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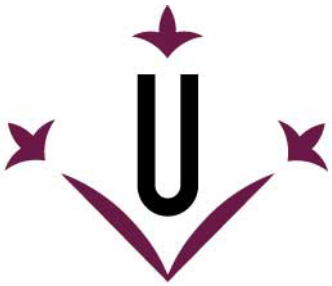

Universitat de Lleida

TESI DOCTORAL

Effect of producing type on digestive efficiency, metabolism and microbiota profile in growing and fattening pigs fed diets with different protein concentration

Laura Sarri Espinosa

Memòria presentada per optar al grau de Doctor per la Universitat de Lleida

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CERTIFIQUEN/*CERTIFY:*

Que la present tesi ha sigut presentada per optar al grau de Doctor en Ciència i Tecnologia Agrària i Alimentaria per la Universitat de Lleida.

That this thesis has been submitted in fulfilment of the requirements for the degree of Doctor of Philosophy in Agricultural and Food Science and Technology by the University of Lleida

La recerca, que es presenta, s'ha realitzat sota la supervisió dels directors i consideren apta per la seva presentació.

The research, which is presented, has been carried out under the guidance of the doctoral supervisor and they consider suitable for presentation.

Lleida, Setembre del 2022

Firmado por Joaquim Balcells Teres - DNI ***3127** (TCAT) el día 27/09/2022 con un certificado emitido por EC-SectorPublic

This thesis has been developed as part of an EU Framework Program for Research and Innovation Horizon 2020 (Feed-a-Gene, Grant agreement no: 633531), which main aim was to better adapt different components of monogastric livestock production systems (i.e., pigs, poultry and rabbits) to improve the overall efficiency and to reduce the environmental impact. In particular, it received funding from the European Union's H2020 program under National Institutes of Health (see above), and also from the Spanish Ministry of Economy and Competitiveness (AGL2017-89289-R). The PhD student Laura Sarri Espinosa received a research training grant from the Generalitat de Catalunya-European Social Funds (2019 FI_B 00416).

Als que hi són sempre, els meus pares

Antonio i Mercè, i germanes Gemma i Núria.

I a l'Albert, per estimar-me tal com soc.

"*Nos reparten unas cartas, mejores o peores, pero son las que tenemos y hay que jugarlas lo mejor posible*"

(Marian Rojas Estapé)

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La comunitat científica va creure durant molt de temps que l'ésser humà naixia amb pràcticament totes les neurones i que aquestes anaven morint irremeiablement amb l'edat, disminuint contínuament en número i connexions fins a la mort. Per sort, aquesta teoria va ser desafiada en el segle passat, i avui dia sabem que es generen noves neurones i connexions neuronals durant l'edat adulta. Això em porta al fet que de la mateixa forma que no soc la mateixa ni penso exactament igual que fa un mes, tampoc ho soc com quan vaig començar aquest procés que ha durat més de tres anys. No ens parem a pensar com d'important és de que això sigui així, que seguim creixent, aprenent i madurant sense gairebé fi. Aquest desenvolupament es deu a la suma de totes les experiències, successos, i per descomptat a les persones que ens acompanyen al llarg d'aquest llarg camí. Per això voldria agrair a les que m'han acollit durant la realització de la meva tesi doctoral:

Als meus directors de tesi Joaquim Balcells i Gabriel de la Fuente, per la vostra dedicació, guia, confiança i comentaris valuosos que m'han aportat seguretat. A Reza Ahmad Seradj i Marc Tor, per tots els vostres coneixements, temps i predisposició a ajudar, us tinc gran afecte.

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SUMMARY

This doctoral thesis is part of a European research program aimed at improving production efficiency and reducing the environmental impact of livestock farming. For this purpose, two trials were proposed for the study of protein and lipid metabolism at different stages of development, using two types of pigs intended for the production of different meat products. On the one hand, crossbred pigs (3W) selected for the production of lean fresh meat, and on the other, castrated Duroc pigs destined for the production of high-quality processed products (e.g., cured ham and loin). Understanding how factors such as age, producing type or dietary crude protein level affect different aspects of protein and lipid metabolism, as well as the structure of the intestinal microbiota, is necessary to be able to optimize nutritional requirements, and thus design strategies that respond to the proposed objectives. In the first study, fractional rates of protein synthesis (FSR) were measured in muscle (*longissimus dorsi* and *biceps femoris*), liver and duodenum, showing a generalized decrease in FSR with age, with the exception of *biceps femoris* muscle. Regarding pig producing type, the lean 3W pig showed higher FSR during the growing phase, thus explaining its higher protein deposition, however, unlike the Duroc pigs, 3W pigs presented a lower adaptation to the reduction of the protein level. In relation to lipid metabolism, the administration of labeled stearic acid (d_{35}-C18:0) allowed studying its direct incorporation in distinct tissues through the fractional incorporation rate (FIR). While FIR decreased in intramuscular fat with animal maturity, it increased in liver. The differences in fat deposition between the two pig producing types possibly reflect a greater role of endogenous synthesis in Duroc pigs. Analysis of oleic acid (d_{33}-C18:1 *c*9) synthesis from d_{35}-C18:0 by means of the stearoyl-CoA desaturase (SCD) enzyme determined that its activity increases with age and is more active in pigs carrying the T allele of the *SCD* gene. Finally, the analysis of the structure of the intestinal microbiota in ileum, cecum and distal colon determined a more stable and mature microbial community in the fattening phase, as well as a more diverse, stable and interconnected microbiota in the leaner 3W pigs. The protein-limiting diet induced a more complex microbial network, robust and adapted to maintain its activity.

RESUMEN

Esta tesis doctoral se enmarca en un programa de investigación europeo que tiene por objetivo mejorar la eficiencia productiva y reducir el impacto ambiental de las explotaciones ganaderas. Para ello, se plantearon dos ensayos para el estudio del metabolismo proteico y lipídico en distintas fases del desarrollo, utilizando dos tipos de cerdos destinados a la obtención de diferentes productos cárnicos. Por un lado, cerdos cruzados (3W) seleccionados para la producción de carne fresca magra, y por otro, cerdos Duroc castrados destinados a la producción de productos elaborados de alta calidad (p. ej., jamón y lomo curado). Comprender cómo afectan factores como la edad, el tipo productivo o el nivel de proteína bruta en la ración en distintos aspectos del metabolismo proteico y lipídico, así como la estructura de la microbiota intestinal, es necesario para poder optimizar las necesidades nutricionales, y con ello diseñar estrategias que respondan a los objetivos propuestos. En el primer estudio se midieron las tasas fraccionales de síntesis proteica (FSR) en músculo (*longissimus dorsi* y *biceps femoris*), hígado y duodeno, mostrando una disminución generalizada de la FSR con la edad, a excepción del *biceps femoris*. En cuanto al tipo productivo, el cerdo magro 3W mostró mayores FSR durante la fase de crecimiento, explicando así su mayor depósito proteico, sin embargo, a diferencia de los cerdos Duroc, presentó una menor adaptación a la reducción del nivel de proteína. En relación con el metabolismo lipídico, la administración de ácido esteárico marcado (d_{35}-C18:0), permitió estudiar su incorporación directa en distintos tejidos a través de la tasa de incorporación fraccional (FIR). Mientras la FIR disminuyó en la grasa intramuscular con la edad, en hígado aumentó. Las diferencias en la deposición grasa entre ambos tipos productivos, refleja posiblemente un mayor papel de la síntesis endógena en los cerdos Duroc. El análisis de la síntesis de ácido oleico (d_{33}-C18:1 *c*9) a partir del d_{35}-C18:0 por medio de la estearoil-CoA desaturasa (SCD), determinó que su actividad incrementa con la edad y es más activa en los cerdos portadores del alelo T del gen de la *SCD*. Finalmente, el análisis de la estructura de la microbiota intestinal en íleo, ciego y colon distal determinaron una comunidad microbiana más estable y madura en la fase de cebo, así como una microbiota más diversa, estable, e interconectada en los cerdos magros 3W. La dieta limitante en proteína indujo una microbiota con interacciones más complejas, robusta y adaptada para mantener su actividad.

RESUM

Aquesta tesi doctoral s'emmarca en un programa de recerca europeu que té per objectiu millorar l'eficiència productiva i reduir l'impacte ambiental de les explotacions ramaderes. Per a això, es van plantejar dos assajos per a l'estudi del metabolisme proteic i lipídic en diferents fases del desenvolupament, utilitzant dos tipus de porcs destinats a l'obtenció de diferents productes carnis. D'una banda, porcs creuats (3W) seleccionats per a la producció de carn fresca magra, i d'altra banda, porcs Duroc castrats destinats a la producció de productes elaborats d'alta qualitat (p. ex., pernil i llom curat). Comprendre com afecten factors com l'edat, el tipus productiu o el nivell de proteïna bruta en la ració en diferents aspectes del metabolisme proteic i lipídic, així com en l'estructura de la microbiota intestinal, és necessari per a poder optimitzar les necessitats nutricionals, i amb això dissenyar estratègies que responguin als objectius proposats. En el primer estudi es van mesurar les taxes fraccionals de síntesi proteica (FSR) en múscul (*longissimus dorsi* i *biceps femoris*), fetge i duodè, mostrant una disminució generalitzada de la FSR amb l'edat, a excepció del *biceps femoris*. En quant al tipus productiu, el porc magre 3W va mostrar majors FSR durant la fase de creixement, explicant així el seu major dipòsit proteic, no obstant això, a diferència dels porcs Duroc, va presentar una menor adaptació a la reducció del nivell de proteïna. En relació amb el metabolisme lipídic, l'administració d'àcid esteàric marcat (d_{35}-C18:0), va permetre estudiar la seva incorporació directa en diferents teixits a través de la taxa d'incorporació fraccional (FIR). Mentre la FIR va disminuir en el greix intramuscular amb l'edat, en fetge va augmentar. Les diferències en la deposició grassa entre tots dos tipus productius reflecteix possiblement un major paper de la síntesi endògena en els porcs Duroc. L'anàlisi de la síntesi d'àcid oleic (d_{33}-C18:1 *c*9) a partir del d_{35}-C18:0 per mitjà de l'enzim stearoil-CoA desaturasa (SCD), va determinar que la seva activitat incrementa amb l'edat i és més activa en els porcs portadors de l'al·lel T del gen de la *SCD*. Finalment, l'anàlisi de l'estructura de la microbiota intestinal en ili, cec i còlon distal van determinar una comunitat microbiana més estable i madura en la fase d'engreix, així com una microbiota més diversa, estable, i interconnectada en els porcs magres 3W. La dieta limitant en proteïna va induir una microbiota amb interaccions més complexes, robusta i adaptada per a mantenir la seva activitat.

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Abbreviation list

3W	Three-way crossbred
AA	Amino acids
ADG	Average daily gain
AID	Apparent ileal digestibility
ASR	Absolute synthesis rate
ATTD	Apparent total tract digestibility
BW	Body weight
CP	Crude protein
D	Deuterium
DM	Dry matter
EE	Ether extract
FA	Fatty acids
FDR	Fractional degradation rate
FIR	Fractional incorporation rate
FSR	Fractional synthesis rate
FUR	Fractional unsaturation rate
GHG	Greenhouse gases
GP	Growth phase
IMF	Intramuscular fat
LP	Low crude protein
MS	Mass spectrometer
MPE	Molar percent excess
MRM	Multiple reaction monitoring
NDF	Neutral detergent fiber
OM	Organic matter
PT	Producing type
SC	Subcutaneous adipose tissue
SCD	Stearoyl-CoA desaturase
UPLC	Ultra-performance liquid chromatography

INTRODUCTION

1. What challenges do society face?

1.1. Social challenges

Despite the commitment to achieve the 'zero hunger' target by 2030, Goal 2 of the 2030 Agenda for Sustainable Development, current data on world hunger and the prevalence of undernourishment indicate insufficient progress (FAO et al., 2021). It was estimated that between 720 and 811 million people were affected by hunger in 2020, mainly in Asia, Africa and Latin America and the Caribbean (FAO et al., 2021), and inevitably, the COVID-19 pandemic and the current conflict between Russia and Ukraine, are substantially worsening the situation. Additionally, the expected substantial growth of the global population along with the increasing purchasing power, constitutes a major challenge due to the dramatic increase in demand for food, feed, and energy in the coming decades. According to the United Nations et al. (2019), the size of the global population is expected to increase from the current 7.7 billion to 9.7 billion in 2050, reaching almost 11.2 billion at the turn of the 22nd century. This demographic growth is motivated by improved survival prospects, especially for the reproductive-age population, together with increasing life expectancy at birth, growing urbanization, and accelerating migration, despite the estimated decline in global human fertility. Consequently, increased agricultural productivity is necessary to meet global needs, as demand for livestock products is projected to double by 2050 (Rojas-Downing et al., 2017; Zappaterra et al., 2022), with China, Brazil and the United States leading the production trend.

1.2. Environmental challenges

Along with the increase in efficiency, sustainability should be the primordial pillar in food production in order to not compromise the feasibility of future generations. The previous demographic predictions, however, are compounded by the limited availability of new land areas dedicated to crop production and land and soil degradation. Intensive agriculture management and deforestation contribute to soil erosion, loss of biodiversity, and soil organic carbon deficit, among other nutrients (Leclère et al., 2020; Prudêncio da Silva et al., 2010). The negative and progressive effects of climate change, including extreme weather events and freshwater scarcity,

are also responsible for lessening natural resources availability and threatening the human future (FAO et al., 2021).

1.2.1. Climate change

In livestock, the main effects of climate change are the decrease of production efficiency in terms of fertility, growth performance, health and welfare in response to heat stress, which negatively impacts the economy of the production sector (Liu et al., 2021). High environmental temperature during certain seasonal periods lead to decreased feed consumption, along with impaired intestinal barrier function and inflammatory response (Liu et al., 2021; Pearce et al., 2013). Moreover, the incidence of vector-borne diseases and parasites is expected to spread in the near future in connection with the changing geographical distribution of vectors (Steinfeld, 2004; Thornton, 2010). Moreover, livestock is a relevant contributor to environment soil and aquifer pollution through organic and mineral load (i.e., nitrogen, phosphorous and heavy metals) and emissions of ammonia (NH_3) and greenhouse gases (GHG), including carbon dioxide (CO_2), nitrous oxide (N_2O) and methane (CH_4).

1.2.2. Gaseous load

According to a global livestock environmental assessment model, livestock contributes 14.5% of total anthropogenic GHG emissions (Gerber et al., 2013), and global meat production, estimated at 328 Mt, is responsible for 54% of total GHG emissions from the agricultural sector, which is expected to increase by 5% by 2030 (OECD-FAO, 2021). Pig production is the second contributor to global livestock GHG emissions (13%), only behind cattle production (70%), with an emission of 448.4kg CO_2equiv. for each slaughtered pig (Philippe and Nicks, 2015).

Emission of CO_2 is primarily associated with animal respiration, estimated at 1.55 kg CO_2 per day in a model pig of 70 kg of body weight (BW) (Philippe and Nicks, 2015). Moreover, manure also releases CO_2 as a result of aerobic putrefaction and/or anaerobic fermentation of organic matter depending on the manure storage medium, as well as through hydrolysis of urea by the enzyme urease (Chmielowiec-Korzeniowska et al., 2022; Philippe and Nicks, 2015). Ammonia is a toxic gas characterized by its pungent odor that produces negative effects on animal behavior, health and productivity (Xia et al., 2021). This air pollutant originates from nitrogen

excreted in manure in form of urea and non-digested organic matter (e.g., proteins) converted to NH_3 by microbial and urease enzymatic activity, and evaporated soon after excretion and during its decomposition (Kriz et al., 2021). Ammonia deposition in the environment is responsible for acidification and eutrophication of ecosystems (Krupa, 2003), and is also a necessary precursor of a GHG, the N_2O (Schauberger et al., 2018).

The two non-CO_2 GHG, N_2O and CH_4, have a global warming potential 310- and 34-times higher than CO_2 over 100 years, respectively (IPCC, 2013). Nitrous oxide is released during incomplete nitrification/denitrification processes conducted by microorganisms, whereby NH_3 is converted into nitrate (NO_3^-) during nitrification, and NO_3^- is subsequently reduced into molecular N_2 by denitrification. When there is insufficient oxygen during nitrification or surplus of NO_3^-, N_2O is synthesized. Furthermore, the accumulation of this GHG in manure is promoted by low presence of degradable carbohydrates, and the coexistence of aerobic and anaerobic conditions, so this process generally occurs on the surface of the slurry crust (Philippe and Nicks, 2015), or during manure application on fields (Rojas-Downing et al., 2017). Methane derives from bacterial degradation of organic matter, particularly carbohydrates, in the anaerobic conditions of the pig hindgut and manure. About 65-75% of CH_4 production from slurry occurs during its storage, while it may also originate during its application to fields (Chmielowiec-Korzeniowska et al., 2022). The amount of gas production will vary depending on temperature, proportion of degradable organic matter or carbon compounds, moisture, pH, redox potential, among others.

