pH lower than the pK_a (4.8) shifts the equilibrium towards the non-dissociated butyric acid, whereas the increasing pH shifts the equilibrium towards the dissociated butyrate ions. The SCFA and MCFA are weakly acid with pK_a values around 4.8 as it summarized in **Table 1.3** (Bach and Babayan, 1982; Zentek, 2011).

Table 1.3 pKa values of the most used short- and medium-chain fatty acids (Kanicky, 1999; Nguyen 2020; Gomez-Osorio et al., 2021).

Fatty acid	pK_a value
C1:0	3.75
C2:0	4.76
C3:0	4.88
C4:0	4.82
C5:0	4.82
C6:0	4.83
C8:0	4.89
C10:0	4.89-4.90
C12:0	5.13-5.30

1.2.2 Sources

The SCFA are mainly originated from the intestinal bacterial fermentation of dietary carbohydrates (Bergman, 1990; Pryde et al., 2002; Leeson et al., 2005). In chicks, the SCFA production is described in the distal small intestine and cecum, meanwhile in pigs, as the rest of mammals, SCFA fermentation are highly detected in colon. So, the total SCFA concentration is lower in the upper tract of the intestine and gradually increases in the lower part. Besides that, it has been described that dietary supplementation with probiotics, also called direct-fed microbials, may be a strategy to produce large amounts of SCFA in the gut of the animals. In this sense, Yang et al. (2012) evaluated *Clostridium butyricum*, a butyric acid bacterium. Moreover, the SCFA are natural substances also present in the milk of most mammals, except human and sow's milk with few traces of butyric acid (Guilloteau et al., 2010; Stinson et al., 2020; Vieira et al., 2020).

Table 1.4 Fatty acid composition of coconut oil and palm kernel oil (Adapted from Gervajio (2005) and Gibon (2012)).

Fatty acid profile, %	Coconut oil	Palm kernel oil
Saturated fatty acids		
C6:0	0.2-0.8	0-1
C8:0	6-9	3-5
C10:0	6-10	3-5
C12:0	46-50	44-51
C14:0	17-19	15-17
C16:0	8-10	7-10
C18:0	2-3	2-3
Monounsaturated fatty acids		
C18:1n9c	5-7	12-19
Polyunsaturated fatty acids		
C18:2n6c	1-2.5	1-2

Regarding MCFA, they are also found as triglycerides in milk lipids of various animals, although their concentration differs between species: the milk of mouse, rat, rabbit, goat, horse, and elephant contain high concentrations of MCFA, meanwhile in cow, sheep and human milk small amounts are detected. Few traces can be found in the milk of the sow, the camel and the guinea pig (Witter and Rook, 1970; Decuypere and Dierick, 2003). Also vegetable oils such as oils from certain species of *Cuphea* (*Lythraceae*), coconut and palm kernel are sources of MCFA (Zentek et al., 2011). High content of MCFA was found in *Cuphea* genus from the *Loosestrife* family, with notable diversity in composition between species (Graham et al., 1981). In fact, the supplementation of piglet diets with *Cuphea* seeds as a source of MCFA was related with

improved growth performance and gut histomorphometry (Dierick et al., 2003). Nevertheless, the two most common sources of MCFA are coconut and palm kernel oil. **Table 1.4** shows the FA composition of coconut oil and palm kernel oil. Most FA are saturated FA, and among them, lauric acid (C12:0) is the most prominent. Therefore, these oils (coconut and palm kernel oils) are also so-called lauric oils (Petrauskaitè et al., 2000; Gervajio, 2005).

Focusing on coconut and palm kernel oils, they must to be refined to become acceptable for human consumption (Erickson, 1995; Gibon, 2012). There are two main refining process methods: chemical refining and physical refining, the latter being the most commonly used for lauric oils, and consists in the following steps: degumming, dewaxing, bleaching, and deodorization, to obtain refined oil. (Petrauskaité et al., 2000; Gibon, 2012). As the aim of deodorization is to reduce the levels of free FA and to remover odors and other volatile components, this step generated FA distillates as by products from the oil refining process (Čmolík and Pokorny, 2000). These FA distillates are characterized to have high levels of free FA (> 90%) with the same composition of the original oil (Gervaijo, 2005; Nuchi et al., 2009). Therefore, their use in animal nutrition promotes the circular economy revalorizing by-products and reducing the residuals and the associated environmental impacts of the refining oil industry.

1.2.3 Digestion and absorption of short- and medium-chain fatty acids

In the present section, the processes of digestion and absorption of SCFA and MCFA are exposed to understand why new forms of presentation of butyric acid and MCFA as feed additives are under research. For this purpose, a general overview of fat digestion and absorption is described first, trying to mention the particularities for broiler chickens and pigs.

Since fat is insoluble in water and an aqueous medium exists in the gastrointestinal tract, emulsification of large fat globules is required prior to hydrolysis. Fat emulsification starts in the gizzard or the stomach (of broiler chickens or pigs, respectively), where lipid globules are broken into droplets by gastric motility and acidity. Moreover, bile acids synthetized in the liver play a key role in the emulsification process, reducing the tension at the oil-water interphase due to their detergent-like properties. However, it has been described that hydrolysis of SCFA and MCFA glycerides may occur without previous emulsification due to their higher water solubility in contrast to LCFA (Greenberger et al., 1966; Caliari et al., 1996).

In poultry, although pancreatic lipases may be present in the gizzard due to anti-peristaltic movements, no lipolytic action has been observed there. Therefore, hydrolysis is characterized by starting in the small intestine (Moreau et al., 1988; Leeson and Summers, 2001). In pigs' case,

the enzymatic digestion of fat is previously favored by the partial lipolysis due to gastric lipase activity (Krogdahl, 1985; Bauer et al., 2005).

As most fat enters in the duodenum in triglycerides forms, pancreatic lipase hydrolyzes the primary (sn-1 and sn-3) ester bonds of triglycerides to sn-2 monoacilglycerol and the corresponding free FA in the duodenum (Mattson and Beck, 1956). The enzyme activity of lipase is influenced by the presence of other active compounds (Wealleans et al., 2021). On one hand, colipase, which is secreted by the pancreas and consists of amino acids hydrophobics and hydrophilics, protects lipase from denaturation and acts as an anchor to reach the inner core of the triglycerides (Borgström et al., 1979; Delorme et al., 2011). In the absence of colipase, bile salts and other surface-active compounds compete with lipase for available surface area at the interface, reducing the efficacy of hydrolysis (Borgström, 1975). The accumulation of monoglycerides and free FA also reaches to inhibit lipase activity (Reis et al., 2009). In addition, the efficiency of lipases gradually increases according to the polarity of the glycerides. In general, lipases break efficiently ester bonds from triglycerides with chains of 16 carbons or more. Hence, pancreatic lipase is inefficient to separate butyrate (4 carbons long) from the glycerol (3 carbons long) in the case of monobutyrin due to its low polarity (Mallo et al., 2012). Besides that, the absence of hydrolysis in the absorption of medium-chain triglycerides has been also described (Guillot et al., 1993; Zentek et al., 2011).

Therefore, the free FA and monoglycerides cross the intestinal epithelium and reach different body tissues to be metabolized (Wang et al., 2013). In general, the hydrolysis products are converted into more polar derivatives with higher aqueous solubility by incorporation into bile-salt micelles (Bauer et al., 2005). The formation of these micellar structures occurs in close collaboration with the colipase-lipase complex at the droplet interphase (Wealleans et al., 2021). Early studies described that monoglycerides, MCFA, and unsaturated LCFA were the first compounds incorporated into bile-salt micelles due to their amphiphilic properties. Then, the entry of more water-insoluble compounds as saturated LCFA and esters can occur (Krogdahl, 1985). More recently, other authors explained that SCFA and MCFA can also be solubilized as individual components in the gut lumen (Caliari et al., 1996). So, SCFA, MCFA and micelles are then transported towards the enterocytes of the small intestine to be absorbed (Wealleans et al., 2021). The major intestinal site of fatty acid absorption in broilers and pigs is the jejunum, although the ileum also plays an important role (Freeman, 1984; Rodriguez-Sanchez et al., 2018). Concerning the duodenum, and according to the literature, FA absorption in this anterior segment of the intestinal tract is expected. However, previous authors suggested that FA digestibility coefficients observed in anterior parts of the intestine in broilers might be related

to the antiperistaltic refluxes (Hurwithz et al., 1973; Krogdahl, 1985; Rodriguez-Sanchez et al., 2018).

To date, knowledge of lipid absorption is not fully understood. However, it is known that it is influenced by the length of the carbon chain. The absorption process is more efficient with SCFA and MCFA, instead of LCFA (Decker, 1996, Van den Borne et al., 2015). The SCFA and MCFA can be absorbed by simple diffusion across the enterocyte membrane, although LCFA requires and active, protein-mediated process to be absorbed (Wealleans et al., 2021). In fact, Ockner and Manning (1974) reported that FA binding protein has higher affinity for long-chain unsaturated fatty acid than for saturated ones. Regarding SCFA and MCFA, lower affinity it has been described. Therefore, although more details are required to understand and distinguish the absorption of SCFA and MCFA, it is known that SCFA are absorbed with higher efficiency than the other FA.

Therefore, although the exact digestion and absorption processes of SCFA and MCFA are a room to elucidate, it seems clear that both processes determine the amount of free FA present throughout the gastrointestinal tract that may impact on the gut barrier of the animals. Therefore, different strategies to increase the presence of SCFA and MCFA throughout the intestine are going to be presented in the following section.

1.2.4 Strategies to increase the efficacy of short- and medium-chain fatty acids to impact on the gut barrier

Many feed additives based on SCFA and MCFA are commercially available with the aim to impact on the gut barrier of broilers and pigs. Among them, there are different presentation forms, besides the use of mixtures and combinations of various SCFA, MCFA or other types of functional additives under research. The main purpose of these strategies is to avoid limiting physical and chemical properties of free FA to promote their potential maintaining and improving gut health. Some of these different strategies are presented below.

1.2.4.1 Derivatives with other chemical forms

Free FA can chemically react with other molecules, resulting in derivative forms with new properties. Salts forms are the chemical derivative presentation that contain a metallic atom, usually sodium, or calcium, instead of the hydrogen of –OH group of the acid (EFSA, 2018). When FA are transformed into a salt (**Figure 1.4**), the metallic cation must have high solubility to bind to the anion that proceed from the FA without the proton (H+).

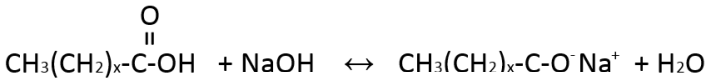

Figure 1.4 General reaction from fatty acid to sodium salt of fatty.

The salts of FA are generally odorless and less corrosive than free FA. In fact, salts can be easier to handle in the feed manufacturing process because of their solid and less volatile form. Besides that, they may be more soluble in water. Among the different FA salts, sodium and potassium ones are more soluble than calcium salt in water and ethanol (Ettle et al., 2004; Huyghebaert et al., 2011; Ahsan et al., 2016; Kaczmarek et al., 2016; EFSA, 2018). Therefore, it seems that the salt forms commercially produced increase palatability and bioavailability in the intestine of birds and pigs (Manzanilla et al., 2009; Melaku et al., 2021).

The FA salts are converted into free FA after the ingestion and remains in non-dissociated form in the gizzard or stomach due to the acidic pH (bird or pig, respectively). Indeed, some studies showed that the presence of FA salts is restricted to the upper gastrointestinal tract (Bolton and Deward, 1965; Manzanilla et al., 2006; Ahsan et al., 2016). Nonetheless, other authors observed that the use of salt forms affected the gut barrier in more distal section of the intestine, in contrast to the use free FA (Ettle et al., 2004; Raga and Korany, 2016).

Another way to supplement the diet with SCFA and MCFA is using glycerides: a glycerol esterified with free FA (Weete, 1974). **Figure 1.5** shows a schematic overview of the esterification reaction to obtain a triglyceride. The hydrogen atom of the FA from -OH group reacts with the hydroxyl group of the glycerol molecule, resulting in the formation of an ester (covalent) bond between the oxygen atom of the FA and the carbon atom of the glycerol molecule, as well as a water molecule. The product of the esterification reaction depends on the number of free FA molecules included, resulting in a monoglyceride, diglyceride or triglyceride plus 1, 2 or 3 molecules of waters, respectively. Among monoglycerides, there are α-monoglycerides with a FA linked to the sn1-position of glycerol via an ester bond. These are the only monoglycerides with antimicrobial effects (Gomez-Osorio et al., 2021).

Glycerides of FA have the advantage that neither have the stringent smell associated with free FA (Namkung et al., 2011). Particularly, Leeson et al. (2005) associated a mild buttery type odor to butyrate glycerides that mask the rancid odor of free butyric acid. On the hand, Zentek et al. (2011) shown that piglets accept higher level of medium-chain triglycerides dietary inclusion in comparison with free MCFA due to their negative sensory properties.

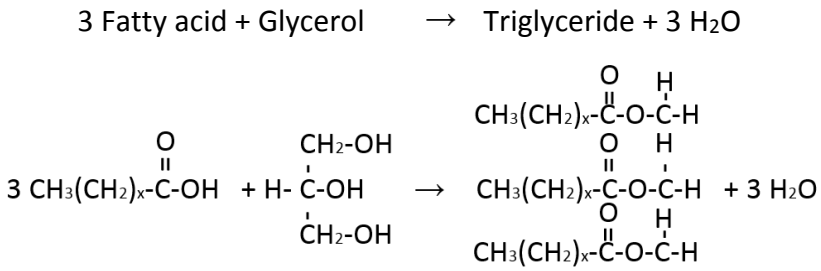

Figure 1.5 General reaction of esterification from three fatty acids reacting with glycerol to form triglyceride plus three molecules of water.

The ester bond between the acid and the glycerol molecule makes them active regardless of pH. As mentioned in the previous section "2.3 Digestion and absorption of short- and medium-chain fatty acids", lipases are required to obtain free FA from glycerides. Therefore, in this context, free FA from glycerides can impact on the gut barrier in posterior parts after the hydrolysis process. In any case, different studies comparing the efficacy of glycerides forms and their respective free FA, showed that the first ones were more efficient impacting in the gut barrier throughout the intestinal tract and, in addition, in the performance parameters (Dierick et al., 2002; Leeson et al., 2005). Also, Makowski et al. (2022) observed that dietary supplementation with glycerides forms improved feed efficiency due to an increased intestinal absorptive surface area as well as reach lower parts of intestinal tract to exert a stronger antibacterial effect than sodium butyrate. The reason why glycerides have this greater effect may be due to the fact that the non-dissociated form of the FA lasts longer in this form of presentation (Warnecke et al., 2005).

1.2.4.2 Protected forms

In turn, free FA, as well as their respective salt forms, can be protected to improve their bioavailability and increase the intestinal region exposed to the acid molecule (Smith et al., 2012; Mallo et al., 2012; Liu et al., 2017a). Therefore, microencapsulation technology has been used in the food and pharmaceutical industries since 1954. There are many reasons that could motivate the protection of the FA and their salts (do Amaral et al., 2019; Nguyen et al., 2020). In general terms:

1. First, the cover reduces the reactivity of FA, the core that is usually quite susceptible to external conditions. In this regard, the advantage of encapsulated acids is that they are generally less corrosive improving their handling in the feed manufacturing process. On the other hand, they are also less irritable for the gut (Desai and Jin Park, 2005; Nedovic et al., 2011).

2. Encapsulation is also used to conceal the flavor of active ingredients (Putnam and Garrett, 2005). In animal nutrition, the protection of organic acids counteracts their unpleasant odors such as rancid odor of butyric acid or intense goat-like odor of caprylic and capric acids (Decuypere and Dierick, 2003; Mroz et al., 2006; van der Aar et al., 2017; Nguyen et al., 2020). In this line, encapsulation should allow minimizing the risk of possible negative impact on feed intake in pigs that have low tolerance to taste changes (Decuypere and Dierick, 2003; Zentek et al., 2011; Li et al., 2015). This advantage is not so much attributed to chickens, as their feed acceptance is mostly determined by particle size (FEDNA, 2018).

3. The protection of FA can slow down the release of FA (mainly SCFA) in the gut tract. In addition, the core material release may be more controlled to achieve the proper delay in the gastrointestinal environment. Recently, it has been noted that SCFA microencapsulated in a lipid shell have effects throughout the gastrointestinal tract because they are slowly released during the digestion, bypassing the low pH in the stomach and the enzymatic activity in the duodenum (Putnam and Garrett, 2005; Fernández-Rubio et al., 2009; Liu et al., 2017a). Consequently, FA can reach more distal sections of the intestine in appreciable and effective amounts to promote intestinal integrity contrary to free salt forms, as sodium butyrate, that mainly affects upper gut tract (Van Immerseel et al., 2004; Piva et al., 2007; Panda et al., 2009; Makowski et al., 2022).

Regarding the protecting material, it must be easy to handle during the encapsulation process, not react with the core material while maintaining a correct level of the active core, and control the releasing time (Temiz and Öztürk, 2018). Furthermore, it should be noted that regulations in animal nutrition are very restrictive. In these context, substances such as hydrocarbohydrates, proteins, and lipids can be used to protect active ingredients. Nonetheless, most of the organic acids are protected by lipid-compounds. They provide better resistance to the acidic pH, which can be targeted to the small intestine where they are degraded by pancreatic lipases and then release the active compound (Piva et al., 2007; Nedovic et al., 2011; Khanvilkar et al., 2016). In fact, vegetable fats are the usual method to protect butyrate salts (Van den Borne et al., 2015; Liu et al., 2017a). Some authors described the efficiency of sodium butyrate partially protected by vegetables fat throughout the gastrointestinal tract because it was slowly released during digestion. In addition, it has been observed that protected forms were more effective than free sodium butyrate in reducing Salmonella shedding in broilers challenged with *Salmonella* e*nteritidis*-infected broilers (Fernández-Rubio et al., 2009; Mallo et al., 2012).

About the protection methods, there are many available techniques to protect active agents such as SCFA and MCFA. The first step in any protection process consists of mixing the active material with the encapsulating material. The mixture is then dried, producing microcapsules of different sizes and forms depending on the method and materials used. Encapsulation techniques are divided in three groups: physical, chemical, or physicochemical processes, as it shown in **Table 1.5** (do Amaral et al., 2019).

Table 1.5 Some microencapsulation techniques according to their nature process.

	Encapsulation techniques
Physical methods	Spray-drying, spray-chilling, spray-coating, air suspension, fluidized blend, extrusion, centrifugation, co-crystallization, lyophilization
Chemical methods	Molecular inclusion, interfacial polymerization, *in situ* polymerization
Physicochemical methods	Coacervation, liposomal wrapping, evaporation of the solvent

Finally, the active ingredients must be easily mixed in small quantities and still achieve uniform distribution. According to the final product size, the encapsulation can be expressed as nanoencapsulation (less than 0.2 µm), microencapsulation (0.2 to 5 µm) or macroencapsulation (more than 5 µm). Microencapsulation is the most common application, although terms of encapsulation and microencapsulation are often used interchangeably (Cosco, 2006). In addition, the protected product can be a single, multi-wall, multi-core or irregular matrix but many times there is not described in the literature (Vasisht, 2014; Temiz and Öztürk, 2018).

1.2.4.3 Mixtures and combinations

Mixing SCFA and MCFA, and combining them with other additives represents another strategy to improve performance and health in monogastric animals. The use of acid blends may show a synergetic effect between SCFA and MCFA (Den Hartog et al., 2005; Nguyen et al., 2020). Improved growth performance parameters and intestinal morphometry have been described with mixtures of FA, reducing clinical effects under enteric disorders. In addition, mixtures or combinations usually include FA with different pK_a values, which may be related to a broader spectrum of antimicrobial activity (explained in the section 1.3.2 Effects on the gut barrier). Indeed, previous authors observed higher efficiency using a mixture of FA than using a single FA (Del Alamo et al., 2007; Hanczakowska et al., 2011; Mathis et al. 2005; Kuang et al., 2015; Lei et al., 2017; Polycarpo et al., 2017). However, other authors described no differences in the gut barrier or growth performance by using the FA alone or mixed (Zentek et al., 2013; Khatibjoo et al., 2018).

The combination of SCFA-MCFA with other nutritional strategies as supplementation with other feed additives has been under research in many studies. Nonetheless, the reported results can be widely variable. The combination with probiotics allows a joint action, modulating the microbiota population in different segments of the gastrointestinal tract that promotes beneficial effects for the host (Bhandari et al., 2010; Rodríguez-Lecompte et al., 2012; Barbieri et al., 2015). In addition, other studies evaluated the combination of organic acids with prebiotics as mannan oligosaccharides (Pelicano et al., 2005).

Furthermore, there is evidence that dietary supplementation with a mixture of organic acids and phytogenic additives as essential oils or natural extracts improved growth performance and gut health of broiler chickens and pigs (Grilli et al., 2010; Jerzsele et al., 2012; Liu et al., 2017a; Yang et al., 2019; Zhang et al., 2020). In addition, other compounds such as enzymes have been considered with variables results, not always being successful (Lückstädt and Mellor, 2011; Smulikowska et al., 2009; Vieira et al., 2018).

Therefore, different strategies to improve SCFA-MCFA efficacy can be found in the literature. Among them, using other chemical forms such as salts or glycerides or protected forms instead of free FA seems to have a great potential to counteract some physical and chemical limitations. In addition, the combination of these FA making use of the aforementioned strategies becomes important. However, further research is required to clarify possible strategies to promote the efficacy of SCFA-MCFA as feed additives in monogastric nutrition. Hence, the efficacy of dietary supplementation of butyric acid, as SCFA, and MCFA in broiler and swine diets evaluated in previous studies will be summarized in the following section.

1.3 Use of butyric acid and medium-chain fatty acids in broiler and piglet diets

The present section aims to summarize the effects described in the literature of dietary butyric acid and MCFA on performance parameters, the gut barrier and digestibility capacity in broilers and weaned piglets. The following tables attempt to outline the significant effects found in the literature, differentiating the form of presentation of butyric acid and MCFA (free FA, salts, glycerides and protected forms, as well as mixtures of FA and combination with other feed additive). Note that studies are ordered according to the supplementation dose: in order of decreasing dose of butyric acid first and MCFA second. Also, challenges in experimental design are also reported as a factor that can determine the efficacy of the FA.

1.3.1 Effects on performance parameters

The effect of butyric acid and MCFA on performance parameters of broilers and piglets was evaluated in numerous studies due to its importance for any producer. Previous reviews considered these FA as useful dietary strategies to enhance performance in poultry and swine production (Hanczakowska et al., 2016; Polycarpo et al., 2017). Nonetheless, some works showed contradictory effects, at the same time that others found no improvement in performance parameters. It is worth to note that although there are many studies, it is difficult to compare results because of different FA and combinations, different presentations and doses, as well as different experimental conditions. **Table 1.6** and **1.7** show the significant effects of butyric acid and MCFA on growth performance of broilers and piglets, respectively.

According to the reviewed studies, it has been described that supplementation with butyric acid and/or MCFA, regardless of their form of presentation, does not affect the intake of broilers or piglets. However, some authors observed higher feed intake in broilers using free and glycerides of MCFA, or protected sodium butyrate, and in piglets using MCFA glycerides, non-protected sodium butyrate or protected MCFA (Piva et al., 2002; Lu et al., 2008; Liu et al., 2015; Sikandar et al., 2017; Baltić et al. 2018; Letlole et al., 2021; Liu et al., 2021). In all cases, it has been hypothesized that butyric acid and/or MCFA stimulate the appetite. Particularly, Lu et al. (2008) suggested that butyric can regulate gastrointestinal emptying patterns in pigs by reducing tumor necrosis factor-α (**TNF-α**) released and, consequently, increasing appetite. In addition, Nishi et al. (2005) described that MCFA have the ability to activate ghrelin, which, in turn, stimulates appetite in mammals. Note that mixing butyric acid or MCFA with other organic acids also increased feed intake in broilers and piglets, even under challenging conditions (Kuang et al., 2015; Pereira et al., 2015; Lei et al., 2017).

In contrast, other authors (Mikhail et al., 2019; Islam et al., 2018) described lower feed intake in broiler fed diets with butyric acid or MCFA as free FA and it may be because ghrelin can also act as an intake inhibitor in broilers (Kayika et al., 2007). Another possible reason for the reduced feed intake may be the unpleasant odor of free fatty acids, both butyric acid and MCFA (Zentek et al., 2011; Wu et al., 2018).

Concerning this property, and knowing that glycerides have a more pleasant aroma (butter aroma instead of rancid odor of free fatty acids), Ali et al. (2014) showed that butyrate glyceride can be used up to 2 kg/t without affecting the feed intake of broiler chickens, but higher doses such as 4 kg/t already compromised dietary intake. Nonetheless, feed odor does not condition feed acceptance in broilers (FEDNA, 2018), leading to further research. On the other hand, as

Table 1.6 Effects of dietary supplementation with butyric acid and medium-chain fatty acids on growth performance of broilers.

Reference	Feed additive	Dose, kg/t	Age, d	Challenge	Treatment effects (Compared to Control)		
					FI	BWG	FCR
		Free fatty acids					
Kamal and Ragaa (2014)	butyric acid	30	0-42	No	NS	↑	↓
Mikhail et al. (2019)	butyric acid	5, 10	7-42	No	↓	↑	↓
Raza et al. (2017)	butyric acid	3, 4	0-42	No	NS	↑	NS
Cave (1982)	caprylic, capric, lauric acids	30	0-56	No	↓	↓	NS
Khosravinia (2015)	MCFA including caproic, caprylic, capric, lauric acids	2	0-49	No	NS	↑	NS
Islam et al. (2018)	MCFA	2/1.5/1 0-10 d/ 11-20 d/ 21-28 d	0-28	No	↓	↑	↓
Baltić et al. (2018)	MCFA including caproic, caprylic, capric, lauric acids	1.6/1.2/1 0-10 d/11-21 d/22-42 d	0-42	No	↑	↑	↓
Chotikatum et al. (2009)	MCFA	1,1000 L/ 1:2,000 L 0-35 d/ 36-45 d	0-45	*Salmonella Enteritidis*	NS	↑	NS
		Salts of fatty acids					
Qaisrani et al. (2015)	sodium butyrate	2	0-35	No	NS	↑	↓
Hu and Guo (2007)	sodium butyrate	0.5, 2	0-42	No	NS	↑	NS
Zou et al. (2010)	sodium butyrate	0.2	0-35	No	NS	NS	↓
		Glycerides of fatty acids					
Antongiovanni et al. (2007)	butyric acid glycerides	2, 3.5, 5, 10	0-35	No	NS	↑	↓
Ali et al. (2014)	butyric acid glycerides	4	0-42	*Eimeria maxima*	↓	↑	↓
Yin et al. (2016)	butyric acid glycerides	3	8-20	No	NS	↑	↓
Van Gerwe et al. (2010)	caprylic and capric triglycerides	10	0- 19	*Campylobacter jejuni*	NS	↑	NS
Lin et al. (2021)	butyric, caprylic and capric monoglycerides	1/1, 1/0.65, 1/-, 3/1 0-21/ 21-42 d	0-42	No	NS	↑	NS
Liu et al. (2021)	capric and lauric monoglycerides	0.3	29-56	No	↑	↑	NS
		Protected forms					
Mátis et al. (2019)	protected sodium butyrate	3	0-42	No	NS	↑	NS
Lan et al. (2020)	protected sodium butyrate (54% sodium butyrate)	1.2	0-21	No	NS	↓	↑
Zhao et al. (2022)	protected sodium butyrate	1	0-42	No	NS	↑	↓
Mallo et al. (2021)	protected sodium butyrate (70% sodium butyrate)	1	0-42	No	NS	↑	↓
Smulikowska et al. (2009)	protected sodium butyrate (30% sodium butyrate)	1	8-21	No	NS	NS	↓

Table 1.6 *Cont*.

Protected forms							
Letlole et al. (2021)	protected sodium butyrate (25% sodium butyrate)	1/0.75/0.25 0-14 d/ 15-28 d/ 29-35 d	0-35	No	↑	↑	NS
Edmonds et al. (2014)	protected sodium butyrate	0.9/0.45 0-14 d/ 15-46 d	0-41	No	NS	↓	↑
Edmonds et al. (2014)	protected sodium butyrate	0.9/0.45 0-14 d/ 15-46 d	0-46	No	↑	↑	↓
Song et al. (2017)	protected sodium butyrate (30% sodium butyrate)	0.8	0-35	*Eimeria, Clostridium perfringens*	NS	↑	↓
Riboty et al. (2016)	partially protected sodium butyrate (30% protected)	0.7	0-42	No	NS	↑	↓
Chamba et al. (2014)	partially protected sodium butyrate (30% protected)	0.7	0-42	No	NS	↑	↓
Lan et al. (2020)	protected sodium butyrate (54% sodium butyrate)	0.6	0-21	No	NS	↑	↓
Liu et al. (2017a)	protected sodium butyrate	0.5, 1	0-11	*Salmonell Typhimurium*	NS	↑	↓
Sikandar et al. (2017)	protected sodium butyrate (30% sodium butyrate)	0.5, 1	0-42	No	↑	↑	↓
Lan et al. (2020)	protected sodium butyrate (54% sodium butyrate)	0.3, 0.6, 1.2	0-35	Hot climatic conditions	↑	↑	NS
Imran et al. (2018)	protected calcium butyrate (50% calcium butyrate)	0.25, 0.35, 0.45	0-35	No	NS	↑	↓
Zou et al. (2010)	protected sodium butyrate	0.2	0-35	No	NS	NS	↓
Levy et al. (2015)	protected calcium butyrate (50% calcium butyrate)	0.1, 0.2, 0.3, 0.4, 0.5	0-42	No	NS	↑	↓
Levy et al. (2015)	protected calcium butyrate (50% calcium butyrate)	0.1, 0.2, 0.3	0-42	No	NS	↑	↓
Mixtures and combinations							
Abdullahi et al. (2020)	calcium butyrate + fumaric + benzoic acids	10, 20	0-22	*Eimeria tenella*	NS	↑	NS
Pereira et al. (2015)	butyric + lactic + acetic acids	8/6/4 1-21 d/ 22-35 d/ 36-43 d	0-42	*Clostridium perfringens*	↑	↑	NS
Khatibjoo et al. (2018)	butyric acid + caprylic and capric triglycerides	4	0-12	No	NS	NS	↑
Jerzsele et al. (2012)	protected sodium butyrate + essential oils	1.5	15-25	*Clostridium perfringens*	NS	↑	NS

MCFA = medium-chain fatty acid; FI = feed intake; BWG = body weight gain; FCR: feed conversion ratio; NS = not significant.

Table 1.7 Effects of dietary supplementation with butyric acid and medium-chain fatty acids on growth performance of piglets.