1.2.3. Mineral load

Although pig manure serves as an accessible organic fertilizer resource in agriculture, the application of excessive amounts of manure in certain areas of highly intensive swine production generates tremendous environmental impacts on soil, water and crops (Zhang et al., 2017). These impacts are basically explained by the high nitrogen and phosphorous content in the form of nitrate (NO_3^-) and phosphate (PO_4^{3}), respectively, which exceed the requirements of most crops and reach ground and surface waters by leaching and runoff losses, causing eutrophication (Aarnink and Verstegen, 2007). The human health implications of high NO_3^- levels in water

resources, above the 50 mg/L target, have raised great concern (Moeini and Azhdarpoor, 2021), with the strategies mainly focused on improving feeding and management practices in farms. Nitrogen is mostly contained in the crude protein (CP) of animal production feeds. Therefore, optimization of this compound is a principal objective, which is partially achieved by lowering CP diets and supplementing them with crystalline amino acids (AA) (Yuming Wang et al., 2018) to comply with the European Union Directive 1991/676/EEC on water protection against nitrates pollution.

As for phosphorous excretion, it is mostly caused by the limitation in the digestibility of a large part of this mineral present in the plant-based ingredients of the feed, in form of phytate; for this reason, the addition of phytase to the diet has been the main strategy to increase digestibility and decrease inorganic phosphorous supplementation (Lei et al., 2013; Muji et al., 2018). Moreover, all this is accompanied by the accumulation of trace minerals (e.g., zinc and copper), which have long been used to promote feed consumption, nutrient utilization, and intestinal health at certain swine growth stages. Soil and water contamination with these heavy metals is responsible for developing ecotoxicity to plants and microorganisms, reducing crop growth and weakening the ecology function of microorganisms (Ding et al., 2021).

1.3. Production costs

Feed costs represent the main expense in animal production, accounting for more than 60% of overall production costs, particularly in swine sector, with most of the total feed consumed during the growing-fattening period. In a globalized market for feed ingredients, among others, prices also depend on conflicts between countries, such as the ongoing Russian invasion of Ukraine, which has increased the cost of most raw materials, including grains and vegetable oils. Both countries were major producers of important crops such as cereals and sunflower, representing about 30 and 20% of global wheat and corn exports over the last three years, respectively (FAO, 2022). The price of the most commonly used ingredients such as wheat, barley, corn and soybean meal (47%) in Spain has increased by 58.06%, 64.50%, 43.46% and 30.42%, respectively, between March 2021 and March 2022. Thus, the pig feed cost has risen by around 40% in the same period of time (MAPA, 2022), which has forced the

increase in the price of pig carcasses in recent weeks. To all this must be added the rising cost of energy resources due to a worldwide energy crisis resulting from dependence on fossil fuels. The impending lack of Russian gas supplies to Europe through the Nord Stream 1 pipeline next fall-winter, due to European Union sanctions packages, has pushed up the global price of gas (BBC, 2022). To combat the shortage, however, it forces the affected countries diversify their energy supply, hopefully leading to a push for cleaner and more efficient energy sources.

2. Animal efficiency

In response to this scenario, there is an urgent need to improve the efficiency of feed resources. Meat is the most important animal-based product in livestock production that provides human with high quality protein, fat and fatty acids (FA), including the essential omega-3 FA and carbohydrates in form of glycogen, but also minerals and vitamins such as copper, iodine, iron, magnesium, manganese, potassium, selenium, sodium, zinc, and vitamin A and B-complex (Ahmad et al., 2018). Moreover, livestock farming systems promote food security, employment and rural economies, forest-fires control measures, cultural identity, and social services. In addition, livestock species, especially pig and poultry, have already reached high-performance levels, and the meat production increased worldwide by 44% between 2000 and 2019 (FAO, 2021). However, the efforts to promote the sustainability of this production sector must be intensified to guarantee its continuity and promote the consumer confidence.

Nutrient losses via gas emissions or mineral load are associated partly with the inefficiency of the production system, including animals and manure management, and the oversupply of dietary nutrients to the animals. The conversion efficiency of dietary nutrients into animal products is relatively low, especially for CP that is about 30% (Dourmad et al., 1999; Han et al., 2001), being one of the most expensive ingredient in the current feeds. However, there are inevitable losses that must be considered derived from the physiological functions of digestion and metabolism, associated with catabolism (i.e., whole-body turnover), intestinal cell-desquamation and endogenous secretions (NRC, 2012; van Milgen et al., 2008).

Current strategies to improve livestock production sustainability are focused on genetic selection and precision livestock farming. From a nutritional perspective, precision feeding systems aim to increase the animal utilization of dietary nutrients by optimizing diets to the estimated nutrient requirements among individuals and over time (Pomar and Remus, 2019). Considering that concentration of most nutrient requirements decrease proportionally throughout the growing-finishing period, the current swine feeding system consists of a multi-phase feeding management (NRC, 2012, 1998). This feeding system consists of changing the diet at certain growth stages to adjust the nutritional content according to the new needs, thus maximizing animal efficiency and minimize nutrient excretion by over-supplementation. The three-phase feeding system is generally implemented in the Europe countries to cover the entire growing-finishing period (Aarnink and Verstegen, 2007; Agostini et al., 2014), however, this method shows inefficiencies due to the relatively long supply time. The amount of nutrients received from most of the animals exceeds their real requirements, and these nutrients will be later excreted in the environment. Indeed, when Pomar et al. (2014) employed a daily multi-phase feeding system, there was an 11.7% reduction in nitrogen excretion compared to a three-phase feeding management promoted by the gradually reduction in protein supply (–7.3% protein intake). In addition, the daily multi-phase feeding resulted in a 1% reduction in the simulated feeding costs, although no effects in pig production performance were detected (Pomar et al., 2014). However, as the number of feeding phases increases, so does the difficulty in its management and the economic cost of adapting the facilities (Pomar et al., 2014).

Besides BW or age of the animals, sex condition, genetics (Brossard et al., 2019) or health status, there is a large and relevant individual variation in nutritional requirements, which contributes to the differential efficiency in which each pig utilizes nutrients for productive purposes (Remus et al., 2021; van Milgen et al., 2008). This variability is not accounted for in the current feeding management and formulation process. Growing-finishing pigs are commonly reared in large size groups with varying number of individuals per pen, all receiving the same feed and through the same feeding system (e.g., manual or automatic feeders, individual or group feeders). In addition, formulation is a large-scale process of designing feeds that cover the entire feeding-phase group of a given farm, or even several farms, in which current

estimates of nutrient requirements are based on standardized mathematical models, considering as reference the average pig model (Andretta et al., 2021).

In particular, the estimation of AA requirements is based on the ideal protein criteria (van Milgen and Dourmad, 2015) that consists of firstly determining requirements of lysine, the first-limiting AA in swine, calculated as a function of several production indicators (e.g., average daily feed intake, average daily gain, BW, protein deposition). The requirements of the remaining AA are then predicted as a fixed rate relative to lysine (FEDNA, 2013; NRC, 2012). In detriment to this method, differences in lysine requirements between individuals have been found (van Milgen and Dourmad, 2015). Moreover, the dose-response studies employed to determine AA requirements has major drawbacks associated with the AA interactions (Remus et al., 2021; van Milgen and Dourmad, 2015), and the variable estimates on AA requirements due to the varying analytical procedures and sample preparation methods (National Research Council, 2012). Therefore, different models have proposed to be used to determine nutrient requirements in swine.

Methodologies centered in the study of protein turnover, including protein synthesis and protein degradation, aim to investigate the physiology of both processes that regulate the protein metabolism and total protein mass, and thus implicates AA and energy requirements. The application of stable isotope tracers make possible the evaluation of the fractional synthesis rates (FSR) to quantify the amount of AA consumed by biological pathways and the rates at which this occur (Wilkinson et al., 2021), thus, it would be used to quantify protein and AA requirements. However, in addition to synthesize proteins, other functions of AA such as cell signaling, gene expression, and metabolic regulation must be considered (Wu et al., 2014). Although the implementation of these methods to feed formulation requires careful consideration, the FSR study in this thesis was redirected to evaluate potential differences in these molecular mechanisms between two distinct producing types of pigs, and the tissues or protein pools responsible for this variation.

3. Meat quality

Besides improving animal efficiency, the pork industry must satisfy consumer demands and preferences in order to guarantee the continuity of meat product

consumption. However, enhancing organoleptic properties of pork is a relatively recent goal. Prior to the 21st century, the predominant focus was on economic performance, where genetic selection for faster growth rates, higher lean-to-fat ratio and feed price minimization were prioritized (Ngapo and Gariépy, 2008). These strategies induced the decline of intramuscular fat (IMF) levels, fat quality and meat taste, and there was a consequent demand by consumers to increase meat flavor to return to that of meat consumed previously (Ngapo and Gariépy, 2008). Quality is currently a critical issue, which requires obtaining safe meat products with a more desirable taste, texture, and nutritional properties. Furthermore, these preferences depend on the animal species and the sociocultural background of consumers, and thus differ between countries or even between regions within the same country (Joo et al., 2013). Therefore, obtaining adequate meat quality requires recognition of the essential traits and the regulating factors.

Although it is not easy to define meat quality, fat is one of the components commonly associated with health and eating value, and its content and characteristics are important determinants of product acceptability (Font-i-Furnols and Guerrero, 2014). In terms of fat content, meat preferences differ between market segments. As for fresh products, leaner meat is generally demanded, which is attributed to the increasing concern for a healthier lifestyle (Papanagiotou et al., 2013). However, the use of lean meat for premium dry-cured products has clear disadvantages, including higher seasoning losses, higher salt intake, lower oxidative and proteolytic stability, and lower sensory quality (Čandek-Potokar and Škrlep, 2012). This type of meat products are especially important in the Southern Europe or Mediterranean countries, where IMF, also referred as marbling, is one of the most important traits associated with increased sensory quality attributes, such as taste, tenderness, and juiciness (Papanagiotou et al., 2013; Wood et al., 2008), as well as water holding capacity (Čandek-potokar et al., 1998). However, in some regions or for some groups of population the perception of marbling is misunderstood, or not perceived as positive quality trait (Cilla et al., 2006; Liu et al., 2021; Ngapo et al., 2013).

Fatty acid composition of meat is also of great importance from a sensory, nutritional, and technological perspective. Saturated and monounsaturated FA have been associated with favorable flavor preference (Cameron et al., 2000). However, the

negative effects of saturated FA on the human cardiovascular health lead professional health authorities to promote minimizing their consumption, replacing them with unsaturated FA, especially monounsaturated FA (Hammad et al., 2016). Moreover, the greater the unsaturation rate of FA, the greater the oiliness and softness of meat, and tendency to oxidation and rancidity of fat (Wood et al., 2008).

Therefore, to establish strategies to improve and optimize the fat deposition profile and FA composition it is necessary to understand the mechanisms involved in fat metabolism and develop a dynamic model of lipid development. Adipose tissue develops mostly during the later stages of pig growth, mainly through adipocyte hypertrophy promoted by the progressive storage of FA as triglycerides (Kouba and Sellier, 2011). The first fat depot to develop is the visceral fat, followed by subcutaneous (SC), intermuscular, and IMF (Bosch et al., 2012). Deposited FA derive from two sources, directly from dietary FA or from *de novo* synthesis, this latter being the main source of incorporated fat (around 80 %) (Dunshea and D'Souza, 2003; Kloareg et al., 2007). Certain FA (e.g., most of polyunsaturated FA) are unchanged during digestion, absorbed in the intestine and incorporated into the blood stream through which they reach the tissue where they will be stored (Wood et al., 2008). Fat biosynthesis in pigs, occurs mostly in adipose tissue (O'Hea and Leveille, 1969), in which FA are synthesized *de novo* from glucose (i.e., lipogenesis), from pre-formed FA (e.g., non-esterified FA) or from the transformation of other FA (Dunshea and D'Souza, 2003). These latter processes rely on the activity of specific lipogenic and lipolytic enzymes, of which the Stearoyl-CoA desaturase (SCD) lipogenic enzyme is highlighted in this work.

Deposited monounsaturated FA can come either from feed or by desaturation of saturated FA. The monounsaturated oleic acid (C18:1 *c*9) is the main FA in meat and can result from desaturation at Δ^9 position of the saturated stearic acid (C18:0) by the catalytic activity of the SCD enzyme (Dunshea and D'Souza, 2003; Wood et al., 2008). The SCD activity is modulated by a polymorphisms in the promoter region of the *SCD* gene (rs80912566), in which the *SCD*_T allele enhances FA desaturation in comparison with the alternative allele *SCD*_C (Estany et al., 2014), without modifying the IMF content.

4. Intestinal microbiota

With the aim to improve feed efficiency in farm animals, the intestinal microbiota has received significant attention, as it is recognized to play relevant roles in nutrient digestion and metabolism (Gardiner et al., 2020; X. Wang et al., 2019), immune system development, and regulation of host gene expression (Niederwerder et al., 2016; Richards et al., 2005). Therefore, the huge potential of its modulation, especially through nutrition, can contribute to economic and environmental improvements in swine production. The intestinal microbiota is a complex and dynamic ecosystem composed of fungi, viruses, archaea, but predominantly thousands of microbial species, which inhabit the gastrointestinal tract and constantly adapt to the physiological status of the host and its environment (Park et al., 2014).

Microbiota is responsible for the fermentation of certain dietary fiber carbohydrates (i.e., non-starch polysaccharides, non-digestible oligosaccharides, resistant starch, and lignin) and proteins that resist enzymatic digestion in the proximal intestine, occurring mainly in the cecum and colon compartments. Bacterial populations degrade the complex polymers through bacterial enzymes and ferment the resulting sugars and AA into organic acids, mostly short chain FA, and produce CH_4 and CO_2 gases. The fermentability of the dietary fiber varies among feed ingredients, which in turn, affects microbial diversity and function, as well as the resulting metabolites (Williams et al., 2017). Short chain FA are predominantly composed of acetate, propionate and butyrate, with an approximate proportion of 60%, 25% and 15%, respectively (Li et al., 2021). These compounds are responsible for most of the beneficial effects attributed to dietary fiber, such as intestinal health, whole-body energy homeostasis, and nutrition (De Vadder et al., 2014; Li et al., 2021).

Associations between intestinal microbiota and body fat composition have also been found (He et al., 2016; Yue Wang et al., 2018) due to its influence on fat metabolism and energy harvest. Several aspects have been studied to evidence this connection, including the predominance of two phylum-level bacteria (Firmicutes and Bacteroidetes), diversity indices, and the appearance of specific bacterial taxa (Xiulan Guo et al., 2008). He et al. (2016) estimated that traits associated with fatness, such as backfat thickness and fat mass, are explained by 1.55 – 2.73% by gut microbiome,

and recognized various microbial taxa related to these traits. Furthermore, Fang et al. (2017) also identified microbial taxa associated specifically with IMF content. Interestingly, some bacterial taxa associated with fatness traits in pigs had been identified with their involvement in the fermentation of non-digested dietary carbohydrates and the production of short chain FA, especially butyric acid (He et al., 2016). In this regard, short chain FA are identified as key signaling molecules that modulate adiposity and energy expenditure, by which fat deposition is reduced (He et al., 2016; Jiao et al., 2020). In terms of energy harvest capacity, short chain FA are considered an important source of energy for both bacteria and host, contributing to 10 – 13% of the total energy requirements of pig (Bai et al., 2022). Butyrate serves primarily as energy source for colonocytes (60 – 70% of energy requirements) when hydrolyzed by the same cells (Chambers et al., 2015; Williams et al., 2017), and also activates intestinal gluconeogenesis. Propionate and acetate are substrates for hepatic lipogenesis and gluconeogenesis in the intestine and liver through different metabolic pathways (De Vadder et al., 2014; Williams et al., 2017). These two latter compounds also participate in the appetite regulation through release of certain hormones, mostly leptin, an adipose-derived hormone responsible for satiety through gut-brain neural circuits (Jiao et al., 2020). Moreover, acetate downregulates the expression of genes related to FA biosynthesis (e.g., FA synthase, acetyl-CoA carboxylase and sterol regulatory element binding protein 1c), whereas butyrate upregulates the expression of genes that promotes FA oxidation (e.g., lipase hormone-sensitive and carnitine palmitoyl-transferase-1α) (Jiao et al., 2020).