Reference	Feed additive	Dose, kg/t	Weaning day, kg	Age, d PW	Challenge	Treatment effects (Compared to Control)		
						FI	BWG	G:F
		Free fatty acids						
Dierick et al. (2002)	MCFA	50	ND, 5.8 kg	0-28	No	NS	↑	NS
Hanczakowska et al. (2016)	capric acid	3	28 d, ND	0-70	No	NS	↑	NS
Hanczakowska et al. (2011)	caprylic and capric acids	2	35 d, 8.6 kg	0-84	No	NS	↑	↑
		Salts of fatty acids						
Hanczakowska et al. (2014)	sodium butyrate	3	35 d, 7.6 kg	0-84	No	NS	↑	NS
Feng et al. (2018)	sodium butyrate	2	21 d, ND	0-7	No	NS	↑	↑
Chiofalo et al. (2014)	sodium butyrate	1.5	21 d, 5.7 kg	0-45	No	NS	NS	↑
Lu et al. (2008)	sodium butyrate	1	21 d, 6.7 kg	0- 30	No	↑	↑	↑
Piva et al. (2002)	sodium butyrate	0.8	28 d, 9.2 kg	14-70	No	↑	NS	↓
		Glycerides of fatty acids						
Sotira et al. (2020)	butyric acid triglycerides	2	28 d, 8.7 kg	7-47	No	NS	↑	↑
Li et al. (2015)	MCFA triglycerides	7, 14, 21	21 d, 7.5 kg	0-28	No	↑	↑	↑
Hong et al. (2012)	caprylic and capric triglycerides	5.5/3.2 0-7 d/ 8-14 d	21 d, 6.9 kg	0-14	No	NS	↑	NS
Hong et al. (2012)	caprylic and capric triglycerides	5.5/3.2 0-7 d/ 8-14 d	28 d, 10.2 kg	0-35	No	NS	↑	↑
		Protected forms						
Wang et al. (2017)	protected sodium butyrate (30% sodium butyrate)	1.5	28 d, 8.5 kg	0-14	No	NS	↑	NS
Chiofalo et al. (2014)	protected sodium butyrate (30% butyric acid)	1.5	21 d, 5.7 kg	0-45	No	NS	↑	↑
Upadhaya et al. (2018)	protected organic acids (1.2% caprylic and capric)	1, 2	28 d, 6.5 kg	0-42	No	NS	↑	NS
Huang et al. (2015)	protected sodium butyrate	1	28 d, 10.2 kg	0-28	No	NS	↑	↑
Upadhaya et al. (2020)	protected sodium butyrate (40% sodium butyrate)	0.5, 1.5/0.75, 3/1.5 0-21 d/ 22-42 d	28 d, 7.0 kg	0-42	No	NS	↑	↑
Lin et al. (2020)	protected sodium butyrate (30% sodium butyrate)	0.3, 0.45	28 d, 8.5 kg	0-14	No	NS	↑	NS
Han et al. (2011)	protected MCFA	1	24 d, 6.0 kg	0- 28	No	↑	↑	NS

Table 1.7 *Cont*.

Mixtures and combinations								
Kuang et al. (2015)	capric, lauric acids + calcium formate, calcium lactate, citric acid, myristic acid	3	21 d, 6.4 kg	7-28	No	↑	↑	↑
Lei et al. (2017)	caprylic, capric acids + fumaric, citric malic acids	2, 4	28 d, 6.2 kg	0-21	ETEC	↑	↑	↑
Long et al. (2018)	mixture MCFA, protected butyric acid, formic, acetic, propionic, citric acids, phenolic compounds	2, 3	ND, 8.6 kg	0-28	No	NS	NS	↑
Devi and Kim (2014)	protected caprylic, capric acids (58%) + *Enteroccus faecium*	2 + 0.1	ND	0-42	No	NS	↑	↑
Hanckzakowska et al. (2013)	caprylic, capric acids + fumaric, propionic acids	2 + 5	35 d, 7.9 kg	7-84[1]	No	NS	↑	↑
Wei et al. (2021)	sodium butyrate + benzoic acid	0.35, 0.7, 1.05 + 5	21 d, 6.9 kg	0-35	No	NS	↑	↑
Wei et al. (2021)	sodium butyrate + benzoic acid	0.35 + 5	21 d, 4.7 kg	0-40	No	↑	NS	NS

[1]d of age; MCFA = medium-chain fatty acid; PW = post-weaning; FI = feed intake; BWG = body weight gain; G:F = gain-to-feed ratio; NS: not significant; ND: not determined; ETEC = enterotoxigenic *Escherichia coli*.

mentioned in the previous section, different authors indicated that the protection of FA aims to overcome any negative effect on feed intake (Han et al., 2011; Li et al., 2015). This fact is well documented in **Table 1.6** where protected forms did not decrease broiler intake in any study, while free FA and glycerides did.

For piglets, no significant reductions in feed intake by any form of these FA have been observed in the literature consulted, although MCFA can stimulate the release of cholecystokinin and insulin that acts as satiating factors (Ooyama et al., 2009; Han et al., 2011; Hejdysz et al., 2012; Figueroa et al., 2019). Therefore, according to Lu et al. (2008), it seems that the mechanism of action of butyric acid and MCFA on feed intake required further research in monogastric nutrition.

In terms of weight gain, in overall, animals fed diets with butyric acid and/or MCFA had higher growth. In fact, this improvement in weight gain has been observed using each of the different forms of presentation (free FA, salts and glycerides, as well as protected forms) of butyric acid and MCFA in broilers and weaned piglets under healthy and even challenging conditions. Concerning the doses, it appears that high doses (up to 30 or 50 kg/t) of free butyric acid or MCFA have the same growth promoting effect that lower doses such as 2 kg/t (Dierick et al., 2002; Hanczakowska et al., 2011; Kamal and Ragaa, 2014; Khosravinia, 2015). However, it should be noted that the protected forms, in general, improve the growth of broilers and piglets using the lowest doses, even under challenging conditions (Liu et al., 2017a; Sikandar et al., 2017; Lin et al., 2020; Upadhaya et al., 2020). Finally, it is observed that the combination of butyric acid or MCFA with other organic acids also often improves growth performance in poultry and swine production (Hanzakowska et al., 2013; Pereira et al., 2015; Lei et al., 2017).

This enhancement in weight gain usually results into improved feed efficiency (generally referred to as feed conversion ratio (**FCR**) for broilers, and gain-to-feed ratio (**G:F**) for piglets) and is often related to the effect of these acids on the intestinal barrier and its functionality (Abdelli et al., 2021). In addition, some authors observed that mixing butyric acid or MCFA with other organic acids or combining them with other functional feed additives, such as probiotics and essential oils, can synergistically improve the gut health of broilers and piglets, and consequently, increase performance (Jerzsele et al., 2012; Devi and Kim, 2014; Lei et al., 2017; Wei et al., 2021).

Furthermore, it has been described that the aforementioned activation of ghrelin by MCFA stimulates the release of growth hormone resulting in higher BW at the same time as it regulates fat mass deposition (Nishi et al., 2005; Heppner et al., 2012). Indeed, this fat deposition-reducing

potential of MCFA is gaining interest in animal production, a scenario in which animals are being genetically selected to reduce their fat content. Therefore, some studies evaluated the effect of MCFA on fat mass deposition of broilers and pigs. Furthermore, the effect of butyric acid on fat deposition has also been assessed. Some authors observed the ability of butyric acid as well as MCFA to reduce body fat deposition of broilers and piglets by regulating gene expression, which is involved in reducing synthesis, storage and transport, and enhancing lipid and FA oxidation (Iyer et al., 2012; Yin et al., 2016; Jiao et al., 2020). In particular, reduced abdominal fat deposition was described supplementing bird diets with free butyric acid at 2 to 6 kg/t or MCFA glycerides at 1 to 4 kg/t (Panda et al., 2009; Shokrollahi et al., 2014; Saeidi et al., 2016). For piglets, Jiao et al. (2020) observed that weaned piglet receiving an oral infusion of SCFA (including butyric acid) had lower fat deposition. Nonetheless, few authors have evaluated the effect of dietary supplementation with butyric acid and/or MCFA on fat deposition. Therefore, further research is required to be able to stablish relationship between the effect of these FA (butyric acid and MCFA) on gut health and fat deposition in broilers chickens and pigs.

1.3.2 Effects on the gut barrier

As mentioned above, animal performance is closely related to the intestinal health, which is determined by a complex interaction involving the integrity of the intestinal epithelium and microbiota. Therefore, many works have tested the effect of butyric acid and/or MCFA on the gut barrier. **Table 1.8** and **1.9** show the significant effects of butyric acid and MCFA on histomorphological parameters, while **Table 1.10** and **1.11** summarize the impact on the intestinal microbiota in broilers and piglets, respectively.

1.3.2.1 Effects on the intestinal epithelium

Concerning the intestinal epithelium, the effect of butyric acid or MCFA on its architecture (assessing villus height and crypt depth) has been widely studied. In general, the use of these FA increased villus height in broiler chickens and weaned piglets. Note that free FA increased duodenal villus (Panda et al., 2009; Adil et al., 2010; Baltić et al., 2018), while their chemical derivatives (salts and glycerides) reach to impact villi of more distal intestinal regions such as the jejunum and ileum (Lu et al., 2008; Tonel et al., 2010; Wen et al., 2012; De Keyser et al., 2019; Amer et al., 2021). Furthermore, protected forms seems to effect villus height throughout all the small intestine of broilers and piglets. This fact may be due to the slow release of butyric acid from the protection in the feed additive (Chiofalo et al., 2014; Liu et al., 2017b; Upadhaya et al., 2020; Letlole et al., 2021).

Anyway, the increase in villus height has been related to the stimulation of epithelial cell proliferation by butyric acid and MCFA (Kien et al., 2007; Le Gall et al., 2009, Lacorn et al., 2010). In fact, it is well documented that SCFA, especially butyric acid, can serve as an important energy source for intestinal epithelial cells (Isolauri et al., 2004; Pan and Yu, 2014). Besides that, MCFA was also considered as another direct source of energy for enterocytes (Zentek et al., 2011). Furthermore, other authors justified this improvement with the reduced competence for nutrients due to antimicrobial effect of these FA. Thus, it has also been reported that the use of FA mixtures and the combination with others products as essential oils, oregano or methyl salicylate increased the villi height throughout the small intestine in broilers and piglets, probably due to a potential synergistic effect on the epithelium and the gut microbiota (Jerzsele et al., 2012; Pereira et al., 2015; Zhang et al., 2020; Letlole et al., 2021).

In the case of crypt depth, more heterogeneous results have been observed. Some authors showed increased crypt depth in different sections of the small intestine in broilers receiving all the above-mentioned presentations of butyric acid (free FA, salt, glyceride and protected form) (Antongiovani et al., 2007; Panda et al., 2009; Czerwiński et al., 2012; Qaisrini et al., 2015). In piglets, deeper crypts were described using sodium and glycerides butyrate, as well as protected sodium butyrate (Manzanilla et al., 2006; Chiofalo et al., 2014; De Keyser et al., 2019). Note that supplementation with protected forms increased crypt depth at lower doses that non-protected forms. Additionally, mixture of capric acid with fumaric and propionic acids also increased crypt depth (Hanczakowska et al., 2013). Therefore, deeper crypts, considered a sign of higher cell turn-over (Choct, 2009), were observed throughout the intestine: the use of free FA, salts or glycerides acids increased the crypt depth in more proximal segments of the small intestine as the duodenum or jejunum, while the use of protected acids or mixtures reached the ileum.

On the other hand, a reduction in crypt depth of broiler chickens and weaned piglets receiving these FA has been also described. Supplementation with lauric acid glycerides (Letlole et al., 2021), protected sodium butyrate (Mallo et al., 2010; Kaczmarek et al., 2016; Liu et al., 2017a) and mixtures of butyric acid with monolaurin (Letlole et al., 2021) reduced the crypt depth in the duodenum, jejunum and ileum of broilers resulting in higher VH:CD ratio. However, in the case of reviewed literature about piglets, reduced crypt depth by protected sodium butyrate (Huang et al., 2015) and mixture of capric acid with others organic acids at 2 and 5 kg/t (Dierick et al., 2003; Hanczakowska et al., 2013), respectively, have been described without affecting the VH:CD ratio.

However, others studies did not describe any effect on villus height, crypt depth neither the ratio. Some authors suggested that the no impact of butyric acid or MCFA supplementation on intestinal morphology may be because animals were under healthy conditions and intestine is fully developed (Del Alamo et al., 2007; Levy et al., 2015; Liu et al., 2017a). Nonetheless, the present review shows heterogeneous results using butyric acid and/or MCFA, as these can affect the intestinal microarchitecture of broilers and piglets under non- and challenged conditions. Further research is required to clarify the impact of dietary supplementation with butyric acid and MCFA. However, in general, and according to the reviewed literature, protected forms of butyric acid can affect intestinal morphometry throughout the small intestine at lower doses that non-protected forms.

Beyond this, and focusing on the cells of the intestine, few studies observed significant effects of butyric acid or MCFA on them. Occasionally, it has been reported that the number of goblet cells, which primary function is the production and stimulation of mucus (Wu et al., 2018), was affected by butyrate and MCFA supplementation in broiler and piglet diets. In broilers, only the use of protected sodium butyrate affected the number of goblet cells, increasing their concentration in the jejunum and ileum (Sikandar et al., 2017; Wu et al., 2018; Letlole et al., 2021). In weaned piglets, the results reviewed are more varied. An increase in the number of these secretory cells has been observed using chemical derivative forms of butyric acid and MCFA (salts and glycerides, respectively) as well as sodium butyrate protected with MCFA salts (Manzanilla et al., 2006; De Keyser et al., 2019; Cui et al., 2020; López-Colom et al., 2020). Therefore, these FA seem to reinforce the mucus layer, preventing the attachment of pathogens to the epithelium (Opapeju et al., 2009; Melaku et al., 2021). In this sense, some authors particularly described *in vitro* the ability of butyric acid to increase the expression of mucin genes in the goblet cells (Gaudier et al., 2004; Burger-van Paassen et al., 2019).

Nonetheless, from a contrary perspective, López-Colom et al. (2020) showed a reduction in the number of ileal goblet cells in weaned piglets fed protected heptanoate with salts of MCFA at 3 kg/t. The aim of this study was to assess the potential of the additive against ETEC. Therefore, the reduction in the number of goblet cells was presented as a protective strategy to reduce the possibilities of the pathogen to adhere to the epithelium. In this line, Hermans et al. (2010) also described the mucus layer as a protective and supportive environment for *Campylobacter* infections, complicating the eradication of the bacteria from the intestinal tract of broilers. Thus, more research is needed to determine the relationship between the use of butyric acid and MCFA, the number of goblet cells, as well as the gastrointestinal development and pathogenic infections (Letlole et al., 2021).

Table 1.8 Effects of dietary supplementation with butyric acid and medium-chain fatty acids on intestinal histomorphometry of broilers.

Reference	Feed additive	Dose, kg/t	Site and age (d) of sampling	Age, d	Challenge	Treatment effects (Compared to Control)
			Free fatty acids			
Panda et al. (2009)	butyric acid	4, 6	duodenum, 35	0-35	No	↑ VH, CD
Zeitz et al. (2015)	lauric and myristic acids	46.3	jejunum, 35	0-35	No	↓ VH:CD
Baltić et al. (2018)	MCFA including caproic, caprylic, capric, lauric acids	1.6/1.2/1 0-10 d/11-21 d/22-42 d	duodenum, 42 ileum, 42	0-42	No	↑ VH, villus width, VH:CD ↓ VH, villus width, VH:CD
			Salts of fatty acids			
Qaisrani et al. (2015)	sodium butyrate	2	duodenum, 35	0-35	No	↑ CD, VH:CD
Hu and Guo (2007)	sodium butyrate	2	jejunum, 21	0-42	No	↑ VH:CD
			Glycerides forms			
Antongiovanni et al. (2007)	butyric acid glycerides	2, 3.5, 5, 10	jejunum, ileum, 35	0-35	No	↓ VH
Antongiovanni et al. (2007)	butyric acid glycerides	2	jejunum, 35	0-35	No	↑ CD
Amer et al. (2021)	lauric acid monoglyceride	3	duodenum, 35	0-35	No	↑ VH
Amer et al. (2021)	lauric acid monoglyceride	1	duodenum, 35 jejunum, 35	0-35	No	↑ VH ↑ VH, mucosa thickness
Letlole et al. (2021)	lauric acid monoglyceride	1	duodenum, jejunum, 20, 33 ileum, 20, 33	0-35	No	↑ VH, VH:CD ↓ CD, ↑ VH:CD
			Protected forms			
Mallo et al. (2010)	protected sodium butyrate (30% sodium butyrate)	2.34/1.17 0-21d/22-42 d	jejunum, 42	0-42	No	↓ CD, ↑ VH:CD
Deepa et al. (2018)	protected sodium butyrate (30% sodium butyrate)	1.8	jejunum, 42	0-42	No	↑ VH, villus width VH:CD
Jerzsele et al. (2012)	protected sodium butyrate (70% sodium butyrate)	1.5	jejunum, 25	0-25	*Clostridium perfringens*	↑ VH
Letlole et al. (2021)	protected sodium butyrate (25% sodium butyrate)	1/0.75/0.25 0-14 d/ 15-28 d/ 29-35 d	duodenum, ileum, 20, 33 jejunum 33	0-35	No	↑ VH, VH:CD ↑VH:CD, goblet cells
Mallo et al. (2010)	protected sodium butyrate (70% sodium butyrate)	1/0.5 0-21 d/ 22-42 d	jejunum, 42	0-42	No	↑ VH, ↓ CD, ↑ VH:CD
Mallo et al. (2021)	protected sodium butyrate (70% sodium butyrate)	1	ileum, 21	0-42	No	↑ VH:CD
Sikandar et al. (2017)	protected sodium butyrate (30% sodium butyrate)	1	duodenum, jejunum, 21, 35 ileum 21, 35	0-35	No	↑ VH, goblet cells ↑ goblet cells
Czerwiński et al. (2012)	protected sodium butyrate (30% sodium butyrate)	1	jejunum, 28	8-28	No	↑ VH, CD

Table 1.8 *Cont*.

Protected forms						
Zhao et al. (2022)	protected sodium butyrate	1	duodenum, jejunum, ileum, 42	0-42	No	↑ VH:CD
Song et al. (2017)	protected sodium butyrate (30% sodium butyrate)	0.8	jejunum, 21 jejunum, 29	0-35	*Eimeria*, *Clostridium perfringens*	↑ VH, CD ↑ VH:CD
Chamba et al. (2014)	partially protected sodium butyrate (30% protected)	0.7	jejunum, 14, 42	0-42	No	↑ VH
Liu et al. (2017a)	protected sodium butyrate	0.5, 1	ileum, 11	0-11	*Salmonella typhimurium*	↑ VH
Liu et al. (2017a)	protected sodium butyrate	0.5, 1	duodenum, 21 jejunum, 21 ileum, 21	0-42	No	↑ VH ↑ VH, ↓CD ↓CD, ↑ VH:CD
Wu et al. (2018)	protected sodium butyrate	0.2, 0.4, 0.8, 1	jejunum, ileum, 42	0-42	No	↑ goblet cells
Kaczmarek et al. (2016)	protected calcium butyrate (35% butyric acid)	0.2, 0.3, 0.4	ileum, 35	0-42	No	↑ VH
Kaczmarek et al. (2016)	protected calcium butyrate (35% butyric acid)	0.2	ileum, 35	0-42	No	↓ mucosa thickness, CD, ↑ VH:CD
Mixtures and combinations						
Pereira et al. (2015)	butyric + lactic + acetic acids	8/6/4 1-21 d/ 22-35 d/ 36-43 d	ileum, 21	0-42	*Clostridium perfringens*	↑ VH, VH:CD
Letlole et al. (2021)	protected sodium butyrate (25% sodium butyrate) + lauric acid monoglyceride	1/0.75/0.25 + 1 0-14 d/ 15-28 d/ 29-35 d	duodenum, jejunum, 20, 33 ileum, 20, 33	0-35	No	↑ VH, VH:CD ↓ CD, ↑ VH:CD
Jerzsele et al. (2012)	protected sodium butyrate (70% sodium butyrate) + essential oils	1.5	jejunum, 25	0-25	*Clostridium perfringens*	↑ VH, VH:CD

MCFA = medium-chain fatty acid; VH = villus height; CD = crypt depth.

Table 1.9 Effects of dietary supplementation with butyric acid and medium-chain fatty acids on intestinal histomorphometry of piglets.

Reference	Feed additive	Dose, kg/t	Weaning day, kg	Site and age (d PW) of sampling	Age, d PW	Challenge	Treatment effects (Compared to Control)
			Salts of fatty acids				
Manzanilla et al. (2006)	sodium butyrate	3	18-22 d, 6.0 kg	jejunum, 21 colon, 21	0-21	No	↑ CD ↑ goblet cells
Chiofalo et al. (2014)	sodium butyrate	1.5	21 d, 5.7 kg	jejunum, 45	0-45	No	↑ CD
Wen et al. (2012)	sodium butyrate	1	21 d, 6.4 kg	duodenum, jejunum, ileum, 21	0-21	No	↑ VH, VH:CD
Lu et al. (2008)	sodium butyrate	0.5, 1	21 d, 6.7 kg	duodenum, jejunum, ileum 30	0-30	No	↑ VH, VH:CD
Tonel et al. (2010)	sodium butyrate	0.5	21 d, ND	jejunum, 35 ileum, 35	35	No	↑ villus width ↑ VH
Tonel et al. (2010)	calcium, sodium, potassium salts butyrate	0.5	21 d, ND	ileum, 35	35	No	↑ VH
Wen et al. (2012)	sodium butyrate	0.5	21 d, 6.4 kg	jejunum, 21	0-21	No	↑ VH, VH:CD
			Glycerides forms				
Cui et al. (2020)	lauric acid monoglyceride	2	21 d, 6.0 kg	jejunum, 14	0-14	Reduced dietary CP	↑ goblet cells
De Keyser et al. (2019)	caproic + caprylic glycerides	1.75	35 d, 8.0 kg	jejunum, 42 ileum, 42	0-42	*Escherichia coli* lipopolysaccharide	↑ CD, goblet cells, ↓ VH:CD ↑ VH, VH:CD
			Protected forms				
López-Colom et al. (2020)	protected sodium butyrate (50 % sodium butyrate)	3	21 d, 5.8 kg	ileum, 16	0-16	ETEC	↑ goblet cells
Chiofalo et al. (2014)	protected sodium butyrate (30% butyric acid)	1.5	21 d, 5.7 kg	duodenum, jejunum, ileum, 45	0-45	No	↑ VH, CD
Wang et al. (2017)	protected sodium butyrate (30% sodium butyrate)	1.5	28 d, 8.5 kg	jejunum, 14	0-14	No	↑ VH, VH:CD
Huang et al. (2015)	protected sodium butyrate	1	28 d, 10.2 kg	jejunum and ileum, 28	0-28	No	↓ CD
Upadhaya et al. (2020)	protected sodium butyrate (40% sodium butyrate)	0.5, 1.5/0.75, 3/1.5 0-21 d/ 22-42 d	28 d, 7.0 kg	duodenum, jejunum, ileum, 42	0-42	No	↑ VH
López-Colom et al. (2020)	protected sodium heptanoate (50% heptanoate)	3	21 d, 5.6 kg	ileum, 12	0-16	ETEC	↓ goblet cells

Table 1.9 *Cont*.

Mixtures and combinations							
Hanckzakowska et al. (2013)	caprylic acid + fumaric, propionic acids	2 + 5	35 d, 7.9 kg	ileum, 56	7-84[1]	No	↑ villus width, CD
Hanckzakowska et al. (2013)	capric acid + fumaric, propionic acids	2 + 5	35 d, 7.9 kg	ileum, 56	7-84[1]	No	↑ VH, villus width, ↓ CD
Hanczakowska et al. (2013)	capric acid + fumaric, propionic acids	2 + 5	35 d, 8.6 kg	ileum, 56	0-84	No	↑ VH, VH:CD
Long et al. (2018)	mixture MCFA, protected butyric acid, formic, acetic, propionic, citric acids, phenolic compounds	2, 3	ND, 8.6 kg	jejunum and ileum, 28	0-28	No	↑ VH,VH:CD
Zhang et al. (2020)	tributyrin + oregano	0.6 + 1.4	21 d, 8.8 kg	duodenum, 28 ileum, 28	0-28	No	↑ VH ↓ CD, ↑ VH:CD
Zhang et al. (2020)	tributyrin + methyl salicylate	0.6 + 1.4	21 d, 8.8 kg	duodenum, 28 ileum, 28	0-28	No	↑ VH ↓ CD, ↑ VH:CD

MCFA = medium-chain fatty acid; PW = post-weaning; VH = villus height; CD = crypt depth.

As for intestinal local immunity, scarce information is available about the effects of butyric acid and MCFA on the number of IEL in the intestine of broilers and piglets. Dierick et al. (2003) described a significant reduction in the number of IEL in the small intestine of weaned piglets fed MCFA triglycerides combined with lipase and it was considered a protective effect. However, there is a controversial discussion in the literature concerning the number of IEL because a lower number of these effector cells, in turn, may indicate a down-regulation of local immune response (Ferrara et al., 2016). In fact, some studies attributed to butyric acid and MCFA down-regulation of pro-inflammatory pathways as well as stimulation of regulatory cytokine expression. Other authors described no effects on the IEL number in the intestine of broilers as well as piglets (López-Colom et al., 2019a; Abdelli et al., 2020; López-Colom et al., 2020). Therefore, it seems that more studies are required to elucidate their effect on the intestinal immunity (Kuang et al., 2015; Yu et al., 2019).

Lastly, this section of the review also highlights the effect of butyric acid and MCFA on the expression of tight junctions among the epithelial cells. Particularly, the literature consulted refers to piglets because of the relationship between the intestinal permeability (straightly regulated by tight junctions) and frequent post-weaning enteric disorders (Xiong et al., 2019). Some authors observed that salt, glycerides and protected forms of butyric acid or MCFA increased the expression of tight junctions, limiting the entry of pathogens by reducing the intestinal permeability (Hou et al., 2014; Huang et al., 2015; Feng et al., 2018; Cui et al., 2020). This improvement may be explained via activation of protein kinases by butyric acid and MCFA (Yan and Ajuwon, 2017; Suzuki, 2019). In parallel, some authors directly reported increased intestinal permeability due dietary supplementation, using different analytical methods as calculating urinary lactulose to mannitol ratio, paracellular flux of dextran or the transepithelial electrical resistance (Huang et al., 2015; Wang et al., 2017; Lin et al., 2020). Other authors, evaluated fecal consistency, observing better fecal scores in piglets supplemented by mixtures that include MCFA (Lan et al., 2018). However, different studies have not observed significant effects on fecal score, suggesting that further research would provide more information to clarify the effect of these FA on the intestinal barrier, especially in combating post-weaning diarrhea.

1.3.2.2 Effects on the intestinal microbiota

Following the impact of butyric acid and MCFA on the gut barrier, several authors studied the effect of these FA on the microbiota composition. The results showed that these effects are observed throughout the intestinal tract by different forms of presentation of butyric acid and

MCFA on broiler chickens and weaned piglets (**Table 1.10** and **1.11**). For years, studies concerning the effects of butyric acid and MCFA on the gut microbiota are focused on specific bacteria. As general premise, butyric acid has a strong antimicrobial activity against both gram-positive and gram-negative bacteria (Xiong et al., 2019). In the case of MCFA, strong microbial activity has been described against some gram-negative bacteria, such as *Escherichia coli*, and especially against gram-positive (Abdelli et al., 2020). In fact, a higher efficacy in the inhibition of gram-positive bacteria than gram-negative bacteria by MCFA has been observed *in vitro* (Setianto et al., 2017).

According to the literature, the microbiota modulation capacity of FA can be explained by either direct or indirect processes (Çenesiz and Çitfci, 2020). On one hand, the direct effect is observed when FA are in non-dissociated form. As discussed in previous sections of the review, free FA are in non-dissociated form when intestinal pH is lower than their pK_a. As glycerides and protected forms mostly need to be digested to obtain free fatty acids, the non-dissociated forms from glycerides and protected forms are usually observed in more distal intestinal regions. Therefore, FA in the non-dissociated form penetrate directly the bacterial cell membrane and enter into the cytoplasm. Once inside, the acid at the neutral pH of the cell is dissociated into anions and protons. Consequently, the accumulation of anions results toxic for the bacteria cell (Fernández-Rubio et al., 2009; Ahsan et al., 2016). As well as, an intracellular acidification occurs, disrupting metabolic process of the bacteria. Some studies shown that MCFA diffuse through the bacterial cell membrane and create transient or permanent pores, resulting in altered membrane permeability and cell death (Vanrolleghem et al., 2019). In this line, Den Hartog et al. (2005) suggested a synergetic effect between SCFA and MCFA. The MCFA may act disrupting the cell wall membrane of the microorganisms and helping the SCFA to enter into the cytoplasm where they act.

Therefore, it is important to keep in mind the pK_a value of each FA (aforementioned in **Table 1.3**) and the gastrointestinal pH of each region (**Table 1.12**) to understand the direct antimicrobial activity of butyric acid and MCFA. In the upper gastrointestinal tract, butyric acid and MCFA are in non-dissociated form, so they have the ability to penetrate directly the membrane of bacteria. However, as intestinal pH increases in distal segments, the equilibrium non-dissociated: dissociated form of the acids (determined by the pK_a value) shifts to the dissociated form, limiting their ability to cross the membrane of bacteria. Consequently, it has been believed for many years that the effect of SCFA is restricted to the upper gastrointestinal tract (Thompson and Hinton, 1997), while MCFA seems to have an extended scenario in the gut

Table 1.10 Effects of dietary supplementation with butyric acid and medium-chain fatty acids on intestinal microbiota of broilers.

Reference	Feed additive	Dose, kg/t	Site and age (d) of sampling	Age, d	Challenge	Treatment effects (Compared to Control)
			Free fatty acids			
Panda et al. (2009)	butyric acid	4, 6	small intestine, cecum, 22	0-35	No	↓ *Escherichia coli*
Raza et al. (2017)	butyric acid	3, 4	cecum, 28, 42 excreta, 42	0-42	No	↓ *Lactobacillus*, Coliforms total bacteria
Solis de los Santos et al. (2008)	caprylic acid	3.5, 5.27, 7, 8.75, 10.5, 12.24, 14	jejunum, cecum, 10	0-10	*Campylobacter jejuni*	↓ *Campylobacter jejuni*
Gracia et al. (2016)	caprylic, capric acids	6	cecum, 35	22-42	*Campylobacter jejuni*	↓ *Campylobacter*
Baltić et al. (2018)	MCFA including caproic, caprylic, capric, lauric acids	1.6/1.2/1 1-10 d/11-21 d/22-42 d	duodenum, 42 ileum, 42 cecum, 42	0-42	No	↑ Lactic acid bacteria, *Enterococcus, Escherichia coli*, ↓*Staphylococcus aureus* ↓ Lactic acid bacteria, *Enterococcus* ↓ Lactic acid bacteria
Chotikatum et al. (2009)	MCFA	1,1000 L/ 1:2,000 L 0-35 d/ 36-45 d	cecum, 17	0-45	*Salmonella Enteritidis*	↓ *Salmonella Enteritidis*
			Salts of fatty acids			
Hu and Guo (2007)	sodium butyrate	2	jejunum, 21	0-42	No	↓ *Lactobacillus*
Bortoluzzi et al. (2018)	sodium butyrate	1	cecum, 28	0-28	No	change phylotype frequencies
Fernández-Rubio et al. (2009)	sodium butyrate	0.92	crop, ceca, 42 feces, 6, 13, 34, 41	0-42	No	↓ *Salmonella*
Makled et al. (2019)	sodium butyrate	0.6	ileum, 21 cecum, 21	0-21	No	↑ *Lactobacillus* ↓ aerobic bacteria
			Glycerides forms			
Yang et al. (2018)	butyric acid glycerides	3	cecum, 21	0-20	No	↑ *Bifidobacterium*
van Gerwe et al. (2010)	caprylic and capric acid triglycerides	10	cecum, 9, 19	0- 19	*Campylobacter jejuni*	↓ *Campylobacter*
Gracia et al. (2016)	caprylic and capric acid monoglycerides	8	cecum, 35, 42	22-42	*Campylobacter jejuni*	↓ *Campylobacter*
			Protected forms			
Onrust et al. (2020)	protected sodium butyrate	3	cecum, 21	0-21	*Salmonella Typhimurium*	↓ *Lactobacillaceae*

Table 1.10 *Cont*.