OBJECTIVES

This thesis **aims to investigate the effects of pig producing type, growth phase and a moderate dietary crude protein restriction in the digestive efficiency, protein and fat metabolism, microbiota profile and emission of pollutant gases**.

To reach this general objective, the following specific goals were developed to test the effects of a) producing type, b) growth phase and c) moderate crude protein restriction:

1. To evaluate the digestibility, absorption, and subsequent utilization of amino acids for protein synthesis in selected tissues, including fractional and absolute rates, along with nitrogen balance to estimate variation in its retention or waste.

2. To characterize the process of direct deposition of dietary fatty acids by monitoring their ingestion, digestion, absorption, and incorporation in several tissues, along with *de novo* synthesis by SCD enzyme activity through supplementation of a D-labelled fatty acid in the diet.

3. To analyze possible differences in the gut microbiome structure along the three intestinal segments, ileum, cecum, and distal colon, in addition to test the degree of interaction between genera.

4. To measure pollutant gas emissions, including ammonia and greenhouse gases, along with the characterization of microbial composition and its correlation with performance parameters and/or nutrient digestibility.

METHODOLOGY

This section outlines the methodology used in the assays intended for this thesis, as it is described in more detail in each of the chapters that compose it.

1. Animals, diets and experimental design

The care and use of the animals were in accordance with the Spanish Policy for Animal Protection Real Decreto 53/2013, which complies with the European Union Directive 2010/63 on the protection of animals used for experimental purposes. In addition, all experimental protocols and procedures were approved by the Ethics Committee for Animal Experimentation of the University of Lleida, under Project License CEEA 09-05/16.

The pigs used in this thesis belonged to two pig producing types (PT), the lean type and fatty type. The lean type consisted of entire males from the crossbreeding of Pietrain sires and Landrace × Duroc dams, while the fatty type consisted of purebred Duroc barrows. All pigs were genotyped by allelic discrimination assay (Estany et al., 2014), and Duroc pigs were selected to be homozygous for the *SCD*_T and *SCD*_C allele for the *SCD* rs80912566 genotype. The experimental procedures were performed identically on animals of two different growth phases (GP), when pigs were in their growing phase (84.17 ± 1.269 days of age; μ ± SE) with an average BW of 28.42 ± 0.861 kg; and when pigs were in their fattening phase (153.58 ± 2.054 days of age) with 87.40 ± 1.256 kg BW.

For each GP, two experimental diets were formulated containing a difference of two percentage units of CP concentration. The two experimental diets were isocaloric and met the nutrient requirements recommended by the Fundación Española para el Desarrollo de la Nutrición Animal (FEDNA, 2013), being supplemented with crystalline AA to ensure a similar concentration of essential AA between experimental groups. In addition, the diets contained titanium dioxide (TiO_2) as an indigestible marker to determine nutrient digestibility, and deuterium (D)-labeled stearic acid (d_{35}-C18:0); the latter only during the last six experimental days.

After the diet adaptation period, pigs were weighted individually, catheterized in the right external jugular vein, and allotted in individual metabolic cages for 6 days. During the 6-day period, feed and water intake, together with fecal and urinary excretion were measured daily, and feces and urine (in H_2SO_4) were collected on the

last 3 days. Blood samples were also obtained daily for the last 5 days. On the last day, pigs were subjected to a flooding dose technique to determine protein FSR (*Chapter II*). Immediately after the last blood sampling, the pigs were euthanized, weighed, and samples from *longissimus dorsi*, *biceps femoris*, *semimembranosus*, and SC were collected from the left half carcass, along with samples of liver, duodenum, and digesta contents from the ileum, cecum, and colon segments. The loin and leg of the right half carcass were cut and frozen for further dissection.

2. Analytical procedures

2.1. Determination of chemical composition (*Chapter II* and *Chapter III*)

For the determination of apparent total tract digestibility (ATTD; *Chapter II*), dry matter (DM) (ref. 934.01), organic matter (OM) (ref. 942.05), and ether extract (EE) (ref. 920.39) following the procedures of the Association of Official Analytical Chemists (AOAC, 2006), together with the CP (nitrogen × 6.25) content analyzed by Dumas combustion (ISO, 2008), and the proportion of neutral detergent fiber (NDF) determined according to Van Soest et al. (1991), were analyzed in feces and feed. Moreover, for the determination of apparent ileal digestibility (AID; *Chapter II* and *Chapter III*), DM (ref. 934.01) and CP (ISO, 2008) were also analyzed in the ileal digesta, along with AA (Colgrave et al., 2008a; Guo et al., 2013) and FA (Tor et al. (2021)) composition. Ashes from feed, feces and ileum were analyzed by inductively coupled plasma mass spectroscopy following Darambazar (2019), for the determination of TiO_2 as a digesta marker.

Apparent nutrient digestibility was calculated using the nutrient-to-marker ratio, as follows:

$$y = 1 - \left(\frac{\text{marker}_{\text{feed}}}{\text{marker}_{\text{ds}}} \times \frac{Z_{\text{ds}}}{Z_{\text{feed}}} \right)$$

where y represents the apparent digestibility coefficient of a nutrient at a certain segment; Z_{ds} and Z_{feed} represent the nutrient concentration in the digestive segment and in the feed, respectively; $marker_{feed}$ and $marker_{ds}$ represent the concentration of the marker (TiO_2) in the feed and in the digestive segment, respectively.

2.2. Determination of fractional and absolute synthesis rate (*Chapter II*)

Free AA were obtained from plasma (Piraud et al., 2005a), viscera (i.e., liver and duodenum) and skeletal muscles (i.e., *longissimus dorsi* and *biceps femoris*) (Qin et al., 2015a). The remaining pellet of the tissue samples was subjected to hydrolysis to obtain protein-bound AA (Colgrave et al., 2008a). After centrifugation, aliquots of the hydrolyzed sample were reserved to be evaporated under vacuum, diluted in water/acetonitrile (15/85 v/v), and filtered through a hydrophilic PTFE membrane. Amino acid analysis were performed by ultra-performance liquid chromatography (UPLC) coupled to a triple quadrupole mass spectrometer (MS) following Guo et al. (2013). The multiple reaction monitoring method (MRM) included phenylalanine and $^{2}H_5$-phenylalanine transitions, previously checking the absence of cross signal between their channels. The results were processed using QuanLynx software.

The percentage of tissue protein synthesized per day (FSR, %/day) was calculated with the following equation:

$$\mathrm{FSR} = \left(\frac{\mathrm{MPE_{bound}}}{\mathrm{aveMPE_{free}}} \times \frac{100}{\mathrm{t}} \right)$$

where MPE_{bound} is the enrichment in $^{2}H_5$-phenylalanine, expressed as molar percent excess (MPE) of protein-bound phenylalanine in tissues; $aveMPE_{free}$ is the average MPE of the free phenylalanine pool in the same tissue, between time 0 and the end of the sampling period; *t* is the labeling time in days.

The liver, *longissimus dorsi* and *biceps femoris* muscles were weighted just after dissection, and the CP (nitrogen × 6.25) content in these same tissues was determined by Dumas combustion (ISO, 2008) for the determination of the absolute synthesis rate (ASR), which is the amount of protein synthesized per day (g/day) and calculated as:

$$\mathrm{ASR} = \frac{\mathrm{FSR}}{100} \times \text{tissue total protein content (g)}$$

2.3. Determination of Allometric tissue growth (*Chapter III*)

The loin and leg from the right half carcass, cut according to Walstra and Merkus (1996) standards, together with the liver, were weighted. The leg was dissected into muscles, bones, skin and SC tissue, intermuscular adipose tissue, and remainder

(blood vessels, ligaments, and tendons). The *gluteus medius* and *semimembranosus* skeletal muscles were reserved for determination of IMF content by Sohxlet method.

The relative growth coefficient (k) of each dissection component was obtained as the slope of the regression in relation to body weight from the log-transformed allometric equation $\left(y = ax^{k}\right)$, as follows:

$$\log\ (y) = k \log\ (x) - \log\ (a)$$

where log *y* is the weight of each leg component, log *x* is the body weight, log *a* is the intercept, and *k* is the allometric growth coefficient.

2.4. Determination of fractional incorporation and unsaturation rate (*Chapter III*)

Freeze dried samples of liver, *longissimus dorsi* and *semimembranosus* muscles, together with SC, and plasma were subjected to total lipid extraction (Folch et al., 1957) and subsequent saponification (Aldai et al., 2006). After neutralizing the KOH in the saponification solution with glacial acetic acid, FA were extracted with petroleum spirit, evaporating the solvent under vacuum. Lipid samples were re-diluted in isopropanol, filtered through a hydrophilic PTFE membrane and analyzed by UPLC coupled to a triple quadrupole MS. The MRM method included the unlabeled and D-labeled stearic acid (C18:0; d_{35}-C18:0) and oleic acid (C18:1 *c*9; d_{33}-C18:1 *c*9), previously checking for cross signal absence between channels. The results were processed with QuanLynx software.

The d_{35}-C18:0 enrichment in plasma and tissues was expressed as MPE. The fractional incorporation rate (FIR; %/day) of d_{35}-C18:0 in fat depots and liver from that available in plasma was calculated as follows:

$$\text{FIR} = \left(\frac{\text{MPE } d_{35}\text{-C18:0}_{\text{tissue}}}{\text{aveMPE } d_{35}\text{-C18:0}_{\text{plasma}}} \times \frac{100}{t} \right)$$

where *MPE* d_{35}*-C18:0*$_{tissue}$ is the enrichment in d_{35}-C18:0 of stearic acid in tissues; *aveMPE* d_{35}*-C18:0*$_{plasma}$ is the average d_{35}-C18:0 enrichment of stearic acid in the plasma pool during the labeled diet consumption; and *t* is the labeling time in days.

Apparent and real fractional unsaturation rates (FUR) in SC were calculated as the percentage of C18:0 and d_{35}-C18:0 unsaturated per day (%/day) to C18:1 *c*9 and d_{33}-C18:1 *c9*, respectively. Calculated as follows:

$$\text{Apparent FUR} = \left(\frac{\text{C18:1 c9}_{\text{tissue}}}{\text{C18:0}_{\text{tissue}}} \times \frac{100}{t}\right)$$

$$\text{Real FUR} = \left(\frac{\text{d}_{33}\text{-C18:1 c9}_{\text{tissue}}}{\text{d}_{35}\text{-C18:0}_{\text{tissue}}} \times \frac{100}{t}\right)$$

Where $C18{:}1\ c9_{tissue}$ and $C18{:}0_{tissue}$ of apparent FUR are the enrichment in C18:1 c9 and C18:0 FA in the same tissue, respectively; while in the real FUR, d_{33}-$C18{:}1\ c9_{tissue}$ and d_{35}-$C18{:}0_{tissue}$ are the enrichment in d_{33}-C18:1 c9 of oleic acid and d_{35}-C18:0 of stearic acid in the same tissue, respectively; and *t* is the labeling time in days.

2.5. DNA extraction, sequencing and bioinformatics

Microbial genomic DNA extraction from freeze dried digesta of ileum, cecum and distal colon was performed using DNeasy PowerLyzer PowerSoil Kit (Qiagen, Hilden, Germany). Mock community DNA was included as a positive control for library preparation. Primers 341F and 805R were used for amplification of samples from the V3-V4 region of bacterial and archaeal 16S rRNA. PCR products were purified using AMPure XP beads (Beckman Coulter, Nyon, Switzeland). The pair-end sequencing was conducted following an Illumina Miseq sequencing 300 × 2 approach. DADA2 software was used for quality control filtering and OUT binning of the resulting sequences; and taxonomic assignment of phylotypes was performed using Bayesian classifier trained with Silva database. Taxonomic assignment of phylotypes and sequencing of DNA and bioinformatic procedures were carried out by Microomics Systems, S.L.

CHAPTER I

Protein turnover in swine: A review of interacting factors

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Abstract

Protein metabolism is a complex process involving multiple elements and characterized by the balance of two continuous energy-consuming processes, synthesis and degradation, which is defined as protein turnover that determines protein deposition in tissues. Each type of protein has its own structure, function and lifespan, which explains the differences in protein turnover rates between tissues. Although the liver and intestine have been studied extensively for their important role in protein digestion, absorption and metabolism, the study of protein metabolism has focused mainly on skeletal muscle tissue to understand the basis for its growth. Due to the high adaptability of skeletal muscle, its protein turnover is greatly affected by different intrinsic and extrinsic factors, contributing to carcass lean-yield and animal growth. Amino acid labeling and tracking using isotope tracer methodology, together with the study of myofiber type profiling, signal transduction pathways and gene expression, allow the analysis of these mechanisms from different perspectives. Deepening the understanding of modifying factors and their possible interaction may contribute to optimize animal nutrient requirements (i.e., protein and amino acids), and thus advance the precision feeding strategy.

Keywords

amino acids, mechanistic target of rapamycin, muscle, pig, protein synthesis, protein turnover

1. Introduction

Tissue protein is continuously submitted to a balance between two energy-consuming processes, synthesis, and degradation. Both processes, in turn, are subjected to anabolic and catabolic stimuli and their balance is defined as protein turnover, regulating the protein mass and potential size changes. When the balance is favorable to protein synthesis, protein accretion and tissue hypertrophy occurs. However, protein loss or tissue atrophy results from either increased rates of degradation or reduced rates of protein synthesis (Millward et al., 1976; Ten Have et al., 2019). Moreover, each protein has its own structure, function, lifespan, and once synthesized, can undergo structural changes, such as being added, reduced in size, or even destroyed. Rothman (2010) proposed that the stable concentration of manufactured and destroyed proteins may be the equilibrium by mass between the physiologically matured proteins, and those forms predisposed to degradation (i.e., modified or altered proteins).

Interest for protein metabolism and its research began in the late 1930s in order to understand the basis of tissue growth or loss, but also because of its relationship with amino acid (AA) and energy requirements. It should be considered that protein synthesis is the most costly biosynthetic process, accounting for approximately 17% of the metabolic rate of the whole animal energy cost, about 0.8 kcal/g of protein for pigs (Garlick et al., 1976). Furthermore, neonatal pigs are considered the most suitable model for pediatric nutrition and metabolism, and therefore, the study of its protein turnover has been an important issue of research in clinical medicine for decades (Odle et al., 2014).

The application of stable isotope tracers (generally containing ^{13}C, ^{15}N, or ^{2}H) has contributed to the monitoring of transport, downstream metabolism, and turnover, as these molecules are chemically and functionally identical to the tracee (i.e., unlabeled molecules). The different number of neutrons in the atomic nucleus between tracee and tracer permit them to be distinguished by their differential molecular weight, through analytical platforms such as gas or liquid chromatography coupled to mass spectrometry, or nuclear magnetic resonance spectroscopy (Violante et al., 2019). The essential AA phenylalanine has been one of the preferred tracers, especially in muscle,

since its metabolism basically consists of its protein incorporation and release, with almost absence of intermediary metabolism (Wilkinson et al., 2021). Furthermore, the precursor-product approach is considered the 'gold standard' method for measuring protein metabolism and consists of the administration of a stable isotope tracer, flooding the AA pool and reaching the isotopic steady state (i.e., constant labeling pattern). This molecule will act as a metabolic substrate, and subsequent tissue sampling is needed for measurements of the amount of tracer and the rates at which it reaches the tissue of interest or 'product', as well as the pool where the polymer or 'precursor' is manufactured that is the muscle fluid pool or blood. With this approach, fractional synthesis rate (FSR) is calculated and defined quantitatively as the fraction of protein synthesized per unit of time (%/t), and these estimations would allow to define basal protein and AA requirements (Wilkinson et al., 2021), although other essential functions of AA (cell signaling, gene expression, and metabolic regulation) should also be taken into account (Wu et al., 2014). In parallel, signal transduction pathways associated with muscle growth regulation and microarray methods allow the identification of genes whose transcription is altered during muscle growth (Ayuso et al., 2016; Benítez et al., 2021; Liu et al., 2018).