Protected forms						
Onrust et al. (2020)	protected sodium butyrate (30% sodium butyrate)	3	cecum, 21	0-21	*Salmonella Typhimurium*	↓ *Salmonella*
Van Immerseel et al. (2005)	protected sodium butyrate (30% sodium butyrate)	2.5	cecum, 8	0-42	*Salmonella Enteritidis*	↓ *Salmonella*
Deepa et al. (2018)	protected sodium butyrate (30% sodium butyrate)	0.9, 1.8	cecum, 42	0-42	No	↓ *Escherichia coli*, *Clostridium perfringens*
Bortoluzzi et al. (2018)	protected sodium butyrate (70% sodium butyrate)	1	cecum, 12-28	0-28	*Eimeria*, *Clostridium perfringens*	stabilize microbiota population
Fernández-Rubio et al. (2009)	partially protected sodium butyrate (30% protected)	0.92	crop, ceca, 42 feces, 6, 13, 34, 41	0-42	No	↓ *Salmonella*
Wu et al. (2018)	protected sodium butyrate	0.8	cecum, 21	0-42	No	↑ *Bacteroidetes*, ↓ *Enterobacteriaceae*
Song et al. (2017)	protected sodium butyrate (30% sodium butyrate)	0.8	cecum, 21, 29	0-35	*Eimeria*, *Clostridium perfringens*	↓ *Escherichia coli, Clostridium perfringens*
Zhou et al. (2017)	protected sodium butyrate (30% sodium butyrate)	0.75	cecum, 19	0-20	*Eimeria tenella*	stabilize microbiota population
Wu et al. (2018)	protected sodium butyrate	0.4	cecum, 21	0-42	No	↑ *Firmicutes*, *Proteobacteria*, *Ruminococcaceae*, ↓ *Lactobacillaceae*
Mixtures and combinations						
Cerisuelo et al. (2014)	sodium butyrate + essential oil	1 + 0.5	cecum, 42	0-42	*Salmonella Enteritidis*	↓ *Salmonella Enteritidis*
Bortoluzzi et al. (2018)	protected sodium butyrate (70% sodium butyrate) + essential oils	0.5 + 0.5	cecum, 12-28	0-28	*Eimeria*, *Clostridium perfringens*	stabilize microbiota population
Nguyen et al. (2018)	protected capric + caprylic + fumaric + citric + malic acids	0.2, 0.3, 0.4, 0.5, 0.6	excreta, 35	0-35	No	↑ *Lactobacillus*, ↓ *Escherichia coli*

MCFA = medium-chain fatty acid.

Table 1.11 Effects of dietary supplementation with butyric acid and medium-chain fatty acids on intestinal microbiota of piglets.

Reference	Feed additive	Dose, kg/t	Weaning day, kg	Site and age (d PW) of sampling	Age, d PW	Challenge	Treatment effects (Compared to Control)
			Free fatty acids				
Hanczakowska et al. (2016)	caprylic acid	3	28 d, ND	jejunum, 60[1]	7-70[1]	No	↓ *Escherichia coli*
Hanczakowska et al. (2016)	capric acid	3	28 d, ND	cecum, 60[1]	7-70[1]	No	↓ *Escherichia coli, Clostridium perfringens*
Zentek et al. (2013)	caprylic acid	1.5	25 d, 6.9 kg	jejunum, 28 colon, 28	0-28	No	↑ *Escherichia-Hafnia-Shigella* ↑ Clostridial cluster XIVa
			Salts of fatty acids				
Lin et al. (2020)	sodium butyrate	1	28 d, 8.5 kg	jejunum, 14	0-14	No	↑ *Lactobacillus:Escherichia coli*
Lu et al. (2008)	sodium butyrate	1	21 d, 6.7 kg	small intestine, colon, 30	0-30	No	↓ *Escherichia coli, Clostridium perfringens*
Wen et al. (2012)	sodium butyrate	1	21 d, 6.4 kg	small intestine, colon, 21	0-21	No	↓ *Escherichia coli, Clostridium perfringens*
Castillo et al. (2006)	sodium butyrate	0.3	18-22 d, 6.0 kg	jejunum, 21	0-21	No	↑ biodiversity
			Glycerides forms				
Sotira et al. (2020)	butyric acid triglycerides	2	28 d, 8.7 kg	feces, 40	7-47	No	↓ *Lactobacillus*, *Bifidobacteria*
			Protected forms				
López-Colom et al. (2020)	protected sodium butyrate (50 % sodium butyrate)	3	21 d, 5.8 kg	ileum, 16	0-16	ETEC	↑ *Enterobacteria*
López-Colom et al. (2020)	protected sodium butyrate (50 % sodium butyrate)	3	21 d, 5.8 kg	colon, 12	0-16	ETEC	↓ *Paraprevotellaceae, Prevotella, Phascolartobacterium*
López-Colom et al. (2019b)	protected sodium butyrate (70% sodium butyrate)	3	21 d, 5.4 kg	ileum, 12	0-16	ETEC	↑ *Escherichia coli*
López-Colom et al. (2019b)	protected sodium butyrate (70% sodium butyrate)	3	21 d, 5.4 kg	colon, 12	0-16	ETEC	↑ *Prevotella*, ↓ *Synergistetes*, *Anaerovibtio, Bilophila*
Lin et al. (2020)	protected sodium butyrate (30% sodium butyrate)	0.3, 0.45	28 d, 8.5 kg	jejunum, colon, 14	0-14	No	↑ *Lactobacillus:Escherichia coli*
Huang et al. (2015)	protected sodium butyrate	1	28 d, 10.2 kg	ileum, colon, 28	0-28	No	↓ *Lactobacillaceae*, ↑ *Clostridiaceae, Rumminococcaceae, Lachnospiraceae, Bacteroidetes*
Upadhaya et al. (2020)	protected sodium butyrate (40% sodium butyrate)	0.5, 1.5/0.75, 3/1.5 0-21 d/ 22-42 d	28 d, 7.0 kg	duodenum, 42 d feces, 42 d	0-42	No	↓ Coliforms ↑ Lactic acid bacteria

Table 1.11 *Cont*.

Protected forms							
Zentek et al. (2012)	protected MCFA (50% caprylic and capric acids)	3	18 d, 4.6 kg	stomach, 28	0-28	No	↑ *Enterobacteriaceae*, Clostridial clusters I, IV, *Lactobacillus*
Upadhaya et al. (2018)	protected organic acids (1.2% caprylic and capric)	2	28 d, 6.5 kg	feces, 21 feces, 42	0-42	No	↓ *Escherichia coli* ↑ *Lactobacillus*
López-Colom et al. (2020)	protected sodium heptanoate (50 % heptanoate)	3	21 d, 5.6 kg	colon, 12	0-16	ETEC	↓ *Enterobacteriaceae*
López-Colom et al. (2019b)	protected sodium heptaonate (70% sodium heptaonate)	3	21 d, 5.4 kg	colon, 12	0-16	ETEC	↑ Coriobacteriaceae, ↓Parapretovella, Lachnospira
Mixtures and combinations							
Hancksakowska et al. (2013)	capric acid + fumaric, propionic acids	2 + 5	35 d, 7.9 kg	ileum, 56[1]	7-84[1]	No	↓ *Clostridium perfringens*
Hancksakowska et al. (2013)	caprylic acid + fumaric, propionic acids	2 + 5	35 d, 7.9 kg	ileum, 56[1]	7-84[1]	No	↓ *Clostridium perfringens*
Hanczakowska et al. (2013)	caprylic, capric acids + fumaric, propionic acids	2 + 5	35 d, 8.6 kg	ileum, 56	0-84	No	↑ aerobic bacteria, ↓ *Clostridium perfringens*
López-Colom et al. (2019a)	mixture of salts of MCFA	3	28 d, 8.1 kg	cecum, 10, 14 feces, 9	0-14	*Salmonella typhimurium*	↓ *Salmonella*
López-Colom et al. (2019a)	mixture of salts of MCFA	3	28 d, 8.1 kg	colon, 14	0-14	*Salmonella typhimurium*	↑ *Fibrobacteraceae*, ↓ *Barnesiellaceae*
López-Colom et al. (2019a)	mixture of salts of MCFA	3	21 d, 5.6 kg	ileum, 15	0-15	ETEC	↓ *Entrerobacteria*, Coliforms
Wei et al. (2021)	sodium butyrate + benzoic acid	0.35 + 5	21 d, 6.9 kg	feces, 40	40	No	↑ Shannon index
Kuang et al. (2015)	capric, lauric acids + calcium formate, calcium lactate, citric acid, myristic acid	3	21 d, 6.4 kg	ileum, rectum, 28	7-28	No	↑ *Lactobacillus*
Long et al. (2018)	mixture MCFA, protected butyric acid, formic, acetic, propionic, citric acids, phenolic compounds	2, 3	ND, 8.6 kg	feces, 28	0-28	No	↓ *Escherichia coli*
Zhang et al. (2020)	tributyrin + oregano	0.6 + 1.4	21 d, 8.8 kg	colon, 28 feces, 28	0-28	No	↑ *Firmicutes:Bacteroidetes* ↑ Lactic acid bacteria
Zhang et al. (2020)	tributyrin + methyl salicylate	0.6 + 1.4	21 d, 8.8 kg	colon, 28 feces, 28	0-28	No	↑ *Firmicutes:Bacteroidetes* ↓ *Escherichia coli*

[1]d of age; MCFA = medium-chain fatty acid; PW = post-weaning; ND = not determined; ETEC = enterotoxigenic *Escherichia coli*.

to be in non-dissociated form due to their higher pk_a values. Nonetheless, despite the heterogeneity of the results summarized in the **Table 1.10** and **1.11**, more intestinal segments are exposed to the antibacterial effect of the FA by using salt, glycerides and protected forms instead of free FA (Fernández-Rubio et al., 2009). In addition, Moquet et al. (2016) suggested that the control exerted on the microbial population in the proximal part of the intestine may sustain the development of a beneficial microbiota in more distal segments.

Table 1.12 Range of pH according to gastrointestinal region of broiler chickens and weaned piglets (Heo et al., 2013; Czerwiński et al., 2012; Marmion et al., 2021).

pH in	Broiler	Weaned piglet
Crop	5.3-7.6	-
Proventriculus/Gizzard	1.4-5.6	-
Stomach	-	2.6-4.5
Duodenum	5.0-6.0	5.5-6.0
Jejunum	6.3-7.0	6.0-6.7
Ileum	5.5-8.0	6.0-7.4
Cecum	5.5-7.4	5.4-6.7
Colon	6.4-8.0	5.6-6.8

Besides the direct antimicrobial ability of these FA, it has also described that butyric acid and MCFA may indirectly modulate the gut microbiota. On one hand, they can inhibit toxin production and the expression of some virulence factors by interfering the signal transduction (Çenesiz and Çitfci, 2020; Gomez-Osorio et al., 2021). On the other hand, a decrease in the intestinal pH may occur limiting acid-intolerant species such as *Escherichia coli*, *Salmonella spp*. and *Campylobacter*, and stimulating the proliferation of lactic acid bacteria, such as *Lactobacillus* and *Bifidobacterium spp*. (Kuang et al., 2015; Ahsan et al., 2016; Gracia et al., 2016; Nguyen et al., 2018; Upadhaya et al., 2018; Zhang et al., 2020). In turn, the increase of this microbial population considered as beneficial bacteria may compete for nutrients and space with pathogenic bacteria, limiting their presence (Ahsan et al., 2016). In this sense, some studies evaluated *Lactobacillus* to *Enterobacteria* ratio as Lin et al. (2020), who demonstrated that supplementation with non-protected sodium butyrate in piglets diets increased the *Lactobacillus*: *Escherichia coli* ratio in the jejunum, while the use of protected sodium butyrate increased the ratio in the jejunum and more posterior sections as colon. Nonetheless, some authors described a reduction in the relative abundance of some lactic acid bacteria in broilers and piglets by butyric acid and MCFA supplementation (Hu and Guo, 2007; Huang et al., 2015; Xu et al., 2016; Baltić et al., 2018; Wu et al., 2018; Onrust et al., 2020; Sotira et al., 2020).

Furthermore, others authors have shown the potential of butyric acid and MCFA in experimental challenge models. In chickens, the reduction of cecum colonization by *Salmonella Enteritis* have

been confirmed in various studies using protected forms of sodium butyrate alone or combined with essential oils in the diets. In addition, Chotikatum et al. (2009) observed the ability of MCFA to control *Salmonella* infection with water supplementation. Other authors described lower cecal *Campylobacter* counts in broilers supplemented with chemical derivatives forms (salts and glycerides) of MCFA. Focusing on coccidiosis and necrotic enteritis disease, stabilization of the microbiota population has been demonstrated by supplementation of protected sodium butyrate and its combination with other feed additives (Song et al., 2017; Zhou et al., 2017; Bortoluzzi et al., 2018). For piglets, experimental infectious models consisted of challenges with enteropathogens as *Escherichia coli* and *Salmonella* that results in intestinal diseases around weaning (López-Colom et al., 2019a). In these scenarios, the authors showed that protected sodium butyrate and protected heptanoate modulated the microbiota of ileum and colon as shown in **Table 1.11** (López-Colom et al., 2019a; López-Colom et al., 2019b; López-Colom et al., 2020).

Additionally, it has been described that mixtures with other organic acids as fumaric, citric, malic or propionic acids also had inhibitory activity against some enterobacteria including *Escherichia coli*, and promoted the proliferation of beneficial bacteria such as *Lactobacillus* in both broiler and piglets (Kuang et al., 2015; Nguyen et al., 2018; Wei et al., 2021). Moreover, the combination with essential oils, which are known to possess antimicrobial properties, showed complementary effects on the intestinal microbiota of both monogastrics by stabilizing the beneficial microbiota population and reducing potential enteropathogens as *Salmonella* and *Escherichia coli* (Cerisuelo et al., 2014; Bortoluzzi et al., 2018). Thus, these results suggest that mixtures including butyric acid or MCFA may achieve additional and even synergetic antimicrobial effects.

1.3.3 Effects on digestibility

The assessment of digestibility is important as it is directly dependent on the integrity of the intestinal barrier, and, in turn, it may determines feed efficiency (Chotikatum et al., 2009; Hong et al., 2012; Li et al., 2015; Riboty et al., 2016; Imran et al., 2018; Upadhaya et al., 2018). Therefore, several studies have been carried out to evaluate the effect of dietary supplementation with butyric acid or MCFA on nutrient digestibility in broilers and weaned piglets. According to the reviewed literature, **Table 1.13** and **1.14** summarize the significant effects of butyric acid and MCFA on digestibility of broilers and piglets, respectively.

Regarding the form of presentation of butyric acid and MCFA, and according to the literature consulted, the use of free FA has improved digestibility in chickens but not in piglets. Particularly,

it has been described the supplementation with butyric acid at 2.5 kg/t in the finisher broiler diet increased the apparent total tract digestibility of CP, ether extract, crude fiber and ash, without affecting the performance parameters (Ndelekwute et al., 2016). On the other hand, the supplementation with MCFA in water enhanced the ileal digestibility of CP and energy as well as the growth performance in chickens under *Salmonella enteritidis* challenge (Chotikatum et al., 2009).

In the case of using salts and glycerides, contradictory effects have been observed. In broilers, the use of butyrate in the diet resulted in higher metabolizable energy values without affecting performance parameters. For piglets, the utilization of MCFA triglycerides improved digestibility of dry matter (**DM**), ether extract, nitrogen and energy, as well as production parameters. Nonetheless, some authors observed reduced digestibility coefficients by supplementing piglet diets with sodium butyrate (Manzanilla et al, 2006; Le Gall et al., 2009). In this sense, the authors suggested that the antimicrobial activity of sodium butyrate could also promote a more stable microbial community that could have prevented the proliferation of some particular bacteria, such as amylolytic bacteria, reducing the digestibility values in weaned piglets. However, performance parameters were not impaired.

Finally, more conclusive results have been observed using protected forms in broilers as well as in piglets. Better digestibility coefficients of DM, organic matter, fat, protein and/or energy have been observed using protected butyric acid or MCFA at doses of 0.2 to 2 kg/t in both species.

Possible mechanisms behind the impact of the different forms of presentations on digestibility lie in the mentioned effects of butyric acid and MCFA on the intestinal barrier. The stimulation of epithelial cell proliferation results in a larger absorptive surface that leaded better nutrient digestibility of DM, CP, fat, fiber and energy (Smulikowska et al., 2009; Kaczmarek et al., 2016; Liu et al., 2017a). Other authors attributed the digestion-stimulating properties of butyric acid and MCFA to the ability to stimulate pancreatic secretion and activity of digestive enzymes such as amylase and trypsin (Sileikiene et al., 2005; Amer et al., 2021).

Another explanation may be the antimicrobial activity of these FA (Hejdysz et al., 2018) that can reduce the microbial competition with the host for nutrients (Dibner and Buttin, 2002; Hong et al., 2012). Therefore, an improvement on the digestibility coefficients of DM, fat, fiber and energy was observed (Hong et al., 2012; Long et al. 2018; Upadhaya et al., 2020).

Table 1.13 Effects of dietary supplementation with butyric acid and medium-chain fatty acids on digestibility of broilers.

Reference	Feed additive	Dose, kg/t	Site and age of sampling	Age, d	Challenge	Treatment effects on digestibility of (Compared to Control)
			Free fatty acids			
Ndelekwute et al. (2016)	butyric acid	2.5	excreta, 56 d	28-56	No	↑ crude protein, ether extract, crude fiber, ash
Chotikatun et al. (2009)	medium-chain fatty acids	1,1000 L/ 1:2,000 L 0-35 d/ 36-45 d	ileum, 48 d	0-45	*Salmonella Enteritidis*	↑ crude protein, energy
			Salts of fatty acids			
Hejdysz et al. (2018)	calcium butyrate	10	excreta, 34 d	0-34	No	↑ AMEn
			Glycerides forms			
Amer et al. (2021)	lauric monoglyceride	3, 5	ileum, 35 d	0-35	No	↓ leucine
Amer et al. (2021)	lauric monoglyceride	1	ileum, 35 d	0-35	No	↑ leucine, ↓ arginine
			Protected forms			
Smulikowska et al. (2009)	protected sodium butyrate (30% sodium butyrate)	1	excreta, 31 d	0-31	No	↑ organic matter, nitrogen
Riboty et al. (2016)	partially protected sodium butyrate (30% protected)	0.7	excreta, 11, 31 d	0-42	No	↑ dry matter, crude protein, ether extract, AME, AMEn
Liu et al. (2017a)	protected sodium butyrate	0.5, 1	ileum, 11 d	0-11	*Salmonella Typhimurium*	↑ energy
Liu et al. (2017a)	protected sodium butyrate	0.5, 1	ileum, 42 d	0-42	No	↑ energy
Imran et al. (2018)	protected calcium butyrate (50% calcium butyrate)	0.45	ileum, 35 d	0-35	No	↑ crude protein
Kaczmarek et al. (2016)	protected calcium butyrate (35% butyric acid)	0.2, 0.3, 0.4	excreta, 35 d	0-42	No	↑ crude fat, AMEn
Kaczmarek et al. (2016)	protected calcium butyrate (35% butyric acid)	0.2	ileum, 14 d excreta, 14 d	0-42	No	↑ crude protein ↑ crude fat
Kaczmarek et al. (2016)	protected calcium butyrate (35% butyric acid)	0.2	ileum, 35 d	0-42	No	↑ amino acids, AMEn
Kaczmarek et al. (2016)	protected calcium butyrate (35% butyric acid)	0.2	excreta, 42 d	0-42	No	↑ crude fat
			Mixtures and combinations			
Nguyen et al. (2018)	protected mixture of capric + caprylic + fumaric + citric + malic acids	0.2, 0.3, 0.4, 0.5, 0.6	excreta, 34 d	0-35	No	↑ dry matter

MCFA = medium-chain fatty acid.

Table 1.14 Effects of dietary supplementation with butyric acid and medium-chain fatty acids on digestibility of piglets.

Reference	Feed additive	Dose, kg/t	Weaning day, kg	Site and age (d PW) of sampling	Age	Challenge	Treatment effects on digestibility of (Compared to Control)
			Salts of fatty acids				
Manzanilla et al. (2006)	sodium butyrate	3	18-22 d, 6.0 kg	ileum, 21 feces, 21	0-21	No	↓ starch ↓ dry matter, starch
Le Gall et al. (2009)	sodium butyrate	3	28 d, ND	ileum, 40[1] feces, 40[1]	5-40[1]	No	↓ nitrogen ↓ dry matter, organic matter, nitrogen
			Glycerides forms				
Li et al. (2015)	MCFA triglycerides	7, 14, 21	21 d, 7.5 kg	feces, 14 feces, 28	0-14	No	↑ dry matter, ether extract ↑ ether extract
Hong et al. (2012)	caprylic, capric triglycerides	5.5/3.2 0-7 d/ 8-28 d	21 d, 6.9 kg	feces, 15-28	0-28	No	↑ energy
Hong et al. (2012)	caprylic, capric triglycerides	5.5/3.2 0-7 d/ 8-14 d	28 d, 10.2 kg	feces, 35	0-35	No	↑ dry matter, nitrogen, energy
			Protected forms				
Upadhaya et al. (2020)	protected sodium butyrate (40% sodium butyrate)	0.5, 1.5/0.75, 3/1.5 0-21 d/ 22-42 d	28 d, 7.0 kg	feces, 21	0-42	No	↑ dry matter
Devi and Kim (2014)	protected MCFA (58% caprylic and capric acid)	2	ND, ND	feces, 42	0-42	No	↑ dry matter, nitrogen, energy
Upadhaya et al. (2018)	protected organic acids (1.2% caprylic and capric)	1, 2	28 d, 6.5 kg	feces, 21, 42	0-42	No	↑ dry matter, energy
Han et al. (2011)	protected MCFA	1	28 d, 6.0 kg	feces, 14	0- 28	No	↑ crude protein, energy, lysine, histidine, phenylalanine, threonine, calcium, phosphorus
			Mixtures and combinations				
Kuang et al. (2015)	capric, lauric acids + calcium formate, calcium lactate, citric acid, myristic acid	3	21 d, 6.4 kg	ileum, 28	7-28	No	↑ amino acids
Hanczakowska et al. (2013)	caprylic and capric acids + fumaric, propionic acids	2	35 d, 8.6 kg	feces, 56-70	0-84	No	↑ crude protein, crude fat
Hancksakowska et al. (2013)	capric acid + fumaric, propionic acids	2	35 d, 7.9 kg	feces, 56-70[1]	7-84[1]	No	↑ crude protein, crude fat, crude fiber
Hancksakowska et al. (2013)	caprylic acid + fumaric, propionic acids	2	35 d, 7.9 kg	feces, 56-70[1]	7-84[1]	No	↑ crude fiber

Table 1.14 *Cont*.

Mixtures and combinations							
Hancksakowska et al. (2013)	caprylic and capric acids + fumaric, propionic acids	2	35 d, 7.9 kg	feces, 56-70[1]	7-84[1]	No	↑ crude fat
Wei et al. (2021)	sodium butyrate + benzoic acid	0.35, 0.7, 1.05 + 5	21 d, 6.9 kg	feces, 40	0-40	No	↓ nitrogen, phosphorus
Devi and Kim (2014)	protected caprylic, capric acids (58%) + *Enteroccus faecium*	2 + 1	ND, ND	feces, 14 feces, 42	0-42	No	↑ dry matter, energy ↑ dry matter, nitrogen, energy
Long et al. (2018)	mixture MCFA, protected butyric acid, formic, acetic, propionic, citric acids, phenolic compounds	2	ND, 8.63 kg	feces, 14 feces, 28	0-28	No	↑ NDF, FAD, phosphorus ↑ ether extract, phosphorus
Long et al. (2018)	mixture MCFA, protected butyric acid, formic, acetic, propionic, citric acids, phenolic compounds	3	ND, 8.63 kg	feces, 28	14-28	No	↑ dry matter, total carbohydrate, fiber

[1]d of age; MCFA = medium-chain fatty acid. PW = post-weaning; ND = not determined; NDF = neutral detergent fiber; ADF = Acid detergent fiber.

Also, combining butyric acid and/or MCFA with other organic acids as well as with other additives such as probiotics seems to have, in general, beneficial effects on digestibility activity. However, some authors observed no differences in digestibility capacity when broilers or piglets received diets supplemented with butyric acid or MCFA (alone or in combination). Therefore, further research is needed to deepen the understanding of the effect of butyric acid and MCFA on intestinal health, including barrier function and digestibility, as well as performance parameters.

CHAPTER 2

Hypotheses and objectives

The present PhD dissertation is part of the LIPOXIFEED project (Research and valorisation of fatty and antioxidant subproducts for the improvement of the nutritional value, quality and useful life of feed and animal food; COMRDI16-1-0033) co-funded by the European Fund of Regional Development of the European Union as part of the FEDER operating program of Catalunya 2014-2020 and managed by ACCIÓ. This project is framed into the research line of our group based on the study of different nutritional strategies aimed to promote gut health in poultry and swine production focused on reducing antibiotics use. As seen in the literature review, butyric acid and medium-chain fatty acids (**MCFA**) appear to be promising candidates for as feed additives in monogastrics. In fact, the opportunity to use MCFA obtained as by-products of the lauric oils refining process has increased their interest in animal nutrition. Therefore, it has been considered important to further the knowledge of the effects on intestinal health by dietary supplementation of butyric acid combined with MCFA using protected forms.

The particular hypotheses were that the dietary supplementation of protected sodium butyrate with MCFA salts:

- Promotes a gradual release of the butyric acid throughout the intestine.
- Improves the gut health of broiler chickens and piglets, especially under challenge conditions.
- Combined with reduced dietary crude protein (**CP**) may be useful to promote and enhance the gut health of weaned piglets.

Therefore, the aim of the present thesis was to investigate the potential of the supplementation with sodium butyrate protected by MCFA to improve the gut barrier and performance of monogastric animals. For this purpose, the following specific objective were established:

- To evaluate the intestinal release of butyric acid from the protected feed additive by performing an updated *in vivo* model.
- To show the effects of sodium butyrate protected by MCFA salts on gut health and performance of broilers reared under optimal conditions or challenged by coccidiosis.
- To assess the effects of supplementation with sodium butyrate protected by MCFA salts supplementation and reduction of dietary CP on the gut barrier and feed efficiency of weaned piglets.

In order to approach the above-mentioned objectives, three *in vivo* trials were performed in broiler chickens and one in weaned piglets. The first trial evaluated the protection of butyric acid from sodium butyrate with MCFA salts in the broilers' intestine (Chapter 3). Besides that, two trials were performed (Chapter 4) to evaluate the effects of the same additive (containing 70%

of sodium butyrate) on the gut barrier and performance of broilers: the first experiment consisted of testing the inclusion of three doses in diets that received broilers reared under optimal conditions, and then, the second experiment evaluated the effect of the additive in broilers housed under challenging conditions (coccidiosis). The last trial (Chapter 5) was carried out in weaned piglets, combining the supplementation of a feed additive (containing 50% sodium butyrate) with two dietary CP levels.

CHAPTER 3

Intestinal release of butyric acid
from protected sodium butyrate

Short communication: Evaluation of intestinal release of butyric acid from sodium butyrate protected by salts of medium-chain fatty acids in broiler chickens

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Meritxell Sadurní [1], Ana Cristina Barroeta [1], Cinta Sol [2], Mónica Puyalto [2] and Lorena Castillejos [1]

[1] Animal Nutrition and Welfare Service (SNiBA), Animal and Food Science Department, Faculty of Veterinary, Universitat Autònoma de Barcelona, 08193 Bellaterra, Spain

[2] Norel S.A., 28007 Madrid, Spain

CHAPTER 4

Dietary supplementation with sodium butyrate protected by medium-chain fatty acids in broiler chickens

Impact of dietary supplementation with sodium butyrate protected by medium-chain fatty acid salts on gut health of broiler chickens

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Meritxell Sadurní [1], Ana Cristina Barroeta [1], Roser Sala [1], Cinta Sol [2], Mónica Puyalto [2] and Lorena Castillejos [1]

[1] Animal Nutrition and Welfare Service (SNiBA), Animal and Food Science Department, Faculty of Veterinary, Universitat Autònoma de Barcelona, 08193 Bellaterra, Spain

[2] Norel S.A., 28007 Madrid, Spain

CHAPTER 5

Dietary supplementation with sodium butyrate protected by medium-chain fatty acids in piglets

Effects of dietary crude protein level and sodium butyrate protected by medium-chain fatty acid salts on performance and gut health in weaned piglets

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Meritxell Sadurní [1], Ana Cristina Barroeta [1], Cinta Sol [2], Mónica Puyalto [2] and Lorena Castillejos [1]

[1] Animal Nutrition and Welfare Service (SNiBA), Animal and Food Science Department, Faculty of Veterinary, Universitat Autònoma de Barcelona, 08193 Bellaterra, Spain

[2] Norel S.A., 28007 Madrid, Spain

CHAPTER 6

Discussion

Research in nutritional strategies to promote gut health has received great attention in poultry and swine production, with reduced use of antibiotics. Focusing on the use of butyric acid and medium-chain fatty acids (**MCFA**), different factors such as the presentation form of the feed additive, its inclusion level, the health status or age of the target animal, as well as the diet composition, can affect their efficacy (Hanczakowska, 2016; Polycarpo et al., 2017). For this reason, in this thesis, we have investigated the potential of dietary supplementation with sodium butyrate protected by salts of MCFA on gut health in broiler chickens and piglets. Particularly, the form presentation has been evaluated in order to determine the availability throughout the intestine of butyric acid fed as MCFA-protected butyrate. In addition, the effects of the feed additive supplementation on gut health have been tested under different broilers raising conditions, on one hand, or combining with another nutritional strategy as reducing the total amount of crude protein (**CP**) in weaned piglet diets, on the other hand. Therefore, the purpose of the present general discussion is to integrate the results of this thesis, in order to provide insights into the health promoting effects of butyrate protected by MCFA in broiler chickens and weaned piglets considering the previous mentioned factors.

6.1 Feed additive form presentation

As it has been mentioned in Chapter 1 (section 1.2.4), there is a wide range of presentation forms for using butyric acid and MCFA to promote gut health in monogastric: free acids, salts or glycerides of butyric acid and MCFA, as well as the protected forms. The last ones, come into limelight to achieve promoting effectiveness throughout the intestinal tract. In this regard, the additives evaluated in the present thesis consisted of sodium butyrate protected by sodium salts of MCFA. Particularly, the feed additive evaluated in broilers trials (Chapter 3 and 4) contains 70% of sodium butyrate, while the supplement fed by the weaned piglets (Chapter 5) consisted in 50% of sodium butyrate.

It should be mentioned that it is generally accepted that fat-protection prevents gastric digestion of butyric acid, increasing its delivery to the small intestine (Moquet et al., 2016). Indeed, predicting of the gastrointestinal tract (**GIT**) segment wherein butyric acid is released has been considered a key point to assess the efficacy of the additives on the gut barrier given the diversity of cell types, microbiota population and pH conditions throughout the GIT (Moquet et al. 2016). Nonetheless, few studies reported it. Furthermore, the description of some parameters that can affect the release kinetics (protective material used, technique developed to protect the active material or the inclusion level) are usually inaccurate in the literature (Moquet et al., 2016; Song

et al., 2017; Upadhaya et al., 2018; Onrust et al., 2020). Therefore, the understanding of butyric acid release and its effect throughout the intestine is limited. In the present thesis, the methodology followed to protect the butyric acid was not discussed in accordance with the manufacturer's confidentiality.