2. Protein turnover

2.1. Protein synthesis

Protein synthesis is a complex process that requires the coordination of a large number of macromolecules, including nucleic acids, enzymes, and regulatory factors. Synthesis stimulation has been predominantly associated with the mechanistic target of rapamycin (mTOR) signaling pathway, which is a highly conserved serine/threonine protein kinase found in two distinct complexes, mTORC1 and mTORC2, as the catalytic subunit. The activity of mTOR is promoted by its phosphorylation at Ser^{2448}. While mTORC2 regulates actin cytoskeleton reorganization for spatial cell growth, mTORC1 mediates cell growth by regulating translation and transcription processes, together with ribosome biogenesis, nutrient transport and autophagy. Along with mTOR, the mTORC1 consists of two required subunits, the Raptor (mTOR- associated regulatory protein) and mLST8 (mammalian lethal with Sec13 protein 8), which let the translocation of mTORC1 to the lysosome

surface where it is subsequently activated (Hall, 2008). Raptor enables AA sensing and the recruitment of two of the major mTOR phosphorylation substrates, by which mTORC1 promotes the translational initiation process, the p70 ribosomal protein S6 kinase 1 (S6K1) and the eukaryotic initiation factor (eIF4E) binding protein 1 (4E-BP1). Phosphorylation of 4E-BP1 prevents its own binding to eIF4E, which would lead to the formation of the inactive 4E-BP1-eIF4E complex and enables the formation of the active eIF4E-eIF4G complex responsible for recruiting mRNA to the 43S ribosomal complex, and the subsequent protein synthesis (see Figure 1). Besides, phosphorylation of S6K1 results in phosphorylation of ribosomal protein S6 (rpS6), which regulates several targets involved in translation initiation and elongation, such as translation of ribosomal proteins and other components of the translational apparatus (Rudar et al., 2019; Saxton and Sabatini, 2017). Further molecular processes are far beyond the scope of this review article; for more details, the reader is directed to excellent reviews on this topic (Rudar et al., 2019; Saxton and Sabatini, 2017).

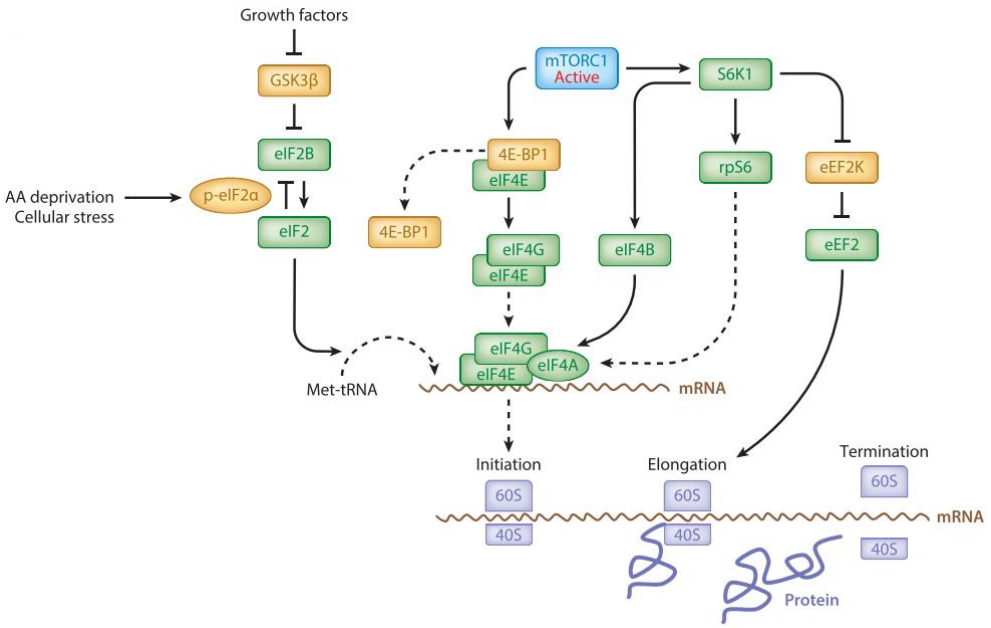

Figure 1. Regulation of translation and protein synthesis downstream of mTORC1 in neonatal pig neonatal pig skeletal muscle. Positive regulators are shown in green; negative regulators are shown in yellow. The dashed arrows indicate movement/transport of the amino acids from outside to inside the cell, or vice versa, and within the cell. Abbreviations: 4E-BP1, eukaryotic initiation factor 4E binding

protein 1; eEF2, eukaryotic elongation factor2; eEF2K, eukaryotic elongation factor 2 kinase; eIF, eukaryotic initiation factor; GSK3β, glycogen synthase-3 β; mRNA, messenger RNA; rpS6, ribosomal protein s6; S6K1, p70ribosomal protein S6 kinase 1; tRNA, transfer RNA (Rudar et al., 2019).

Once synthesized, proteins are released from the synthetic machinery of the ribosome into the cytosol or other cellular compartments. The process of synthesis by the ribosome is discontinuous, with no more than one chain being manufactured in the same ribosome. When the protein is released, the ribosome becomes inactive due to the dissociation of the two major subunits, until a new mRNA molecule arrives to initiate the process. The capacity for protein synthesis depends on the abundance of ribosomes and their efficiency in translating mRNA into proteins; in addition, Garlick et al. (1976) reported a strong correlation (0.77) between RNA content and FSR.

2.2. Protein degradation

Three mechanisms of degradation of old or mistaken proteins exist in eukaryotic cells, (i) the ubiquitin-proteasome system, (ii) the autophagy-lysosome, and (iii) the calcium-mediated calpains and caspases. Degradation of most individual muscular, cytosolic and nuclear proteins is mediated by the ubiquitin-proteasome system and calpains. The 20S proteasomes are complex proteins found in the cytosol and nucleoplasm that contain degradative enzymes and regulatory proteins. Errors in protein structure arising from environmental causes, transcription or translation mistakes are detected by ubiquitin, which are small proteins that bind to specific molecular errors and cause covalent modifications, becoming targets for protein destruction. This labeling system is regulated by certain ligases responsible for the activation of ubiquitin (E1), its conduction to the problematic proteins (E2), and its binding (E3) to make the protein recognized by the proteasome (Figure 2; Brooks and Myburgh, 2014).

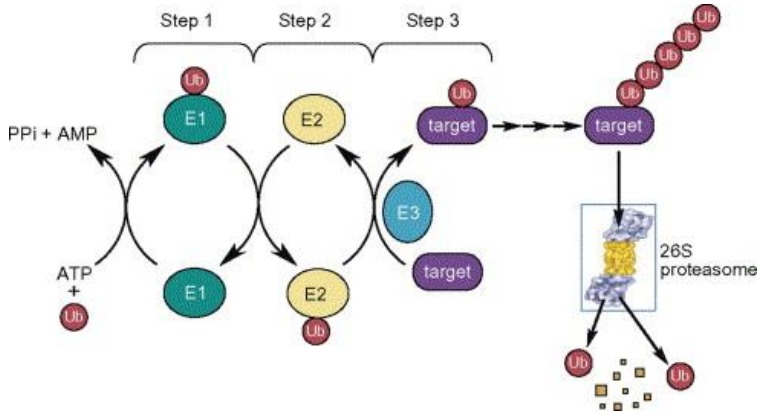

Figure 2. Ubiquitination of proteins targeted to the proteasome. A ubiquitin-activating enzyme (E1) binds ubiquitin, which is then transferred to a ubiquitin-conjugating enzyme (E2); a ubiquitin-protein ligase (E3) helps transfer ubiquitin to the target substrate. Multiple ubiquitin molecules are attached to proteins before recognition and degradation by the 26S proteasome; before degradation, the ubiquitin chain is removed, allowing free ubiquitin molecules to be recycled (Adams, 2003).

On the other hand, calpains are cysteine-proteases that are presented in different isoforms such as μ-calpain and m-calpain. The latter isoforms are ubiquitous in skeletal muscle and mostly inactive under basal conditions, however, their activity depends on certain factors such as free cytoplasmic calcium for their activation, or the presence of the polypeptide calpastatin for their inhibition (Smith et al., 2008).

Degradation of more complex structures (i.e., damaged organelles and protein aggregates), occurs via the autophagy-lysosome system. In this case, the problematic proteins are engulfed into a double-membrane vesicle called autophagosome, which transfers its content into the lysosomes by fusing of membranes of both structures. Lysosomes contains into its lumen lysosomal enzymes and acidic pH (3 – 5) necessary for digestion and degradation (Wesselborg and Stork, 2015). In addition, lysosomes are also capable of sequestering small cytoplasmic residues through invaginations of its membrane giving rise to vesicles, or may even present channels to transport proteins directly into their lumen (Lőrincz and Juhász, 2020). The resulting AA from protein degradation become potential substrates for recycling through biosynthesis process or energy production though catalytically activity.

Oxidative phosphorylation is also a relevant pathway to modulate cellular metabolism, energy homeostasis and cell number. This pathway controls the rates of programmed cell death or apoptosis, and thus protein degradation. Cellular aerobic metabolism regulated by mitochondria generates the production of free radicals such as reactive oxygen (ROS) and reactive nitrogen (RNS) species. These species spontaneously oxidize nucleic acids, lipids, and proteins, modifying its structure, and consequently losing their function or enzymatic activity. Under physiological conditions, cells counteracts this macromolecular damage with antioxidant production or degradation mechanisms, mainly the 20S proteasome system and calpain/caspases (Smuder et al., 2010). Mitochondrial proteins are particularly exposed to this oxidation, and their withdrawal is crucial to maintain cellular integrity. Excessive ROS production due to mitochondrial dysfunction, together with the deficient antioxidant defense of cells, leads to oxidative stress occurring mostly during the aging process or in pathological conditions (Hao et al., 2021). In pig, certain factors that induce oxidative stress have been identified as birth, weaning stress, dietary mycotoxin exposition and other environment or social factors (Hao et al., 2021).

3. Factors influencing protein turnover rates

3.1. Organs and tissue type

The mechanisms responsible for protein turnover vary for each type of protein, resulting in differences among organs and tissues. There is wide evidence that skeletal musculature has a relatively slow turnover rate in comparison with several visceral organs (Bregendahl et al., 2008; Cross et al., 2020; Garlick et al., 1976; Ten Have et al., 2019). According to Garlick et al. (1976), protein FSR of skeletal muscle are 77 – 86% lower than those of spleen, kidney, liver and lungs, and 47% lower than those of the brain. Similarly, skeletal muscle FSR is 76 – 93% lower than liver and 79 – 97% than upper gut sections (duodenum and jejunum), taking in consideration the references collected in Table 1 of the present review. In general, jejunum has the highest FSR, followed by liver, ileum, heart and skeletal muscle (Cross et al., 2020). Nevertheless, skeletal muscle is the major contributor to whole-body protein synthesis in the pig (50%; Huber et al., 2018), as it is the main reservoir of total body mass protein, while the liver contributes about 10% (Garlick et al., 1976).

Early growth and maturation of these more active tissues (e.g., liver, or intestinal tract) have important implications for nutrient availability to peripheral tissues (Reeds et al., 2000). Moreover, increased protein synthesis allows these organs to better adapt to stimuli or environmental changes (Ten Have et al., 2019). However, the higher rates of protein synthesis in specific viscera can also be attributed to its secretory activity, including the synthesis of digestive enzymes by the exocrine pancreas, or the synthesis of mucus or mucins by the mucosal epithelial cells of the gastrointestinal tract (Sans et al., 2004; X. Wang et al., 2007). The latter proteins are released into the intestinal lumen and do not belong to constitutive tissue cells (Reeds et al., 1993). In addition, the mucus and mucins lining the gastrointestinal epithelium are constantly subjected to erosion and proteolytic degradation, which are counteracted by a high protein synthesis activity (130%/day) (Montagne et al., 2004).

Table 1. Fractional synthesis rates (FSR) of skeletal muscle, liver, and upper intestine obtained from pigs of different body weights.

Reference	Body weight (kg)	Genetics [1]	Sex	Method [2]	FSR muscle (%/day) [3]	FSR liver (%/day)	FSR upper gut (%/day)
(Bregendahl et al., 2008)	7.2	YK	gilts	Flooding dose $^{2}H_{5}$-Phe	20.3 (b.f.) 21.8 (l.d.)	91.2	109.5 (prox. jejunum)
(X. Wang et al., 2007)	11.8	LW × LD	Barrows	Flooding dose $^{2}H_{5}$-Phe (i.p)	9.8 (l.d.)	136.8	115.8 (jejunum)
(Deng et al., 2009)	12.0	DU × LD × YK	–	Flooding dose $^{2}H_{5}$-Phe	11.8 (l.d.)	83.5	59.7 (prox. small gut)
(Rivera-Ferre et al., 2005)	23.5	IB	Gilts	Flooding dose $^{2}H_{5}$-Phe	7.8 (l.d.) 8.2 (b.f.)	46.6	60.5 (duodenum)
(Rivera-Ferre et al., 2005)	27.1	LD	Gilts	Flooding dose $^{2}H_{5}$-Phe	6.4 (l.d.) 6.3 (b.f.)	46.0	65.8 (duodenum)
(Sarri et al., 2021)	28.5	DU	Barrows	Flooding dose $^{2}H_{5}$-Phe	6.5 (l.d.) 6.4 (b.f.)	36.0	52.0 (duodenum)
(Sarri et al., 2021)	30.5	PI × LD × DU	Boars	Flooding dose $^{2}H_{5}$-Phe	14.0 (l.d.) 6.9 (b.f.)	57.3	50.2 (duodenum)
(Sève et al., 1993)	64.6	PI × LW	Barrows	Flooding dose ^{13}C-Val	4.1 (l.d.)	43.4	118.9 (duodenum)

Table 1. Continued.

Reference	Body weight (kg)	Genetics [1]	Sex	Method [2]	FSR muscle (%/day) [3]	FSR liver (%/day)	FSR upper gut (%/day)
(Sarri et al., 2021)	86.1	DU	Barrows	Flooding dose $^{2}H_{5}$-Phe	8.7 (l.d.) 7.9 (b.f.)	36.2	47.8 (duodenum)
(Sarri et al., 2021)	91.1	PI × LD × DU	Boars	Flooding dose $^{2}H_{5}$-Phe	9.3 (l.d.) 8.3 (b.f.)	41.9	42.2 (duodenum)
(Huber et al., 2018)	183.0	YK / YK × LD	Sows	Continuous infusion $^{2}H_{5}$-Phe	2.2 (l.d.) 2.4 (g.n)	29.9	–

[1]DU: Duroc; IB: Iberian; LD: Landrace; LW: Large White; PI: Pietrain; YK: Yorkshire; [2]i.p: intraperitoneal; [3]b.f.: biceps femoris; l.d.: longissimus dorsi; g.n.: gastrocnemius

As for the liver, it should be considered that is the main site for AA metabolism (Hou et al., 2020) and it does not only synthesize hepatic proteins, but also plasma proteins such as albumin and acute-phase proteins (McNurlan et al., 1980; Ten Have et al., 2019), although they are released shortly after being synthesized. Furthermore, although skeletal muscle has no secretory activity, a certain proportion of the synthetic activity is applied for the physiological function of the tissue (Reeds et al., 1993). In a recent study, it was found that the elongation process of mRNA translation showed differences of more than 50% between different organs or tissues (Gerashchenko et al., 2021), with the liver being the organ with the highest elongation rate, followed by kidney and skeletal muscle.

Likewise, there are also differences between different types of muscle tissue, with cardiac musculature showing higher rates of protein synthesis than skeletal muscles, which is supported by the continuous and indispensable function of the heart (Garlick et al., 1976; Yuan et al., 2008). It should also be considered that there are differences in protein synthesis rates among different anatomical parts within the same organ, due to distinct structure and functions. In this sense, Garlick et al. (1976) concluded that the medulla of the kidneys synthesized less protein than the cortex, as did the pons of the brain compared to the cerebellum and cerebrum. In addition, differences have been found between segments of the small intestinal in their rates of protein synthesis and degradation, decreasing from proximal to distal regions (Stoll et al., 2000).

3.1.1. Muscle fiber type

There are two main processes by which skeletal muscle mass grows, either by increasing the number of myofibers or by increasing their size, called hyperplasia and hypertrophy, respectively. Myofibers are the functional contractile unit of skeletal muscle, accounting for 75 – 90% of this tissue (Joo et al., 2013). The process of hyperplasia occurs mainly during the fetal period, and the total number of myofibers is considered to be stablished at around 90 days of gestation (Gondret et al., 2020), remaining practically constant during the postnatal period. However, myofiber size appears to remain almost unaltered during the prenatal period, with hypertrophy occurring mainly after birth (Lefaucheur, 2010). Myofiber hypertrophy is accompanied by the appearance of new nuclei that are provided by the satellite cells,

which are myogenic stem cells located under the basal lamina of the myofibers, constantly required for growth and maintenance of muscle tissue. Activation of satellite cells leads to proliferation, becoming myoblasts that differentiate into mononucleated myocytes and further fuse with myofibers or with each other, resulting in multinucleated myotubes and subsequently matured into contractile myofibers (Murach et al., 2018; Zammit et al., 2004). A second population of satellite cells, however, is often in a quiescent state, becoming activated under regenerative or anabolic stimuli such as exercise or damage (Neal et al., 2012), not being influenced by age or sex.

Differences in protein metabolism among skeletal muscles, which differ in their location and functionality, and within the same muscle, have been reported (Beermann et al., 1990; Garlick et al., 1989). It has been attributed to the heterogeneous composition in different types of myofibers, which are characterized by the expression of the myosin heavy chain (MyHC) isoform. Four main types of myofibers (I, IIa, IIb and IIx) are recognized in pigs, which are differentiated by their metabolic enzyme profile, morphological features and contractile properties (Choi and Kim, 2009), although myofibers may also contain more than one MyHC isoform.

Based on the speed of contraction, myofibers are classified into slow-twitch type I fibers and fast-twitch type II fibers (IIa, IIb and IIx), with the following order of shortening speed: I < IIa < IIx < IIb (Fazarinc et al., 2020). Type I fibers are predominantly oxidative and contain a higher number of mitochondria, myonuclei and myoglobin, which is responsible for their red color (Choi and Kim, 2009). These fibers are characterized by a higher resistance to fatigue, the tendency to be smaller and the use of lipids as aerobic metabolic fuel. Type II fibers contain a greater amount of glycogen and glucose, giving them higher glycolytic activity and higher myosin ATPase activity than slow-twitch fibers. While IIb fibers are the most glycolytic type, the IIa and IIx are metabolically intermediate between type I and IIb fibers. The IIb fibers have the most developed T-tubule system, are rich in glycolytic enzymes that allow them faster energy transfer and contraction, although they fatigue easily, and are therefore referred to as fast-fatigable fibers. Type IIx fibers, also called fast glycolytic fiber, exhibit intermediate resistance to fatigue (Schiaffino and Reggiani, 2011), and the fatigue-resistant IIa fibers or fast oxido-glycolytic fibers have higher

oxidative capacity than IIx fiber type. In the pig, deeper postural muscles are generally more oxidative than the more superficial ones, responsible for fast movements, and have higher proportion of type I fibers (Joo et al., 2013).

Although myofiber composition is genetically defined for each individual, myofibers adapt their phenotype and MyHC isoform in response to internal or external factors such as sexual condition, age, nutrition, environment, and physical activity (Fazarinc et al., 2020). In addition, myofibers are dynamic structures and their MyHC expression can be reversibly converted into others by the action of long-non-coding RNAs (lncRNA), as follows: from MyHC I to IIa type, from MyHC IIa to IIx type, and from MyHC IIx to IIb type (Pette and Staron, 2001). The relative composition in myofiber types determines the metabolic properties, and meat characteristics at slaughter of each muscle (Joo et al., 2013; Scheffler and Gerrard, 2007), with protein synthesis rates following this order according to myofiber type: IIb < IIx < IIa ≈ I (Goodman et al., 2011).

On the other hand, Mittendorfer et al. (2005) suggested that other intrinsic factors, such as the amount of RNA and myofiber nuclei, muscle function and location, are more suggestive of the basal rate of protein synthesis. Substantial advances in high-throughput sequencing techniques have identified IGF-1-Akt-mTOR as one of the major pathways regulating, but mostly increases, protein synthesis by modulating translation initiation (Manning and Toker, 2017).

3.2. Developmental stage

Animal growth and development occur continuously beyond reaching sexual maturity, being associated with high rates of protein synthesis and deposition; however, this does not take place uniformly. Whole-body protein synthesis reaches its highest rates during the early postnatal period, related to higher rates in skeletal muscle than in other body tissues (Burrin et al., 1995; Rudar et al., 2019). This phenomenon has been widely documented in different animal species such as pigs (Davis et al., 1996), chickens (Kang et al., 1985), rats (Millward et al., 1975), sheep and humans (Reeds et al., 2000). In addition, neonatal skeletal muscle is characterized by a high glycogen content to ensure sufficient energy in the first moments of the

newborns (Herpin et al., 2002), which decreases rapidly within a few days while lipid and protein content increases.

It is after birth when skeletal musculature is particularly sensitive to anabolic stimuli, such as hormones and nutrient availability (Thivierge et al., 2005), coinciding with the ingestion of colostrum that is highly nutrient-dense and contains a high concentration of growth-regulating compounds (Reeds et al., 2000). Similarly, some vital organs such as the brain and heart have also shown increased rates of protein synthesis when piglets are fed colostrum rather than other substitutes, including mature milk or synthetic formula (Burrin et al., 1997). Increased protein synthesis at this stage has also been associated with a higher RNA-to-DNA ratio and a higher content of cellular ribosome and mitochondria; the later associated with the higher predominance of oxidative fibers in young animals (Skjaerlund et al., 1994). Conversely, many negative regulators are less abundant in the skeletal muscle of these younger pigs (Davis et al., 2008), with mTORC1 suppressing protein catabolism, particularly autophagy (Saxton and Sabatini, 2017). All these aspects result in increased muscle protein deposition and explain the rapid relative growth of these animals, achieving the highest nutritional efficiency for muscle protein accretion. In this sense, Davis et al. (1996) found a decreased FSR in skeletal muscles (longissimus dorsi and gastrocnemius), heart, and liver of piglets between 7 and 26 days of age, whereas no differences were found in the jejunum and pancreas.

Transcriptome studies have indicated that gene expression changes considerably throughout pig life, but especially during the perinatal period (Mohammadabadi et al., 2021). The intense evolution of FSR during this life period is explained by different reasons. First, the decrease in ribosome abundance between birth and weaning (i), together with their lower translational efficiency under feeding stimulation (Reeds et al., 2000; Srivastava, 2017). The significant decrease in the number of satellite cells with age (ii) (Neal et al., 2012). Satellite cells proliferate and differentiate intensively during the postnatal period, contributing to myofiber hypertrophy by fusing with them (Gondret et al., 2020). Finally, modification of the MyHC myofiber profile (iii) (Brocks et al., 2000; Saxton and Sabatini, 2017). During the embryonic and fetal period, the predominant MyHC isoform is slow-twitch type I, although by late gestation the adult fast-twitch types IIa and IIx isoforms appear. It is at birth that a

large part of the type I MyHC isoforms mature to fast-twitch isoforms, and type IIb MyHC isoform also emerge (Gondret et al., 2020). Accordingly, the intensity with which the proportion of slow-twitch myofibers changes to fast-twitch myofibers decreases with pig maturation (Fazarinc et al., 2017).

Regarding muscle protein degradation rates, although they exist in the neonatal period, are much lower than those of synthesis and decay more slowly than synthesis rates as the animal grows (Fiorotto et al., 2000; Skjaerlund et al., 1994).

Skeletal muscle development throughout growth and adulthood is affected by its genetic background and by extrinsic factors to which individuals are constantly subjected (e.g., nutrition, hormones, activity, injury) (Wallace et al., 2016). It is generally suggested that protein synthesis and its regulatory machinery decreases throughout animal maturity (Attaix et al., 1988; Connors et al., 2008). In this sense, the decrease in FSR between growing lambs and mature ewes obtained by Connors et al. (2008) was attributed to downregulation of translation process, with decreased RNA concentration and RNA:protein ratio, which is a measure of synthetic capacity. In this sense, recent studies performed mostly in mice suggest that the decrease in protein synthesis is mainly attributed to changes in different steps of the translation process (Gonskikh and Polacek, 2017), both in the translation initiation and elongation (Anisimova et al., 2020; Gerashchenko et al., 2021). These changes are also evident at gene expression and transcriptome level (Anisimova et al., 2020), by downregulation of ribosome biogenesis and components of the protein synthesis machinery, resulting in a gradual decrease in translation efficiency.

Upregulation of genes associated with inflammation, extracellular matrix (ECM) development and lipid metabolism has been identified as the animal matures, to the detriment of genes involved in myofibers proliferation and differentiation. (Ayuso et al., 2016). Subsequently, Benítez et al. (2021) described that as the maturity progresses, the expression of genes involved in the chronological processes of myogenesis changed, such that upregulated genes in transition pigs were more associated with proliferation and early stages of differentiation, whereas upregulated genes in grower pigs were more involved in advanced differentiation, hypertrophy, and ECM organization. Development of ECM is generally associated with

hypertrophy of muscle growth since it is involved in signaling pathways, activating/inhibiting enzymatic activities, being the site of binding hormones and enzymes, regulating the interaction of several ligands with their receptors, and also regulating the mechanical support between muscle cells (Csapo et al., 2020). To all these changes must be included the accumulation of molecular damage in cells and proteins with aging, preventing their normal functioning, and causing the appearance of aggregates or toxic products (Anisimova et al., 2018; Hipp et al., 2019).

3.3. Genetics

Comparison of protein metabolism between breeds is not a simple issue since they can exhibit differences in growth potential and tissue development, which implies substantial changes in protein turnover and nutritional requirements throughout the different growth stages. Therefore, it is important to work with comparable ages or physiological states. Most of the comparative studies between pig breeds aim to compare protein metabolism between leaner or modern breeds versus fatty or autochthonous pig breeds, due to potential differences in the quality of their meat products.

In an initial study, Rivera-Ferre et al. (2005) found that fatty Iberian pigs had a higher skeletal muscle FSR than leaner purebred Landrace pigs. Considering that Landrace pigs had a larger protein pool than Iberian, they concluded that the higher FSR registered in Iberian pigs was unrelated to higher protein accretion but was associated with higher rates of protein degradation. Consistent with this study, lower protein FSR were reported in fast-growing genotypes in other species such as steers (Lobley et al., 2000). However, in chickens, higher growth rates in fast-growing strains were associated with lower fractional degradation rates (FDR) compared to slower-growing strains (Klasing et al., 1987). Subsequently, when Rivera-Ferre et al. (2006) studied whole-body protein synthesis and degradation based on metabolic body size, they found that it was lower in Iberian than in Landrace pigs at 28 kg BW, without significant breed differences in FSR and FDR. Authors attributed the inconsistency with their previous study to the different body protein mass, with Iberian pigs having 20 – 32% smaller muscles than Landrace (Rivera-Ferre et al., 2005). Our group (Sarri et al., 2021) recently compared two distinct producing types: castrated purebred Duroc

pigs as fatty type against entire male from a leaner crossbreed [Pietrain sires × (Duroc × Landrace)], and obtained that leaner males had higher FSR than the fatty ones, being such effect true in liver and muscle (longissimus dorsi).

Because variation in fiber type composition among genotypes may affect both, meat organoleptic characteristics and protein turnover rates, it has been extensively studied (Chang et al., 2003; Joo et al., 2013). In this sense, intramuscular fat content and backfat thickness correlate positively with the expression of MyHC I and IIa isoforms, as opposed to the expression of MyHC IIb (Qi et al., 2019). Accordingly, the proportion of glycolytic fibers, especially type IIb fibers, increases with those genotypes highly selected for improved growth rate, lean carcass meat and feed efficiency (Fazarinc et al., 2017; Ruusunen and Puolanne, 2004). Examples of this assumption are: Serra et al. (1998) reported a higher proportion of type I fibers in Iberian pigs than in Landrace, while Landrace contained a higher proportion of type IIb fibers. Ryu et al. (2008) noted that Berkshire pigs had greater percentage of type I fibers and lower type IIb fibers than Landrace, Yorkshire and Landrace × Yorkshire × Duroc crossbred pigs. Guo et al. (2011) observed higher mRNA abundance of oxidative myofibers and lower expression of glycolytic myofibers in Jinhua pigs compared to Landrace pigs. Wojtysiak and Połtowicz (2014) also found that autochthonous Puławska pigs presented a higher proportion of type I fibers than muscles from Polish Large White pigs. And more recently, Fazarinc et al. (2017) demonstrated the transition of European wild pig myofibers toward increased expression of oxidative myofibers with maturation, whereas the domestic Large White pig expressed more type IIb myosin heavy chain after the same time period.

Although oxidative type I fibers have a higher transcriptional potential and a greater capacity to synthesize myofibrillar proteins, including a higher density of mitochondria and myonuclei per fiber, higher RNA and mRNA content, higher α-actin mRNA expression and a larger satellite cell population during postnatal development, they exhibit a smaller fiber size and lower cross-sectional area than glycolytic type IIb fibers (van Wessel et al., 2010). This contradiction seems to be related to higher rates of protein degradation and thus, higher protein turnover, as certain degradative machinery is more abundant in oxidative muscle fibers, especially

calpains and calpastatins (Smuder et al., 2010; van Wessel et al., 2010), resulting in lower net protein deposition.

Gene expression is mainly behind the muscle fiber type profile and other phenotypic characteristics, upregulating or downregulating key genes involved in signaling pathways throughout the animal's growth that can alter protein metabolism among pig genotypes. In this regard, the heritability of certain traits such as the percentage of type I ($h^2 = 0.46$) and IIb myofibers ($h^2 = 0.58$) are relatively high (Larzul et al., 1997). According to SanCristobal et al. (2015), the phosphoinositide 3 (PI3) kinase pathway, mostly involved in the regulation of muscle mass growth, is the most relevant signaling pathway showing variable expression between modern pig breeds. In addition, genes associated with muscle growth, muscle anabolism, and cell proliferation and differentiation are overexpressed in leaner breeds (Zhao et al., 2011). Moreover, the higher expression of collagen in this latter type of breeds is also related to their leaner growth and lower intramuscular fat deposition. On the other hand, genes related to fatty acid synthesis, adipogenesis, energy or glucose metabolism, cytoskeleton organization and microtubule dynamics are overexpressed in fatty breeds (Ayuso et al., 2016; Srivastava, 2017). In addition, expression of genes related to growth and development in earlier stages have also been identified in these later breeds, which may be associated with their earlier developmental stage, as fatty and autochthonous breeds are more precocious than leaner breeds. As for genes associated with protein degradation, showed upregulation in fatty breeds (Srivastava, 2017), as well as genes regulating myogenesis inhibition (Zhao et al., 2011).

3.4. Sex condition

Differences in muscle growth and carcass characteristics among sexes have been widely reported (Boler et al., 2014; Kress et al., 2020; Pauly et al., 2008; Suster et al., 2006), being frequently attributed to the effects of androgen hormones, like testosterone (Bonneau, 1998; Claus and Weiler, 1994; JinLiang Xue et al., 1997). An early study looking for differences in protein turnover rates between boars and barrows was conducted by Mulvaney (1984), who through in vitro procedures reported an average reduction of 56% and 9% in FSR and FDR, respectively, in barrows compared to boars in the prepubertal stage (40 kg BW); and 33% and 26%,

respectively, in the pubertal stage (75 kg BW) using the semitendinosus muscle. When Skjaerlund et al. (1994) further studied differential protein turnover rates in younger barrows and boars (1 to 4 weeks old), they found no differences in FSR, FDR or accretion rates, although barrows tended to have lower protein content than boars. Although the authors agreed with other studies (Colenbrander et al., 1978; Ford, 1983) that circulating testosterone picked between the second and third week of age to decrease thereafter, the low levels in this early stage may be insufficient to detect significant effects on protein turnover.

The effect of androgens (e.g., testosterone) on tissue protein balance has been of great interest especially in the human species, to give a solution to different types of hypogonadism in men, which is a clinical condition, congenital or acquired, characterized by reduced production or availability of androgens. Affected patients suffer a decrease in their muscular mass, leading to muscle atrophy or sarcopaenia, and immunocastration in male pigs is serving as a model to study this condition in men (Batorek-Lukač et al., 2022). Supplementation with testosterone and other pharmacological derivates are proposed to reverse the clinical signs, although the underlying molecular mechanisms are complex, with numerous experimental inconsistencies, and remain to be stablished. Existing studies suggest that the anabolic effect of testosterone acts through different pathways, as can be: increasing AA availability, stimulation of myoblast growth and differentiation (Herbst and Bhasin, 2004), activation and proliferation of satellite cells (Rosa-Caldwell and Greene, 2019), stimulation of synthesis through leucine-induced pathway (Jiao et al., 2009), and restoring or increasing protein FSR (Wendowski et al., 2017) activating mTORC1 signaling pathway through upstream effectors (Basualto-Alarcón et al., 2013; Steiner et al., 2017; White et al., 2013). In this sense, Basualto-Alarcón et al. (2013) demonstrated that stimulation of myotube cultures with testosterone resulted in increased hypertrophy through activation of the mTOR/S6K1 pathway via PI3K/Akt signaling.

Males also showed greater mRNA expression associated with differentiation and hypertrophy (myogenin and MyoD) and higher proliferation capacity than females (Lee et al., 2011; Manzano et al., 2011), which is reflected by increased myonuclei number. In addition, it has also been suggested that androgens mediates the increased

expression of their receptors and the activation and proliferation of satellite cells (Mulvaney et al., 1988; Rosa-Caldwell and Greene, 2019). In this regard, uncastrated males exhibit higher satellite cell proliferation and differentiation than females and castrated males (Lee et al., 2011); and male individuals show a higher satellite cell content per fiber than females, particularly those specialized in muscle growth and muscle maintenance (Neal et al., 2012).