Concerning published assays, intestinal release kinetics from protected additives can be evaluated by *in vitro* techniques (Mallo et al., 2012; Omonijo et al., 2018). Briefly, the protected forms of feed additives were exposed to a certain pH and enzyme mixtures that simulate the GIT of the animals, while the concentration of butyric acid is determined at various time points as it was released (Liu et al., 2017). These are rapid experiments lasting few hours that provide an indication of the protected compounds' intestinal release. In fact, an *in vitro* assay (data not shown in previous chapters of the present thesis) was conducted by the present authors at the Animal and Food Science Department (Universitat Autònoma de Barcelona, Bellaterra, Barcelona, Spain) following the procedures described by Boisen et al. (1991) and modified according to Mallo et al. (2012).

In order to evaluate the progressive release of butyric acid from sodium butyrate (70%) partially protected by MCFA salts over time, digesta solutions were collected (duplicates) at 1, 2, 3, 4, 5, 6 h of the *in vitro* digestion, and immediately refrigerated and kept at -20 °C until analysis. The determination of butyric acid was analyzed using HPLC chromatograph (Agilent 1200) with detector UV-DAD (Agilent) and using column Zorbax SB-AG, 4.6 mm by 150 mm by 5 µm by Norel Research and Development Center (Llicà de Vall, Barcelona, Spain). Butyric acid was detected from the beginning of the *in vitro* digestion and could be attributed to the non-protected butyric acid contained in the additive (Mallo et al., 2012). The total release of butyric acid was observed at 3 h, which can correspond to the duodenum, jejunum or ileum of chickens according to the GIT transit time described by different authors (Rougière and Carré, 2010; Ravindran et al., 2013). As the rate of GIT passage depends on different factors (such as the development status of the GIT and the diet) (Rougière and Carré, 2010), determining the intestinal segment in which release occurs based on the elapsed time can be imprecise. Moreover, the available enzymes (pepsin, pancreatin) are from porcine, which may restrict the interpretation of studies focused on simulating broiler chickens GIT.

Furthermore, it should be noted that studies focused on GIT transit time were usually calculated during fasting periods that change physiologic intestinal conditions (as reducing the absorptive surface area) and may perturb the passage time (Gonzales et al., 2003). To avoid this limitation, Liu et al. (2017b) developed a precision-fed digestive rate-of-passage assay using iodinate

contrast as a marker. Thus, the GIT transit time of broiler was determined without fasting, which allowed a more accurate evaluation of feed additive protection considering the *in vitro* release time. However, when comparing the results of *in vitro* and *in vivo* trials, it is known that there are differences among the results using *in vitro* media or intestinal contents (Namkung et al., 2011). In addition, intestinal bacteria or the anti-peristaltic motility in chickens are examples of factors related to broiler and piglet physiology that are also not considered in *in vitro* trials and could cause differences between *in vivo* and *in vitro* studies (Jiménez-Moya et al., 2021).

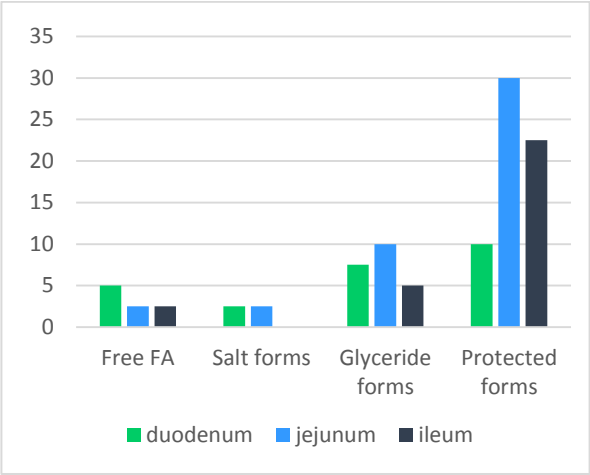
For this reason, a novel *in vivo* approach was performed in Chapter 3 to avoid these issues and evaluate the intestinal release of butyric acid from the protected feed additive. In this context, observing that the highest butyric acid release was in the ileum of non-fasting broilers, the effects of the additives were evaluated in this intestinal segment in Chapter 4 (broilers) and Chapter 5 (weaned piglets). Although broiler chickens and piglets are both monogastric, there are physiological properties such as the gastrointestinal passage rate and the presence or absence of anti-peristaltic refluxes that differ between them. Therefore, future research should follow the same methodology using a dye as a marker to evaluate the intestinal kinetics of butyric acid from the additive evaluated in piglets (Chapter 5).

Anyway, the results obtained are supported by previous authors who compared the efficacy of different forms of butyric acid presentation on the gut health. Supplementation with fat-protected butyrate reached to impact on morphological measurements in the ileum as our results, while the effects of butyrate salt or glycerides forms were restricted to anterior intestinal segments as the jejunum (Chiofalo et al., 2014). Furthermore, the efficacy of butyric acid to modulate microbiota population and intestinal fermentation was also reported in posterior segments by using fat-protected butyrate (Chiofalo et al., 2014; Makowski et al., 2022) supporting the higher ratio *Lactobacillus*:*Enterobacteriaceae* observed in the colon of piglets in the Chapter 5.

Moreover, the works reviewed in Chapter 1 have supported the obtained results. For histomorphometry, the significant results summarized in **Tables 1.8** and **1.9** are shown in **Figure 6.1** and **6.2** (for broilers and piglets, respectively) considering the intestinal segment wherein butyric acid and/or MCFA affected the histomorphometry, and the presentation form of fatty acids (**FA**).

On one hand, for broilers, **Figure 6.1** shows that all forms of presentation affect duodenal histormophometry (20% free FA, 10% salts, 30% glycerides, 40% protected forms). However, to

reach the jejunum and ileum, the use of protected forms appears to be the most effective strategy (66.7% and 75.0%, respectively).

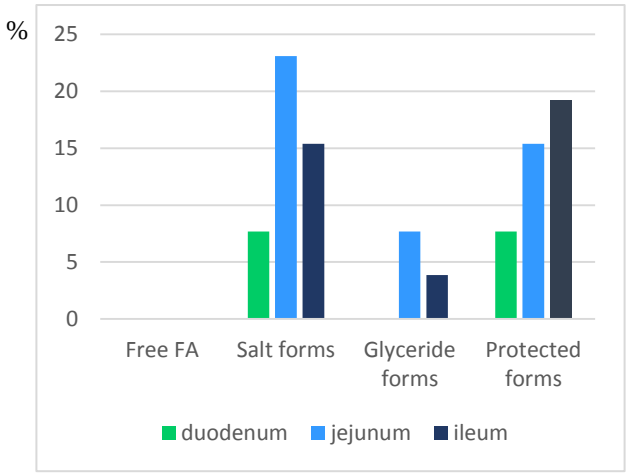


FA = fatty acids.

Figure 6.1 Distribution of histomorphometric results in intestinal segments due to the supplementation of butyric acid and/or MCFA, considering presentation forms (among all the broilers studies summarized in **Table 1.8** of Chapter 1).

This high percentage of efficacy in the ileum of broilers supports the results obtained in the present thesis (Chapter 4), observing a reduction on the number of goblet as well as a tend to increase the number of intraepithelial lymphocytes (**IEL**) in the ileum of broilers that received the additive at 2 kg/t in contrast to lower inclusion doses. Besides that, the use of protected sodium butyrate (70%) by MCFA salts at 1 kg/t in broiler under coccidiosis challenge supported the gut barrier promoting a rapid tissue turnover indicated by deeper crypts, in addition to reinforce the ileal mucosa by restoring the number of goblet cells.

On the other hand, **Figure 6.2** summarizes the results in piglets' small intestine. In this case, no effects on the intestinal histomorphometry of piglets by the use of free FA have been described. This fact may be due to their limiting properties, such as rapid absorption. Furthermore, as piglet intake can be reduced due to the non-pleasant odor of free butyric acid and MCFA, the interest in using these free forms may have been limited (Decuyper and Dierick, 2003). However, focusing on the other forms of presentation, the duodenum was only affected by the use of salts and protected forms, whereas the jejunum and ileum were also affected by glyceride forms. As in broilers, the ileum was the intestinal segment of piglets most affected by the protected forms (50%), which justifies the sampling performed in Chapter 5 of this thesis. Thus, the increase in the number of ileal goblet cells by the sodium butyrate (50%) protected by MCFA salts seems to corroborate the slow intestinal release of butyric acid from the protected feed additive tested in weaned piglets.



FA = fatty acids.

Figure 6.2 Distribution of histomorphometric results in intestinal segments due to the supplementation of butyric acid and/or MCFA, considering presentation forms (among all the piglets studies summarized in **Table 1.9** of Chapter 1).

Concerning the intestinal microbiota, small intestine is a key site to evaluate the interaction of microbiota, gut function, diet, and animal health. Nonetheless, many authors reported antimicrobial effects by butyric acid or MCFA derivatives (mainly in protected forms) in the cecum and colon of broilers or piglets. These posterior intestinal segments were usually evaluated because they hold the highest microbial diversity and the content remains there for longer (Di Marcantonio et al., 2022). In this regard, *Clostridium perfringens* population was counted in the cecum of coccidiosis-challenged broilers (Chapter 4) as common secondary infection of coccidiosis that results in necrotic enteritis. However, no differences in clostridial counts were observed because coccidial infection induced by attenuated oocysts may not have promote the proliferation of this bacteria and, consequently, butyric acid or MCFA antibacterial property was not exacerbated. In contrast, antimicrobial effects were observed in the colon of piglets reducing enterobacteria counts and increasing *Lactobacillus* number by using the feed additive (Chapter 5). These results suggest that, in this case, the butyric acid protection allowed a later intestinal release of butyric acid in weaned piglets. This difference between the trials can be attributed to many factors such as the physiology of each species, the microbiota population evaluated or the composition of the feed additive (Chiofalo et al., 2014; Wu et al., 2018). With regard to this last point, it should be reminded that sodium butyrate protected by MCFA salts contains 50% of butyrate in piglets' case (Chapter 5) instead of the 70% composed for broilers (Chapter 3 and 4), so that a greater amount of butyric acid may be protected and reach more posterior intestinal segments, affecting the tested microbiota population.

6.2 Challenging conditions for gut health

It has been hypothesized that the efficacy of feed additives could be higher when animals are exposed to challenging conditions (Del Alamo et al., 2007; Abdelli et al., 2020). Therefore, experimental designs that can potentially disrupt the gut integrity and health of broilers and piglets was performed in the present thesis to evaluate the effects of sodium butyrate protected by salts of MCFA on gut health and growth performance. For broilers, a coccidiosis-challenge (an infectious model) was conducted due to the intestinal barrier disturbance caused by *Eimeria* spp. and its worldwide impact on poultry production (Belote et al., 2019). Regarding the coccidiosis experimental design, it should be mentioned than there is a wide variety of experimental models in the literature. In some studies, coccidial oocysts were obtained from infected chickens, and after their propagation, the oocysts sporulated and were cleaned to be counted and ensure the required concentration for the inoculation of one *Eimeria* spp. (Leung et al., 2019; Teng et al., 2021). Beyond that, many authors evaluated the effects of feed additives under necrotic enteritis after *Eimeria* spp. and *Clostridium perfringens* infection (Bortoluzzi et al., 2018; Liu et al., 2019). Furthermore, other authors orally inoculated a mixture of sporulated oocysts (mostly *E. acervulina*, *maxima* and *tenella*) because of their importance in broiler chicken production. However, the origin of the oocysts mixture was not mentioned (Amerah and Ravindran, 2015; Barbieri et al., 2015). Since coccidiosis cases are usually co-infection, mixed inoculation of oocytes was developed in the present thesis using a high amount of a commercial anticoccidial vaccine as previous authors (Adedokun and Adeola, 2017; Belote et al., 2019). Considering the properties of the oocysts from a live vaccine (such as lower multiplication capacity and pathogenicity), the concentration for the challenge's inoculation was calculated for a mild infection, which it was corroborated with reduction on average daily feed intake (**ADFI**) and average daily gain (**ADG**) one-week post-inoculation (**PI**) and the gut barrier modulation during the 14 d PI (Cervantes, 2020).

For piglets, weaning was considered the challenge in Chapter 5 because of the importance of this phase in swine production. As mentioned in Chapter 1, weaning is usually performed between 21 and 28 d of age when the intestinal function is not yet fully developed, which often results in post-weaning diarrhea besides compromised performance parameters. In particular, the piglets of the experiment performed in the present thesis were weaned at 21 d of age according to the Directive 2008/120/EC (*Piglets cannot be weaned from the sow before they are 28 days old unless the welfare or health of the dam or the piglet would otherwise be harmed*) because it was hypothesized that nutritional strategies may support the immature intestine development of the animals. In this regard, the results on fecal consistency observed in Chapter

5 supported the previous hypothesis that weaning *per se* can be a challenge model to evaluate the gut function of piglets, and that a nutritional support (supplementation with sodium butyrate protected by MCFA or reducing dietary CP) promote improvements in the intestinal barrier of weaned piglets.

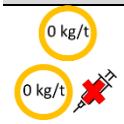
Therefore, in the present thesis, coccidiosis was performed as an infectious-challenge for broilers, and weaning as a non-infectious challenge for piglets. Below, a schematic overview of the results obtained when using the feed additives at 1 kg/t in each experiment is presented in **Table 6.1**. In particular, this table shows the treatments for which the use of 1 kg/t had significant (*) effects or trends (†) in order to compare the results between trials using the same feed additive inclusion dose.

Our results showed that feed additive supplementation at 1 kg/t affected growth gain and feed intake of coccidiosis-challenged broilers in agreement with previous studies (Liu et al., 2019; Amer et al., 2021). In fact, following a factorial arrangement design with coccidiosis challenge and butyrate supplementation as main factors, Hansen et al. (2021) corroborated the present hypothesis by observing that performance parameters were only maintained or improved by the feed additive in broilers under coccidiosis-challenge. However, performance parameters were not affected by feed supplementation in weaned piglets as observed by previous authors (Mallo et al., 2012; Zentek et al., 2013). The large heterogeneity among the published results may be because there are many forms of butyric acid and MCFA used for poultry and swine dietary supplementation, as it could be seen in Chapter 1. In this regard, it has been hypothesized that greater amount of sodium butyrate (0.2 kg/t) that received broiler chickens in contrast to piglets can be useful to improve performance parameters in monogastric animals under challenging conditions. Anyway, it must be considered that the main objective of the present thesis, rather than evaluating the effects on performance, was to study the impact on the gut health of monogastric animals (broilers and pigs) as well as to give an idea of the possible modes of action. Therefore, the number of replicates used in our trials was calculated to evaluate mainly the effects on the gut barrier and function that, consequently, determine performance.

In this sense, the villus height:crypt depth ratio, an indicator of the intestinal absorbance surface, was only affected by the feed additive in broilers challenged with coccidiosis. In particular, the results seem to corroborate the efficacy of combine butyric acid with MCFA to promote intestinal cell turn-over by increasing crypt depth at 7 d PI of coccidiosis. Indeed, our results showed that MCFA-protected sodium butyrate at 1 kg/t is useful against invasion and damage

Table 6.1 Schematic overview of the results by using 1kg/t supplementation obtained in the experiments performed in the present thesis.