However, the lack of differences between young females and males in protein turnover, obtained in multiple studies, indicates that other mechanisms may exist. As for the skeletal muscle fiber type profile, females tend to have a more oxidative profile, especially a predominance of type I MyHC isoform than males (Haizlip et al., 2015). Therefore, females have more mitochondrial content and activity than males, which may contribute to differences in protein metabolism, however, further research is required to confirm that aspect (Rosa-Caldwell and Greene, 2019). In addition, it has been hypothesized that female hormones (e.g., estrogens and progesterone) may compensate their lower levels of testosterone (Hansen, 2018). Both, estrogens and progesterone have been proposed to exert hypertrophic effects and enhance muscle function, with progesterone enhancing protein synthesis and estrogens reducing protein degradation and increasing cell sensitivity to anabolic stimuli (Hansen, 2018; Rosa-Caldwell and Greene, 2019).

Degradative processes also appear to be influenced by sex, with females showing higher autophagy-lysosome related protein degradation, but lower ubiquitin-proteasome activity than males (Rosa-Caldwell and Greene, 2019), although testosterone treatment at the human elderly was seen to decrease protein degradation through ubiquitin-proteasome system (Kruse et al., 2020). In addition, higher calpain expression and lower calpastatin expression were shown in boars in comparison with barrows, evidencing their faster protein synthesis and degradation (dos Santos et al., 2021). Such characteristics are also noticeable in meat between sexes, with boar meat being less fatty but with a higher tenderness and lower shear force than barrows (dos Santos et al., 2021), considering the persistence of degradative mechanisms after the animal slaughter, contrary to synthetics. Finally, testosterone also reduce protein degradation through the hepatic urea cycle (Birzniece et al., 2011; Rossetti et al., 2017). In this sense, testosterone restrict the action of certain enzymes present in the

urea cycle, decreasing the synthesis of hepatic urea and thus, reduces protein degradation and the loss of AA and nitrogen through urine. Therefore, it may increase the amount of AA susceptible for muscle anabolism (Lam et al., 2017).

3.5. Feeding level

It was early noticed that whole body protein synthesis is influenced by feeding and fasting periods (Millward et al., 1975). Colostrum or milk intake in the neonate is the main stimuli responsible for enhancing whole-body protein synthesis by promoting mTORC1 activation, as they provide a high source of nutrients, including AA, which consist of the biochemical building blocks and precursors for protein accretion and energy storage (Burrin et al., 1997). In addition, colostrum contains potential bioactive compounds, including those related to passive immunity, such as immunoglobulins (IgG, IgA and IgM) and leukocytes, bacteriostatic agents, but also hormones and growth factors, such as epidermal growth factor, insulin-like growth factors (IGF-1/2) and transforming growth factors, which have been linked to enhanced intestinal maturation (Xu et al., 2002), and benefit ribosomal RNA synthesis, and thus improve translational efficiency of protein FSR (Burrin et al., 1997, 1995).

Connors et al. (2008) suggested that the increased FSR with feed intake in growing and mature ewes was due to the upregulation of translational initiation rate, which causes an increased proportion of ribosomes actively translating mRNA. However, the effect of feeding level on FSR is not homogeneous over time, decreasing as the postnatal period progresses (Davis et al., 1996). Some studies suggested that skeletal muscles, especially those with predominantly fast-twitch glycolytic fibers, along with the brain, are the tissues most stimulated by feeding (Burrin et al., 1997; Fiorotto et al., 2000). As for visceral tissues, such as liver and intestine, FSR increment to feeding level have also been reported (Burrin et al., 1997; Widdowson et al., 1976), which was suggested to be a result of forcing these organs to a greater metabolic response (Nyachoti et al., 2000). It is noteworthy that, i) 30 – 50% of dietary AA are used by intestinal tissues, and ii) the intestinal tract is directly exposed to dietary nutrients, hormones (i.e., insulin and IGF-1) and growth factors. Therefore, the absence or reduced availability of lumen nutrients that occur in parenterally fed piglets alters protein metabolism in the small intestine (Stoll et al., 2000). In addition, the

dependence on lumen nutrients was greater in the proximal (jejunum) than in the distal (ileum) segments, which is explained by the normal decrease in nutrient availability as nutrients are absorbed along the small intestine. This is consistent with registered changes in intestinal villus height (Stoll et al., 2000).

It has also been proposed that increased postprandial insulin and IGF-1 stimulate skeletal muscle protein synthesis (Davis et al., 2002; Rennie et al., 2004). These two hormones stimulate the phosphorylation of S6K1 and 4E-BP1 (Han et al., 2008), which activates mTORC1 and the subsequent translation initiation process, with an efficient binding of the mRNA to the 43 ribosomal complex. Although the stimulation of protein synthesis by insulin is exclusive to immature skeletal muscle (Reeds et al., 2000), decreased whole-body protein degradation by insulin has been detected at more mature ages as well (Reeds et al., 2000).

Regarding degradation rates, feeding was also reported to reduce whole-body protein degradation in neonates (Thivierge et al., 2005), mainly in visceral tissues, although protein degradation increased or was unaffected in skeletal musculature (Rudar et al., 2019; Wilson et al., 2009). Protein degradation actually plays a critical role in maintaining protein accretion rates, as this process maintains the free AA pool, although the preferred AA for muscle and whole-body protein synthesis are dietary rather those coming from intracellular degradation (Groen et al., 2015). Nevertheless, increased rates of protein degradation are below protein synthesis rates, leading to protein deposition (Y. Zhang et al., 2014).

On the other hand, fasting states inhibit mTORC1 signaling, as well as low ATP levels in order to conserve limited resources (Allen et al., 2010; Saxton and Sabatini, 2017), therefore, skeletal muscle synthesis rates decrease in all myofiber types, but especially in types IIx and IIb (Goodman et al., 2012). Decreases in FSR have also been reported in liver during fasting states, however, no differences were found in myocardial tissue, even though animals were subjected to chronic dietary restriction (Yuan et al., 2008). Under these circumstances, heart showed few reductions in 4E-BP1 and eIF2α phosphorylation.

In addition, upregulation of atrophy-related genes have been identified under prolonged feed deprivation in mice (Allen et al., 2010). As for protein FDR during

fasting states, it was proposed to increase in order to permit the released AA to be converted into glucose through gluconeogenesis, since maintaining the circulating glucose is essential for the proper functioning of certain organs (Allen et al., 2010). Undernutrition during the gestation period results in lower postnatal growth performance, decreasing the number of secondary fibers and muscle growth (Brown, 2014; Du et al., 2010), whereas offspring from overfed gestating sows have also showed lower total number of myofibers (Cerisuelo et al., 2009).

3.6. Dietary CP content

Reducing the dietary CP level by balancing non-bound AA (i.e., synthetic and crystalline AA) is a commonly used feeding strategy in swine production to conform to EU regulations (Council Directive 91/676/EEC, 1991), directed to decrease nitrogen waste through manure. However, the reduction of CP in the diet may compromise the adequate provision of essential AA. Essential AA play a critical role in protein synthesis, and their imbalance could delay or cease the RNA translation process, due to polyribosome disaggregation (Deng et al., 2009; Escobar et al., 2007), thus, adequate supplementation of non-bound AA is crucial (Deng et al., 2009). Understanding the physiological functions of AA and their requirements becomes of great interest to optimize the AA profile in dietary formulations.

Amino acids are signaling regulators that stimulate protein FSR of skeletal muscles and most visceral tissues throughout life (Davis et al., 2008, 2002) by mTORC1 activation (Rennie et al., 2004; Saxton and Sabatini, 2017), however, the response to this stimuli loses intensity with age, which has been linked to a change in the mTOR-associated signaling pathway (Rudar et al., 2019). Previous assays have shown a decrease in protein synthesis with decreasing dietary CP level (Rennie et al., 2004; Rivera-Ferre et al., 2006; Sève et al., 1986). Accordingly, Li et al. (2016) described downregulation of certain AA transceptors when CP level was reduced more than 4%, which modulate the mTORC1 activation pathway, even though crystalline AA were supplemented to cover the limiting essential AA. The same authors also reported that pigs fed this same diet had lower expression of key myogenesis regulatory genes (MyoD and MyoG), and higher expression of genes related to proteolysis.

Moreover, Deng et al. (2009) described a differential sensitivity among tissues to dietary CP concentrations. While gastrointestinal tract (including small intestine and colon) was relatively insensitive in weaning pigs, skeletal muscle (longissimus dorsi), liver, pancreas and kidneys reduced their protein FSR when animals were fed low-CP diets, even though pigs were supplemented with deficient crystalline essential AA. The authors suggested that the underlying mechanisms were related to the reduced phosphorylation of mTOR found in liver and of 4E-BP1 in both muscle and liver. However, the formation of the active eIF4E-eIF4G complex only decreased in liver, but not in muscle, which was associated with a tissue-specific response (Deng et al., 2009). In this sense, scarce or no differences were found in skeletal muscles (longissimus dorsi and biceps femoris) and viscera (duodenum and liver) protein FSR, when moderate differences in CP content were tested in growing (15 vs 17% CP) and fattening pigs (13 vs 15% CP) in our recent study (Sarri et al., 2021). These results agreed with those of Huber et al., (2018), who identified no FSR differences in longissimus dorsi, gastrocnemius, liver or mammary gland protein when milking sows were fed a low-CP diet, although the apparent efficiency of nitrogen utilization for milk protein production was enhanced. In both assays, crystalline AA were supplemented in low CP diets to meet limited AA requirements. The short feeding period or moderate CP variance between treatments in Sarri et al. (2021) could have been the reasons for the lack of effects, since Li et al. (2016) found consistent changes in muscle AA transceptor expression and mTORC1 pathway when the CP level drops below 3% and when the feeding period reached 25 days.

Important signaling roles of certain AA in protein turnover have been identified, mainly mediated through the mTORC1 pathway, especially for leucine, arginine, glutamine, and proline (Rezaei et al., 2013). Leucine stimulates protein synthesis by enhancing mTORC1 activation through phosphorylation of mTOR and its downstream effectors S6K1 and 4E-BP1 (Han et al., 2008; Rudar et al., 2019; Suryawan et al., 2008), especially in neonatal pigs. In addition, leucine downregulates the expression of proteolytic-related genes linked to ubiquitin-proteasome and autophagy-lysosome in skeletal muscle (Nakashima et al., 2005), and some metabolites from its catabolism (α-ketoisocaproate and β-hydroxy-β-methylbutyrate) are also involved in the activation of translation initiation factors (Zheng et al., 2017).

The deficiency in lysine and methionine have also been linked with decreases in both FSR and FDR (Mazor et al., 2018; Rivera-Ferre et al., 2006; Roy et al., 2000), although Palma-Granados et al. (2019) observed an increase in the proportion of oxidative myofibers in pigs fed a lysine-deficient diet. In addition, different sensitivities to a lysine-deficient diet have been found among pig genotypes, being more sensitive the highly selected pigs in comparison with the autochthonous ones (Rivera-Ferre et al., 2006). Besides Lys and Met, the supplementation of low-CP diets with specific non-essential AA, such as glutamine and arginine, have shown maintenance of mTORC1 signaling activation of for optimal protein synthesis (Deng et al., 2009; Rudar et al., 2019).

3.7. Rearing system

The growing consumer concern for animal welfare on farms has increased their preference and demand for meat products coming from organic rearing systems. These livestock systems enable animals access to outdoor areas and/or pastures, which implies regular or spontaneous exercise (*Regulation 2018/848/EU of the European Parliament and of the Council*). Physical activity promotes the use of dietary AA for de novo protein synthesis in muscle (Holwerda et al., 2019), and influences muscle characteristics regulating the myofiber composition. Fazarinc et al. (2020) noticed that Slovenian Krškopolje pigs reared in organic production systems presented lower glycolytic-to-oxidative myofiber ratio. This muscle phenotype corresponded to mRNA expression levels, with upregulation of MyHC I, IIa and IIx isoforms and downregulation of MyHC IIb isoform in organic-reared pigs. According to these results, Qi et al. (2019) also demonstrated that pig production in a semi free-grazing system increased the percentage of type I and IIx fibers than those reared on an indoor farm.

Previous studies also reported increased oxidative capacity when pigs were subjected to treadmill training while reared in conventional indoor facilities (Mcallister et al., 1997; Petersen et al., 1998). Under this same training, Mcallister et al. (1997) found a lower proportion of type IIb fibers and an increased composition in type IIx fibers in the triceps brachii, while Petersen et al. (1998) detected a higher ratio of type IIa-to-IIb/IIx in the longissimus dorsi of pigs. However, correlation between muscular fiber

profile and physical activity is controversial and other authors could not evidence any difference. Gentry et al. (2002) showed no differences in the percentage of muscle fiber types when pigs were reared in long pens with 10 times more space allowance, even though they noticed higher physical activity than those reared in conventional dimensions. Similarly, neither Cross et al. (2020) found differences between inherently low-active and high-active inbred strain mice; while low-active mice had a higher kidney FSR.

In addition to increased physical activity, access to the outdoors may lead to greater exposure to temperature fluctuations. In this regard, prolonged exposure to cold temperatures increased the proportion of oxidative myofibers (Herpin and Lefaucheur, 1992; Mizunoya et al., 2014; Yu et al., 2021), as well as increased protein turnover rates in chicks, which may result in increased nutrient burning for caloric energy (Aoyagi et al., 1988).

3.8. Health status

Protein and AA metabolism is significantly affected by injury, inflammatory states, and the presence of disease (Rudar et al., 2019). Such effects have been studied mainly through sepsis and bacterial lipopolysaccharide models by the administration of endotoxins, live bacteria, and bacterial fragments (e.g., lipopolysaccharides); or through induction of local inflammation by administration of substances such as dextran sulfate, turpentine, or tumor necrosis factor (TNF-α). When the immune system is stimulated, a greater proportion of nutrients that were being destined to maintenance, growth, or reproduction are redirected to support the immune system to enable the production of specific proteins and metabolites (e.g., proinflammatory cytokines and leucocytes). Therefore, AA requirements are modified; for instance, the demand for certain AA (e.g., cysteine, methionine, glutamine, arginine, tryptophan) increases under these conditions, as they act as synthesis precursors for the immune response, such as the synthesis of glutathione, defensins, antimicrobial peptides or albumin (de Ridder et al., 2012; Obled, 2003).

Furthermore, although ileal digestibility appears not to be affected during immune system stimulation (Rakhshandeh et al., 2014), immune-challenged pigs significantly reduce their voluntary feed consumption (de Ridder et al., 2012). Decrease in feed

intake and AA utilization efficiency reduce animal growth and protein retention rates under pathological circumstances (de Ridder et al., 2012; McGilvray et al., 2019). This results in decreased rates of skeletal muscle protein synthesis (McGilvray et al., 2019; Rudar et al., 2019), which is primarily related to a suppression of mTORC1 signaling and changes in local and circulating growth factor levels (Rudar et al., 2019). Although skeletal muscle reduces its contribution (Breuillé et al., 1994), whole-body protein synthesis is generally maintained in comparison with non-challenged animals, as synthesis in some viscera compensates.

Protein metabolism of viscera in response to immune challenge is highly variable and depends on each organ or tissue. Increases in the protein synthesis rate have been described especially in the liver (Breuillé et al., 1998; Mackenzie et al., 2003), due to increased AA demands and energy expenditure directed to the synthesis of acute-phase proteins such as fibrinogen and albumin (Ten Have et al., 2019). The degree of increase in its synthetic rates can vary widely, being more pronounced in septic than in inflammatory models (Breuillé et al., 1998). Along with the liver, the spleen is also involved in the immune response, filtering the blood to remove antigens. This organ undergoes cell hypertrophy and even hyperplasia, as a result of increased protein synthesis (Breuillé et al., 1998; Obled, 2003). Another splanchnic tissue involved in immune response is the gastrointestinal tract, which also undergoes relevant morphological and physiological changes under immune system stimulation, affecting mucins production, epithelial cells and proteins related to intestinal repair and protection against bacterial translocation (Ten Have et al., 2019). Under systemic inflammation caused by acute bacteraemia-induced sepsis (e.g., *Escherichia coli*), increases in FSR of the whole intestine have been found in rats (Breuillé et al., 1998). However, Ten Have et al. (2019) could not confirm previous results and noticed no change in FSR in the ileum during *Pseudomonas aeruginosa*-induced sepsis, along with substantial decreases in FSR (29%) and FDR in the jejunal mucosa. Furthermore, the lungs experienced increased protein synthesis in the early phases of bacteremia-induced sepsis (Breuillé et al., 1998; Ten Have et al., 2019). In addition, states of malnutrition and nutritional imbalances resulting from this state, negatively affect by increasing competition for indispensable AA (Kampman-van de Hoek et al., 2015).

Protein degradation, particularly of muscle or skin, has been described as a potential AA-contributor of acute-phase proteins synthesis, which are plasma proteins produced mainly by hepatocytes that act destroying or inhibiting the pathogens growth, or contributing to the negative feedback of the inflammatory response. In this sense, some authors have reported increased rates of protein degradation in skeletal muscle (Breuillé et al., 1998; Rudar et al., 2019). However, other authors described no changes in whole body protein degradation (Rudar et al., 2017), or even a reduction in whole-body protein degradation rates in immune-challenged pigs (McGilvray et al., 2019; Ten Have et al., 2019). In this regard, increases in calpain activity were reported during sepsis, particularly in skeletal muscle, which has been linked to decreased calpastatin activity inhibition (Smith et al., 2008), whereas increased activity of the ubiquitin-proteasome system has also been observed (Voisin et al., 1996).

Finally, alteration in muscle fiber type profile during disease has also been described (McGilvray et al., 2019), with type I fibers increasing their proportion towards type II, especially type IIx fibers, in immune-challenged pigs. On the other hand, specific muscle pathologies such as muscular dystrophies or cancer cachexia show their inherent muscle fiber type changes (Rosa-Caldwell and Greene, 2019).

3.9. Thermal Stress

The limited number of functional sweat glands, thick subcutaneous fat layer, and intense metabolism prevent pigs from adequate body heat dissipation (Renaudeau et al., 2006), making these animals susceptible to heat stress (HS). Temperatures above the pig's thermoneutral zone significantly reduce their appetite and, consequently, their voluntary feed intake as a biological strategy aimed at avoiding the generation of additional heat from ingestion, absorption, and metabolism (Huynh et al., 2005; Pearce et al., 2013a). Other strategies include intensified respiration rate and reduced physical activity and basal metabolic rate (Collin et al., 2001). The gastrointestinal tract is particularly sensitive to HS, suffering a loss of epithelial integrity (Liu et al., 2009; Pearce et al., 2013b) generated by vasoconstriction that redirects blood flow to peripheral tissues to dissipate heat to the environment, leading to hypoxia and reduced nutrient supply to this organ (Gabler and Pearce, 2015; Ortega and Szabó, 2021).

Therefore, the functions of the intestinal tract may be impaired, leading to lower apparent and standardized ileal digestibility of certain AA (Morales et al., 2016a, 2016b).

Within this framework, reduced growth rates and protein deposition in pigs under these environmental conditions have been associated with lower feeding levels and subsequent potential nutrient deficiency, with a consistent and negative relationship between feed intake and body temperature having been described (Cervantes et al., 2018). However, this hypothesis is currently questioned because other works suggested that HS itself may induce other metabolic alterations caused by intestinal barrier disruption, inflammatory state, postabsorptive metabolism and endocrine responses (Liu et al., 2022; Qaid and Al-Garadi, 2021). In addition, HS seems to cause oxidative stress and mitochondrial dysfunction, increasing ROS production in muscle cells (Kikusato and Toyomizu, 2013; Kim et al., 2021), whose intracellular signal transduction contributes to protein catabolism.

Pearce et al. (2013) demonstrated that, under the same feeding level, growing pigs subjected to HS had distinct performance responses compared to pigs kept under thermoneutral conditions, which would indicate that HS can induce both indirect (reduced feed intake) and direct (metabolic adjustments that alter tissue growth) effects. It has been evidenced that when animals are raised under HS conditions, their carcasses show a significant reduction in lean tissue and an increase in fat deposition (Collin et al., 2001; Liu et al., 2022), while the opposite effect is observed when feed intake is reduced in pigs kept in thermoneutrality. It is noteworthy that pigs with higher genetic potential for lean growth, as well as heavier and older pigs appear to have less tolerance to HS (Renaudeau et al., 2011). Similarly, the effect of HS on carcass composition is also age-dependent, being irrelevant in young pigs but pronounced in older pigs (Christon, 1988). These results have been confirmed in other species such as chicks (Temim et al., 1999; Yunianto et al., 1997) or rodents (Katsumata et al., 1990).

Regarding protein metabolism, the authors of the present review are not aware of studies in pigs describing the direct impact of HS on FSR and FDR, however, there is extensive related literature in chickens, which can be used as a homeothermic animal

model. Broilers are also greatly affected by high ambient temperatures due to their underdeveloped sweat glands and the high body heat expenditure of modern commercial lines. The low growth and protein retention of broilers subjected to chronic HS conditions results from a pronounced depression of FSR (Temim et al., 2000), which has been associated with lower protein synthesis capacity (i.e., RNA-to-protein ratio; Temim et al., 2000), and reduced S6K1 mRNA expression in tissue (Ma et al., 2021). Maharjan et al. (2020) and Temim et al. (2000) noticed that HS affected the FSR to a greater extent than the FDR.

However, in terms of FDR several authors have described the increase of this rate through distinct indicators among which we highlight an increased concentration of circulating markers of muscle catabolism, such as N_{τ}-methylhistidine, creatinine and blood urea nitrogen (Nakashima et al., 2004; Pearce et al., 2013a), higher expression of muscle atrophy genes (e.g., MAFbx; Ma et al., 2021), increment in the activity of calpain, cathepsin D and proteasome (Nakashima et al., 2004), increased postabsorptive concentration of serum AA metabolites (e.g., 3-methyl-Histidine, OH-Pro, OH-Lys; Morales et al., 2016a), higher hepatic deamination through aminotransferase enzymes (e.g., GOT and GPT), and higher corticosterone level in serum (Ma et al., 2021). As seen in the other circumstances where feed intake is negatively affected, skeletal muscle proteolysis is aimed at redirecting AA to hepatic gluconeogenesis for energy provision (Ma et al., 2021), where corticosterone plays a critical role (Lin et al., 2004). High circulating concentration of this glucocorticoid has been shown to accelerate protein degradation, and disrupt protein synthesis (Dong et al., 2007; Liu et al., 2004; Qaid and Al-Garadi, 2021), consequences that could be attributed to any type of stress as the hypothalamic-pituitary-adrenal cortex axis is activated.

4. Conclusions

Along with improving the feed efficiency of animals for meat production, maximizing protein retention continues to be one of the main challenges of science and the meat industry. Protein deposition in muscles is explained by the balance between protein synthesis and degradation processes, which are greatly affected by multiple intrinsic and extrinsic effects, as reviewed. Although much is known about the mechanisms of

protein synthesis and its rates, protein degradation remains difficult to analyze, and appears to be less involved in protein retention than synthesis. Increased accessibility and the growing development of highly accurate and sensitive techniques allow better detailing of the molecular mechanisms involved in these processes and open the door to new advances that can be applied in precision livestock farming.

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CHAPTER II

Evolution of viscera and muscle fractional protein synthesis rate in lean meat selected hybrids and castrated Duroc pigs fed under moderate crude protein restriction

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Abstract

Differences in producing performance and organoleptic meat characteristics among pig genotypes and/or producing types are widely known. These parameters are also subjected to the animal's development, feeding and management. Detailed knowledge of the effects of production phase (**PP**), pig producing type (**PT**), dietary protein availability and their interactions on nutrient digestibility, nitrogen balance and protein metabolism is essential information to improve precision feeding techniques. The experiment was a 2 (PP) × 2 (PT) × 2 (diet) factorial design conducted with 32 male pigs, 16 entire F2 pigs progeny of Pietrain sires and Duroc × Landrace dams, and 16 castrated purebred Durocs belonging to two production phases (growing: 29.5 ± 3.19 v. fattening: 88.6 ± 6.26 kg BW), and assigned to one of two dietary CP levels, either standard (**SP**: 17% in growing and 15% in fattening) or low (**LP**: 15% in growing and 13% in fattening). Viscera and muscle fractional protein synthesis rates (**FSRs**; %/day) were conducted through a single infusion of 15% L−[ring−$^{2}H_{5}$]−phenylalanine, with subsequent blood sampling from 12 to 40 min, and sample collection of liver, duodenum, *biceps femoris* and *longissimus dorsi* skeletal muscles after sacrifice. Fattening animals acquired a greater feed ingestion capacity, average daily gain ($P < 0.01$) and apparent ileal digestibility, whereas growing pigs showed higher FSRs in both viscera (duodenum and liver) and in *longissimus dorsi*. F2 pigs showed higher average daily gain, nitrogen retention rates and FSR in liver and *longissimus dorsi* ($P < 0.01$). Nevertheless, apparent ileal digestibility in all essential amino acids was lower in F2 compared with Duroc pigs ($P < 0.05$). Protein metabolism was barely influenced by dietary CP content, although animals fed LP registered the lowest apparent ileal digestibility for CP and also for most of essential amino acids compared with SP-fed pigs. This information may reveal differences in amino acid requirements between both PTs, with Duroc pigs receiving excess of dietary amino acids.

Keywords: protein synthesis, apparent digestibility, production phase, pig producing type, protein restriction

Implications

Performance parameters and carcass quality are important features in the swine industry that are influenced by different factors such as age, genotype, gender and diet. In the present study differences in protein metabolism, amino acid digestibility and nitrogen balance between two commercially tested productive types of pig were identified, which may evidence differences in their use of nutrients and amino acid requirements. Although further experiments are needed to validate the results obtained, our findings provide valuable information to refine rationing models in livestock precision feeding.

Introduction

Precision feeding systems are tools designed to improve feed efficiency by reducing production costs and environmental load (Pomar and Remus, 2019). They rely on accurately matching nutrients supply to animal's requirements according to its genetic merit, physiological and health status. Due to the significant diversity existing among livestock production systems, there is still a potential to improve efficiency, but it is necessary to identify what causes specific variations over a large range of systems and nutritional scenarios.

The existing literature has consistently described the influence of breed and gender on both performance and carcass quality in swine (Latorre et al., 2003); some breeds have been selected to produce low-cost lean meat, whereas others (i.e. fatty pigs from breeds like Duroc or Iberian) are transformed in high-quality products with some specific features such as higher levels of intramuscular fat (Gol et al., 2019). Diet composition (Ruiz-Ascacibar et al., 2019a) and animal's weight are also factors that might influence growth rates of tissues and carcass composition (Álvarez-Rodríguez and Teixeira, 2019), especially in terms of fat incorporation. For this reason, males chosen to produce fatty pigs and slaughtered at higher weight are submitted to castration to avoid boar taint, whereas leaner breeds usually not. Therefore, commercial pig farms can deliver two distinctive products with a direct impact on the nutritional requirements, needed to fulfill their physiological potential (i.e. fractional protein synthesis rate, **FSR**) and their resilience to nutritional changes (i.e. protein restriction). Moreover, due to the different precocity exhibited in animals subjected to

both types of products, it might be reasonable to expect variations in their use of nutrients depending on their availability (i.e. level of CP) and their interaction with production phase (**PP**; growing or fattening) (Brossard et al., 2019; Poklukar et al., 2020).

In order to assess nutritional requirements in a precision feeding system, it is essential to understand, not only the mechanisms involved with nutrient absorption, but also with the relationship between protein turnover and real amino acid (**AA**) requirements. The present study aims to determine: the influence of two producing types (**PTs**), commercial hybrids selected for producing lean meat against castrated purebred Duroc pigs (raised as fatty animals), at two different PPs (growing v. fattening) on their digestibility, nitrogen balance and FSR, together with the effect of moderate protein restriction in both types of animals.

Material and methods

Animals and experimental diets

The study was conducted at the Swine Research Center, located in Torrelameu (CEP; Lleida, Spain) during the June and July months. Thirty-two male pigs from two PTs and two PPs (growing and fattening) were used, where 16 were entire males [F2: Pietrain sires × (Duroc × Landrace) dams], in growing (n = 8; 30.5 ± 1.36 kg BW; mean ± SD) and in fattening (n = 8; 91.1 ± 1.23 kg BW) periods. Same number of surgically castrated purebred Duroc was used in growing and fattening periods (28.5 ± 1.03 and 86.1 ± 2.74 kg BW, respectively). For each PP, two experimental diets with different CP concentration [standard (**SP**) or low (**LP**)] were formulated to be isocaloric, and to meet the nutrient requirements recommended by the Fundación Española para el Desarrollo de la Nutrición Animal (FEDNA, 2013). Moreover, crystalline amino acids were supplemented to ensure similar concentration of essential AAs in both diets, as well as to avoid potential effects of lowering CP supply in LP treatments. Table 1 shows the ingredients and chemical composition of the different diets given to the animals during the trial.

Table 1. Ingredients and chemical composition (g/kg DM) of the two-phase experimental diets, differing in 2% CP concentration (standard protein, SP v. low protein, LP) for pigs of growing and fattening production phases.

	Growing		Fattening	
	LP	SP	LP	SP
Ingredients				
Corn	294.8	246.5	190.3	99.2
Barley	290.0	287.8	466.0	512.8
Wheat	200.0	200.0	200.0	200.0
Soybean meal	137.6	195.0	65.8	113.6
Beet pulp dehydrated	30.0	30.0	30.0	30.0
Calcium carbonate	13.4	13.2	10.0	10.0
Mono Calcium phosphate	9.4	8.8	7.0	6.4
Soybean oil	9.0	6.3	17.0	17.1
L-Valine	6.8	8.0	0.0	0.0
Sodium chloride	4.6	4.6	4.1	4.1
L-Lysine HCL	4.2	2.4	3.7	2.1
Vitamin- Mineral mix[1]	4.0	4.0	4.0	4.0
L-Threonine	1.6	0.8	1.2	0.5
DL- Methionine	1.0	0.5	0.6	0.3
L-Tryptophan	0.3	0.2	0.2	0.0
Chemical composition				
Dry matter (g/kg FM[2])	900.1	897.1	909.9	908.4
Crude Protein	147.2	166.9	129.27	154.95
Ether Extract	24.9	26.9	39.7	43.2
Ash	65.8	70.3	58.1	52.8
NDF	173.3	179.9	211.8	211.6
ME[3] (kcal/kg)	3 108	3 094	3 185	3 185

[1] Vitamin mineral mixture composition (per kg of complete diet): vitamin A, 2.4 mg A; vitamin D_3, 0.02 mg; vitamin E, 40 mg; vitamin B_1, 0.8 mg; vitamin B_2, 4 mg; vitamin B_6, 1.6 mg; vitamin B_{12}, 2.4 × 10−2 mg; vitamin K_3, 1.2 mg; pantothenic acid, 8 mg; niacin, 16 mg; biotin, 0.08 mg; folic acid, 0.4 mg; citric acid, 0.264 mg; Co ($CoSO_4 \times 7H_2O$); 0.16 mg; Cu ($CuSO_4 \times 5H_2O$), 128 mg; Fe ($FeCO_4$), 72 mg; Mn (MnO_2), 23.8 mg; Zn (ZnO), 80 mg; Se (Na_2SeO_3), 0.24 mg; I (KI), 0.32 mg; choline chloride, 280 mg; 6-phytase, 600 FYT; endo-(1.4)-ß-glucanase, 2 000 BGU; endo-(1.4)-ß-xylanase, 4 800 FXU; ethoxyquin, 0.264 mg.

[2] ME = metabolizable energy.

[3] FM = fresh matter.

During the growing period, four animals from each PT were fed SP diet (17% CP), and four received LP diet (15% CP). Same pattern was conducted during the fattening period, where four animals from each PT were fed SP and the rest LP diets (15% and 13% CP for SP and LP diets, respectively). With the objective of determine their apparent digestibility coefficient, all experimental diets were supplemented with titanium dioxide (TiO_2) as external marker at the rate of 5 g TiO_2/kg DM.

Experimental design

The experiment lasted 11 days for each pig. After 5 days of diet adaptation, animals were individually weighed, catheterized (Drucafit® Splittocan, B-Braun, Melsungen, Germany) into the right external jugular vein and allotted in individual metabolic cages (2 × 1.04 m^2) for 6 days under controlled environmental and antiseptic conditions. Catheters were flushed daily with heparinized saline solution. Water and feed intake, as well as fecal and urinary excretion were measured daily. During the last 3 days of the trial, urine was collected in H_2SO_4 (100 ml; 10% v/v) and fecal spot samples (≈ 50 g) were obtained by rectal stimulation. Fecal samples were stored immediately at −20ºC for further analyses.