	Chapter 4		Chapter 5
	Experiment 1	**Experiment 2**	
Target animal	*Broilers*	*Broilers*	*Weaned-piglets*
Feed additive	DIC	DIC	BM
Challenge	*No*	*Coccidiosis*	*Weaning*
Performance parameters			
↑ ADFI	NS	0 kg/t *	NS
↑ ADG	NS	0 kg/t †	NS
Ileum Histomorphometry			
↑ crypt depth	NS	0 kg/t *	NS
↑ Number of goblet cells	2 kg/t *	0 kg/t *	0 kg/t *
↓ Number of IEL	2 kg/t †	NS	NS
Ileum Microbiota			
↑ Lactic acid bacteria	2 kg/t †	0 kg/t *	NS
↑ Enterobacteria	NS	0 kg/t *	NS
Colon Microbiota			
↑ Lactic acid bacteria	-	-	0 kg/t *
↓ Enterobacteria	-	-	0 kg/t *
↑ Digestibility of DM, OM	-	NS	0 kg/t † *



Differences in contrast to non-supplemented diet
Differences in contrast to non-supplemented and non-challenged

Differences in contrast to basal diet supplemented with 2 kg/t DIC
Differences in contrast to non-challenged and control-challenged (coccidiosis)

NS = non-significant differences

DIC = sodium butyrate (70%) protected by sodium salts of medium-chain fatty acids.
BM = sodium butyrate (50%) protected by sodium salts of medium-chain fatty acids.
ADFI = average daily feed intake; ADG = average daily gain; IEL = intraepithelial lymphocytes; DM = dry matter; OM = organic matter.
* Means significantly differ ($p \leq 0.05$); † Means tend to differ ($p \leq 0.10$)

on intestinal epithelial cells induced by *Eimeria* spp. Considering that the number of replicates (dose-response experiment: n = 6; coccidiosis challenge experiment: n = 12) was calculated to obtain similar statistical power in each experiment, it appears that our results support that supplementation with butyric acid and MCFA exerts additional beneficial effects on the altered intestinal mucosa. Thus, these FAs appears to be a clearly useful energy source for enterocytes. However, no effects on villus height nor crypt depth were observed by the supplementation in the case of the piglet study, according to other authors. In fact, these morphometric parameters

were affected in piglets that received higher doses of butyric acid or MCFA (alone or in combination) for longer periods than the two weeks elapsed in our trial (Manzanilla et al., 2006; Chiofalo et al., 2014; De Keyser et al., 2018; Long et al., 2018).

As for mucin-secreting cells, the results obtained in healthy 10-day-old broilers (Chapter 4) allowed the establishment of a maximum dose (1 kg/t) of butyric acid to avoid its cytotoxic effect reducing the number of these cells, as it was previously described in *in vitro* studies (Barcelo et al., 2000). Furthermore, although no effect on the number of goblet cells was observed in non-challenged broilers supplemented at 1 kg/t in contrast to non-supplemented animals, its efficacy in increasing the number of these mucin-secreting cells was corroborated in broilers challenged with coccidiosis that received the feed additive for two weeks. In this line, piglets supplemented with the feed additive at 1 kg/t for the same period of time also had higher numbers of these cells in the ileum. Furthermore, since no increase the number of *Enterobacteria*ceae, including *Escherichia coli*, or *Clostridium perfringens* was observed in piglets or broiler digesta, respectively, it can be hypothesized that effectively the use of MCFA-protected sodium butyrate at 1 kg/t is an effective strategy to reinforce the intestinal barrier: increasing the number of mucin-secreting cells without promoting the adherence of some bacteria that may be potentially pathogenic in monogastrics.

Concerning the number of intraepithelial lymphocytes (**IEL**), the tendency to have lower number of this T-cell population in broilers with a normal villous architecture by feed 1 kg/t of protected sodium butyrate (70%) instead of receiving 2 kg/t (Experiment 1 in Chapter 4) suggested that the use of the first dose results more comfortable for the animal health. Indeed, an increase of the IEL number is the earliest pathological change observed after challenging conditions (Chang et al., 2015). Therefore, the absence of differences in coccidiosis challenged broilers (Experiment 2 in Chapter 4) and weaned piglets (Chapter 5), in which an increase of these cells could be expected, suggests that further investigation is required about this sentinel immune system cells.

Furthermore, digestibility capacity was assessed in broilers under coccidiosis-challenge and weaned piglets due to the beneficial effects observed by previous authors (Chapter 1). In the present thesis, it must be remembered that dry matter and organic matter digestibility were assessed in both species, and fat digestibility was assessed in broilers because coccidiosis causes an alteration in lipid metabolism (Hansen et al., 2021). On the other side, for weaned piglets, the crude protein (**CP**) digestibility was performed since the dietary CP levels were a main factor in the experimental design. However, only the use of feed additive for 14 days in weaned piglets

improved the digestibility capacity of dry matter (tendency) and organic matter. It should remind that methodology to assess the digestibility was different between the studies: in poultry, excreta collection and hence apparent total tract digestibility (**ATTD**) was evaluated as previous authors who observed increased ATTD of dry matter, organic matter, or fat in broilers supplemented with butyric acid or MCFA (Chotikatum et al., 2009; Smulikowska et al., 2009; Riboty et al., 2016). For weaned piglets, digestibility was tested as apparent ileal digestibility (**AID**) since CP digestibility was evaluated and ATTD does not consider intestinal fermentation and endogenous losses afterwards small intestine. Since improved dry matter and organic matter digestibility was observed in piglets but not in broilers, these differences could be attributed to the methodology. However, it has been described that differences between AID and ATTD are minimal in broiler chickens because fermentation capacity is wide lower than that of pigs, mainly due to their faster rate of passage (Ravindran et al., 2016). Therefore, disturbances in the results due to the performed methodology was ruled out.

On the other hand, the overall results seem to corroborate the hypothesis stated in Chapter 4: the evaluation of digestibility capacity was performed too early to appreciate the disruption by coccidiosis and, in turn, the beneficial support of the additive. In this regard, it should be mentioned that improved digestibility in previous studies was mainly observed in animals supplemented with butyric acid or MCFA for longer periods (from 14 d onwards) (Smulikowska et al., 2009; Devi and Kim, 2014; Adedokun and Adeola, 2017; Upadhaya et al., 2020). According to the reviewed literature, only two cases reported beneficial effect on broilers supplemented with protected butyrate for 11 d. On one hand, Riboty et al. (2016) observed an improvement on digestibility of healthy broilers. On the other hand, Liu et al. (2017a) reported higher digestibility capacity of supplemented broilers that were challenged by *Salmonella* spp. In the latter case, broilers were challenged on d 4 of age, so the beneficial effect of the feed additive was observed at 7 d PI. Therefore, it would be interesting to evaluate digestibility capacity at least 7 d post coccidial inoculation in broilers to check if the challenge compromises intestinal function and, on the other hand, if the additive already represents a beneficial contribution, or the animal should receive it for at least 14 d to see its effect, as occurred in the weaned piglets in Chapter 5. In this line, testing the efficacy of the feed additive in animals reared under challenging conditions for at least 7 d can be useful to elucidate the supporting capacity on the gut function of protected sodium butyrate and its rapid action.

6.3 Combination of different nutritional strategies to promote gut health

Dietary supplementation with butyric acid and MCFA is among a wide and varied array of nutritional strategies focus on promoting gut health in broiler chickens and piglets. Furthermore, some research evaluated the efficacy to combine different nutritional interventions, trying to get synergic benefits (Amer al., 2021; Wang et al., 2021). In this regard, the combination of using sodium butyrate protected by MCFA and reducing dietary CP level was evaluated in piglets at Chapter 5. It should be noted that the reduction of dietary CP content is under research to promote gut health of piglets as well as broiler chickens (Lambert et al., 2022). Indeed, this nutritional strategy also deserves attention because it appears to be a possibility to reduce nitrogen excretion by improving nutrients' utilization (Petrilla et al., 2018; Mátis et al., 2019; Amer et al., 2021). However, the inherent controversy of compromising piglet performance parameters by reducing dietary CP levels (Maynard et al., 2021; Lambert et al., 2022) was corroborated in Chapter 5 when a lower feed efficiency was observed in piglets fed 18.8% instead of 22.2% CP. In this context, our results emphasize that it would be interesting to focus research on the amino acid requirements of piglets (including isoleucine and histidine) to understand their role and to assess the dietary CP levels fully focused on improving animal health and reducing environment impact. Furthermore, these results supported other research lines that were focus to evaluate feed additives as organic acids, phytogenic, enzymes, etc. with reduced-CP diets suggesting that the supplementation reach to improve feed efficiency and reinforce the gut barrier and function (Amer al., 2021; Wang et al., 2021).

However, few studies particularly combined the reduction of CP level and organic acids in piglets or broiler' diets. Focusing on swine nutrition, beneficial effects on gut health has been described by combining restricted CP content with the supplementation of MCFA glycerides (monolaurate or triglycerides of caprylic and capric) or protected organic acids (including fumaric, citric, malic, and phosphoric acids) (Cui et al., 2020; Lee et al., 2021). Nonetheless, the experimental model of these previous studies consisted of a lineal model that did not allow to differentiate the efficacy of each strategy separately. For this reason, a 2 x 2 factorial arrangement was followed in the trial performed in Chapter 5, as previous studies that evaluated both strategies in broiler chickens (Petrilla et al., 2018; Borda-Molina et al., 2021). In neither case interactions were observed, but Borda-Molina et al. (2021) described influences on the same bacterial family (*Bacteoidaceae*) in the intestine of broilers by sodium butyrate supplementation and reduced CP content, suggesting that both strategies contribute to modulate the gut barrier. These previous results in broiler chickens, coupled with the beneficial effects observed on different

parameters throughout the piglets intestine in Chapter 5, guide future research to combine reduced CP level and butyrate and MCFA supplementation in poultry and swine nutrition.

Moving on the other combination tested in the present thesis (mixture of butyric acid and MCFA), some studies corroborated that dietary supplementation with blends containing butyric acid and MCFA promoted the gut function and improved growth performance (López-Colom et al., 2020; Letlole et al., 2021; Sacakli et al., 2023). These authors observed the beneficial effects throughout the small intestine: duodenum, jejunum, and ileum. Furthermore, microbiota population and fermentation were also modified in the posterior intestinal segments. These results along with the gradual intestinal release of butyric acid observed in Chapter 3 provide further direction to also evaluate the effects of the tested feed additive throughout the entire intestinal tract. In fact, it has been hypothesized that the MCFA contained in salt forms protecting the butyric acid in the evaluated additive can be a more immediate source to promote beneficial effects on the gut barrier and function (Liu, 2015; Amer et al., 2020). Thus, combining MCFA to protect butyric acid using salt forms may result in a dual strategy: promoting the gradual release of butyric acid in the intestine (corroborated in Chapter 3) and providing *per se* beneficial effects throughout the gut barrier, as observed in the ileum and posterior intestinal segments in the *in vivo* trials conducted (Chapter 4 and 5).

6.4 Future considerations

This thesis was conducted in order to obtain information about the impact on the gut barrier of butyric acid and MCFA used as sodium butyrate protected by MCFA salts in broiler and piglet diets. So, four *in vivo* trials (Chapter 3, 4 and 5) were performed. The first trial provided valuable data about how to evaluate the intestinal release kinetic of butyric acid from a protected feed additive. Besides that, a general overview of the progressive intestinal release of butyric acid from one of the additive tested in the present PhD dissertation was observed in broiler chickens: sodium butyrate protected by MCFA sodium salts containing 70% of the short-chain FA salt released the highest amount of butyric acid in the ileum. Therefore, it would be interesting to follow the updated methodology to evaluate the other protected feed additive that includes 50% of sodium butyrate to understand the role of the butyric acid amount and its particular protection on the intestinal broiler kinetics and, consequently, its effects on the gut barrier. Furthermore, other questions may be clarified in future experiments such as the impact of the initial gut health status of the animal on the release kinetic by conducting studies at different ages or under diverse rearing conditions in both species (broilers and pigs).

Besides that, as it has been mentioned in the section 6.1, it would be interesting to evaluate the effects throughout the small intestine, sampling tissue and digesta content from each segment: duodenum, jejunum and ileum. According to the results obtained in the present thesis, future research could point out other analytical procedures to deepen the knowledge about the effects on the gut barrier since promised results were described by some authors (Bortoluzzi et al., 2017; Wu et al., 2017; Cui et al., 2019): such as expression of mucins, tight junction, concentration of cytokines, and markers of functionality of the epithelium as AMPK. Concerning the gut microbiota, massive-sequencing technologies should be developed to characterize the microbial population and obtain an overview of microbe-to-microbe interactions that, in turn, exerts protective, structural and metabolic effect to the host (López-Colom et al., 2019). Thus, along with the butyric acid release profile, it would be feasible to further the understanding of the contribution of using MCFA as protective material and the optimal degree of butyric acid protection according to the target intestinal segment.

Another issue to assess is the effect of protected sodium butyrate by MCFA salts on performance parameters in animals under commercial conditions to obtain valuable information for animal production. In this sense, it is important to remark that this PhD thesis is part of a project that, in later steps, included studies to evaluate the efficacy of the additives on performance parameters under experimental conditions more similar to commercial circumstances. Therefore, *in vivo* trials were performed in broiler chickens, laying hens and grower-finisher pigs facilities (bonÀrea, Nial Farm, Guissona, Spain) to assess the effect on feed efficiency and meat or egg quality. It should be highlight that the use of sodium butyrate protected by sodium MCFA salts tended to increase the eggshell thickness and impacted on yolk color of 52 to 62 weeks laying hens. These results may be explained by improved organic matter and fat digestibility, leading future perspectives to further investigate the effect on digestive capacity as mentioned in section 6.2. Altogether, this work provides valuable data about the potential strategy to improve gut health by using protected sodium butyrate and MCFA in poultry and swine diets, as well as providing several recommendation for future research.

CHAPTER 7

Conclusions

From the results obtained in the present dissertation, the following conclusions can be drawn:

1. The novel *in vivo* approach by including a dye in the protected feed additive is a useful strategy to demonstrate qualitatively and quantitatively (by spectrophotometry) the intestinal release of butyric acid from MCFA-protected sodium butyrate.
2. The use of sodium butyrate partially protected by medium-chain fatty acid salts allows a gradual release of butyric acid throughout the gastrointestinal tract of broilers, mainly achieving the ileum.
3. The effects of sodium butyrate protected by medium-chain fatty acid salts (70% sodium butyrate) on the ileal histomorphometry differs among doses. The use of 2 kg/t in contrast to 1 kg/t reduces the number of ileal goblet cells in young chickens and tend to increase the number of intraepithelial lymphocytes at 39 days.
4. The dietary supplementation with sodium butyrate protected by medium-chain fatty acid salts (70% sodium butyrate) at 0.5 to 2 kg/t increases lauric and myristic acid contents in abdominal fat, without affecting the amount of abdominal fat pad and growth performance of broilers.
5. The performed coccidiosis challenge compromises growth performance of broilers during the first week post-inoculation, and disrupts ileal histomorphometry by decreasing the villus height:crypt depth ratio and the number of goblet cells. Furthermore, it modulates ileal microbial counts, increasing the number of lactic acid bacteria and reducing the counts of enterobacterias.
6. Dietary supplementation with sodium butyrate protected by medium-chain fatty acids (70% of sodium butyrate) at 1 kg/t in broilers challenged by coccidiosis does not affect nutrient digestibility, and in the ileum, recovers the reduced villus height:crypt depth ratio and number of goblet cells and increases the number of lactic acid bacteria and restores the enterobacteria counts at 21 d of age.
7. Reducing dietary crude protein levels (from 22.2% to 18.8%) for two weeks post-weaning compromises performance parameters of piglets but improves fecal consistency and organic matter digestibility, reducing ileal crypt depth and modifying the *Lactobacillus* counts in the ileum and the colon.
8. The use of sodium butyrate protected by medium-chain fatty acid salts (50% sodium butyrate) at 1 kg/t in weaned piglets' diets increases the number of ileal goblet cells and improves digestibility of dry and organic matter, as well as increases *Lactobacillus* counts and decreases enterobacterial population meanwhile affects microbial fermentation profile in the colon.

CHAPTER 8

References

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