At the last day of the trial, animals were submitted to the flooding dose technique to determine the FSR, following Garlick et al. (1980). In brief, an initial blood sampling was conducted in order to determine natural phenylalanine enrichment. Then, a flooding dose of phenylalanine was infused continuously for 10 min through the jugular catheter. Infusion of phenylalanine consisted of a 3.7 ml/kg BW of sterilized saline dilution containing 15 mg/kg BW of L−[ring−$^2H_5$]−phenylalanine ($^2H_5$−phenylalanine) (Cambridge Isotope Laboratories CIL; Andover, MA), and 85 mg/kg BW of L−phenylalanine (Sigma Aldrich, Steinheim, Germany). Following the start of the infusion, a series of blood extractions were performed at 12, 15, 20, 25, 30 and 40 min. Then, plasma was obtained by centrifugation (3 000 *g* for 10 min) and kept frozen at −80ºC until analysis. Immediately after the last blood sampling, animals were euthanized with sodium thiopental (Esteve S.A., Oudewater, Netherlands), bodies were weighed, eviscerated and liver, duodenum, *longissimus dorsi* and *biceps femoris* skeletal muscles were sampled from the left half carcass. Ileal digesta samples were also collected. All samples were immediately stored at −80ºC until further

analyses. Both liver and skeletal muscles from the right half carcass were dissected and weighed.

Analytical procedures

Both feces and urine samples were thawed at 4ºC overnight and pooled into one feces and one urine sample for each animal. Both ileal and pooled fecal samples were freeze-dried and ground. Feed samples and feces were analyzed for DM (ref. 934.01), organic matter (**OM**) (ash, ref. 942.05) and ether extract (**EE**) (ref. 920.39) following the proximate analysis procedures described by the Association of Official Analytical Chemists (AOAC, 2006). The proportion of NDF was determined according to Van Soest et al. (1991), using α-amylase but not sulphites, and subtracting ashes from the residue.

The CP (nitrogen × 6.25) content in all samples, including feed, feces, ileal content, urine and tissues, was performed by Dumas combustion (Tru Spec CN; Leco Corporation, St. Joseph, MI, USA) (ISO, 2008). Urine samples were spun (3 500 *g* for 2 min) to discard impurities before determination of the nitrogen content.

TiO_2 as a digesta marker was analyzed in ileum, feces and feed ashes using inductively coupled plasma mass spectroscopy (Agilent Technologies 7 700X model, Tokyo, Japan) following Darambazar (2019) procedure in which sample preparation consisted in a digestion process with 6.5 ml of H_2SO_4 (7.4 M, 1.5 h at 200ºC), a cooling step and the addition of 5 ml of H_2O_2 (30% (v/v)).

Amino acid analyses

Analyses of AAs were performed by means of a Multiple Reaction Monitoring method (**MRM**) implemented in an Ultra-HPLC Acquity system (Waters, Milford, MA) coupled to a triple Quadrupole mass spectrometer (Micromass MS Technologies, Manchester, UK). Plasma free AAs were obtained as described by Piraud et al. (2005). Briefly, 200 µl of plasma were mixed with 800 µl of methanol, vortexed (2 min) and maintained at room temperature for 10 min. The extract was then centrifuged (17 500 *g* for 5 min*)* and 50 µl-aliquot of the supernatant was preserved. Free AAs from viscera tissues (liver, duodenum) and muscles were obtained as described by Qin et al. (2015); briefly, 300 mg of ground freeze-dried

samples were homogenized (in 2 ml of distilled water and 2 ml of methanol (1/1 v/v)), incubated (4ºC for 30 min) and centrifuged (10 000 *g* for 10 min). Finally, 100 µl of the supernatant was preserved.

Amino acids from tissue protein, feed and ileal digesta samples were obtained by hydrolysis of the remaining pellet after free AA extraction, feed and freeze-dried ileal digesta samples, respectively. Hydrolysis was performed following Colgrave et al. (2008), in which samples (75 mg of tissue pellet; 50 mg of feed; 50 mg of ileal digesta) were incubated (5 ml of HCl 6 N (with 0.02% phenol)) during 24 h at 110ºC under nitrogen steam. After hydrolysis, tubes were cooled and centrifuged (3 000 *g* for 30 min), and 50 µl aliquots were then reserved.

All reserved aliquots were evaporated and re-diluted in 500 µl of water/acetonitrile (15/85 v/v), vortexed and filtered through a 0.20 µm hydrophilic PTFE membrane. Ultra-HPLC/MRM analyses were performed following Guo et al. (2013), on a BEH Amide column (2.1 × 150 mm; 1.7 µm). Transitions of MRM were tested in our conditions for alanine, arginine, aspartic acid, cysteine, glycine, glutamic acid, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tyrosine and valine.

To monitor protein synthesis, the transition of $^{2}H_{5}$−phenylalanine was added to the MRM method. Precursor/product ion pairs were determined obtaining the following values: 171.22 > 106.14 / 171.22 > 125.26. Cone voltage and collision energy was optimized for all the transitions, and the absence of crossed signal between phenylalanine and $^{2}H_{5}$−phenylalanine channels was checked.

Calibration curves were constructed from a commercial AA standard mixture (Sigma-Aldrich, St. Louis, MO, USA) spiked with $^{2}H_{5}$−phenylalanine and diluted to a series of appropriate concentrations with water/acetonitrile (20/80 v/v). Tryptophan concentration was not determined due to its partial degradation under our hydrolysis conditions. Results were processed using QuanLynx software (MassLynx, Waters Corporation, Milford, MA, USA).

Calculations

Apparent nutrient digestibility was calculated using the nutrient-to-marker ratio in diet, feces and ileum, as follows:

$$y = 1 - \left(\frac{\text{marker}_{\text{feed}}}{\text{marker}_{\text{ds}}} \times \frac{Z_{\text{ds}}}{Z_{\text{feed}}} \right)$$

where *y* represents the coefficient of apparent digestibility of a nutrient at a certain segment;

Z_{ds} and Z_{feed} represent the nutrient concentration in the digestive segment and in the feed, respectively;

$marker_{feed}$ and $marker_{ds}$ represent marker (TiO_2) concentrations in feed and digestive segments, respectively.

Plasma and tissue $^2H_5$−phenylalanine enrichment was expressed as molar percent excess (**MPE**), where labeled phenylalanine moles are calculated as percentage of the sum of unlabeled and labeled phenylalanine moles. The percentage of tissue protein synthesized per day (FSR, %/day) was calculated from the equation:

$$\text{FSR} = \left(\frac{\text{MPE}_{\text{bound}}}{\text{ave MPE}_{\text{free}}} \times \frac{100}{t} \right)$$

where MPE_{bound} is the enrichment in $^2H_5$−phenylalanine of protein-bound phenylalanine in tissues; $ave\ MPE_{free}$ is the average of the enrichment in $^2H_5$−phenylalanine of the free phenylalanine pool in the same tissue, between time 0 until the end of the sampling period, assuming that free phenylalanine in tissues follows equivalent kinetics than in plasma; *t* is the labeling time in days.

Supplementary Figure S1 shows decreases in the MPE (%) of free phenylalanine in plasma over time, as well as the MPE (%) of free phenylalanine recorded in the target tissues.

Absolute protein synthesis rate (**ASR**) as the amount of protein synthesized per day (g/day) in liver and each muscle, was calculated as follows:

$$\text{ASR} = \frac{\text{FSR}}{100} \times \text{tissue total protein content (g)}$$

Total CP content in liver and muscle was determined after its manual dissection, sampling and nitrogen determinations.

Statistical analysis

All statistical analyses were performed using the MIXED model using SAS statistical software (v9.4; SAS Institute Inc., Cary, NC, USA), in a completely randomized design with a pig as the experimental unit. Nutrient intake, growth performance, apparent nutrient digestibility, fractional and absolute synthesis rate, and nitrogen balance data were analyzed as follows:

$$Y_{ijklmn} = \mu + PP_i + PT_j + DI_k + (PP \times PT)_l + (PP \times DI)_m + (PT \times DI)_n + \varepsilon_{ijklmn}$$

Where Y is the dependent variable, μ is the mean value, PP_i is the production phase (growing and fattening), PT_j is the producing type (Duroc and F2), DI_k is the diet (SP and LP) along with their possible interactions, and ε_{ijklmn} is the error.

The three-way interaction did not affect any parameter and was removed from the final model.

Differences between least square means were assessed using Tukey multiple comparison test. Results were reported as least square means and their standard error. Significant differences and tendencies were declared at $P \leq 0.05$ and $0.05 < P < 0.10$, respectively.

Results

Intake and growth performance

Daily intake of DM, OM, CP, EE and NDF, together with the performance indices such as average daily gain and feed conversion ratio during both PPs are shown in Table 2. Daily intake of DM, OM, CP, EE and NDF increased ($P < 0.01$) with age. Interaction effect between PP and PT tended to be significant. In growing period, DM and OM intake was numerically higher in lean pigs, while in fattening period the opposite trend was true ($P = 0.08$ and 0.09, for DM and OM intake, respectively). As it was expected, CP intake was significantly higher in SP pigs ($P = 0.01$).

Table 2. Average daily intake of dry matter (DM), organic matter (OM), crude protein (CP), ether extract (EE) and neutral detergent fiber (NDF) and growth performance parameters in F2 [Pietrain sires × (Duroc × Landrace) dams] and Duroc pigs at growing (29.50 kg) and fattening (88.62 kg) phases fed two experimental diets, with a standard (SP) and low (LP) CP concentration.

Parameters	Phase (PP)		Producing type (PT)		Diet (DI)			*P*-value				
	Growing	Fattening	Duroc	F2	LP	SP	SEM[1]	PP	PT	DI	PP × PT	PT × DI
Nutrient Intake (g/day)												
DM	824	2 029	1 450	1 403	1 448	1 405	50.4	<0.01	0.55	0.58	0.08	0.15
OM	768	1 916	1 364	1 320	1 361	1 323	47.6	<0.01	0.54	0.60	0.09	0.15
CP	158	321	242	236	222	257	8.6	<0.01	0.65	0.01	0.1	0.15
EE	21.4	83.9	53.9	51.4	51.7	53.6	1.99	<0.01	0.42	0.54	0.13	0.12
NDF	145.7	429.4	293.3	281.8	291.6	283.5	10.33	<0.01	0.47	0.61	0.10	0.14
Performance												
ADG[2] (g/day)	426	933	615	743	744	615	47.6	<0.01	0.09	0.08	0.13	0.03
FCR[3] (g/g)	2.1	2.3	2.3	2.1	2.0	2.4	0.13	0.20	0.28	0.02	0.02	0.20

[1] SEM = average standard error of the means.

[2] ADG = average daily gain.

[3] FCR = feed conversion ratio

Average daily gain increased with age ($P < 0.01$) and F2 males grew faster when LP diet was given (Interaction effect PT × dietary CP level, $P = 0.03$, see Figure 1a). Pigs fed LP diets were more efficient in converting feed than SP ones expressed as feed conversion ratio ($P = 0.02$). F2 pigs showed better feed conversion than Durocs in fattening phase (Interaction effect between PP and PT, $P = 0.02$, see Figure 1b).

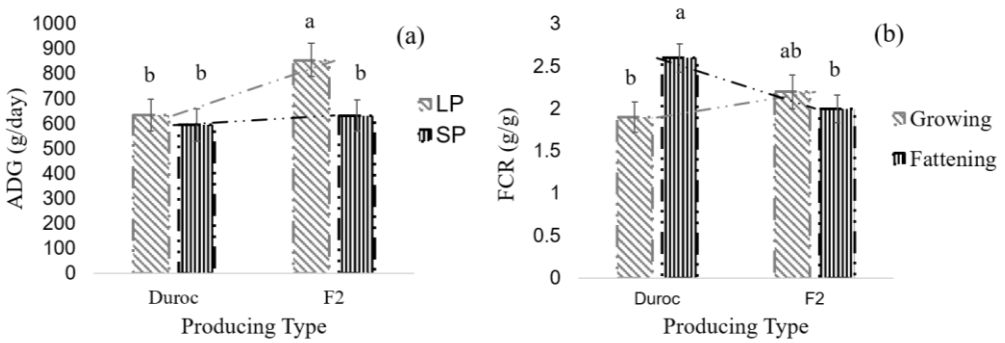

Figure 1. Average daily gain (ADG) affected by the interaction of the productive type (PT; Duroc v. F2) and the dietary CP (standard, SP v. low, LP) (Figure 1a). Feed conversion ratio (FCR) affected by two interactions: production phase (PP; Growing v. Fattening) and pig PT (Figure 1b). Above each bar, different letters (^{a,b}) indicate significant differences ($P < 0.05$). Error bars = SEM.

Coefficients of apparent total tract and apparent ileal digestibility

Effects of PP, PT and dietary CP content on DM, OM, CP, EE and NDF apparent total tract digestibility coefficients (**ATTDs**) are presented in Table 3. Except for EE, which was more digested as animals aged ($P < 0.01$), small differences in ATTD were observed among PPs. Duroc pigs showed higher EE ($P = 0.03$) and NDF (trend, $P = 0.07$) ATTD, compared with F2. Dietary CP content also tended to affect ($P = 0.08$) CP digestibility, where SP diets showed a higher rate.

Table 3. Apparent total tract digestibility (ATTD, %) of dry matter (DM), organic matter (OM), crude protein (CP), ether extract (EE) and neutral detergent fiber (NDF) in animals of 2 different production phases (PP, growing v. fattening) and 2 producing types of pig (F2 crossbred and purebred Duroc) fed with 2 dietary levels of protein (low, LP v. standard, SP).

	Phase (PP)		Producing type (PT)			*P*-value		
	Growing	Fattening	Duroc	F2	SEM [1]	PP	PT	PP × PT
DM	90.6	89.4	90.2	89.8	0.47	0.08	0.63	0.38
OM	91.5	90.4	91.1	90.8	0.42	0.09	0.60	0.51
CP	89.2	87.7	88.4	88.5	0.63	0.11	0.95	0.70
EE	70.0	81.3	78.7	72.6	1.83	<0.01	0.03	0.90
NDF	76.8	74.2	77.1	74.0	1.18	0.13	0.07	0.45

[1] SEM = average standard error of the means.

Table 4 shows the apparent ileal digestibility (**AID**) of DM, CP and essential AAs, while Supplementary Table S1 includes AID of conditionally essential and non-essential AAs. Fattening pigs showed higher AID for DM ($P < 0.01$), and growing F2 pigs the lowest DM digestibility (Interaction effect PP × PT, Supplementary Figure S2). Rates of DM and CP digestibility were greater for Duroc animals respect to F2 ones ($P < 0.01$). Moreover, dietary CP content also affected CP ileal digestibility, where pigs fed SP diets digested more CP in ileum ($P < 0.01$, Table 4). Except for isoleucine, methionine and valine, AID of essential AAs were affected by the PP, being greater ($P < 0.05$) in fattening pigs, but did not have influence on most of conditionally essential and non-essential AAs. Serine ($P = 0.01$) was more digestible in the ileum of fattening pigs; however, glutamic acid showed the opposite effect ($P < 0.01$).

Table 4. Apparent ileal digestibility (AID, %) of dry matter (DM), crude protein (CP) and essential amino acids (AA) of 15 purebred Durocs and 16 F2 pigs, in growing and fattening phases and fed 2 dietary levels of CP (low, LP v. standard, SP).

Parameters	Phase (PP)		Producing type (PT)		Diet (DI)			*P*-value				
	Growing	Fattening	Duroc	F2	LP	SP	SEM[1]	PP	PT	DI	PP × PT	PT × DI
DM	79.2	88.4	87.4	80.3	82.7	85.0	1.17	<0.01	<0.01	0.2	<0.01	0.42
CP	82.3	83.7	85.9	80.1	80.1	85.8	1.42	0.5	0.01	0.01	0.09	0.25
Essential AA												
Histidine	70.9	80.9	81.8	70.0	69.9	81.9	2.45	0.01	<0.01	<0.01	0.01	0.13
Isoleucine	79.5	82.9	85.8	76.7	78.7	83.8	1.47	0.11	<0.01	0.02	0.06	0.17
Leucine	74.5	87.2	86.2	75.5	76.8	85.0	2.46	<0.01	0.01	0.03	0.03	0.41
Lysine	80.1	87.7	86.6	81.2	84.0	83.9	1.72	<0.01	0.04	0.97	0.07	0.18
Methionine	90.1	88.2	91.7	86.6	87.5	90.9	1.43	0.36	0.02	0.11	0.15	0.15
Phenylalanine	80.1	85.2	86.0	79.3	79.7	85.6	1.50	0.03	<0.01	0.01	0.04	0.28
Threonine	66.4	84.3	81.0	69.7	74.4	76.2	2.28	<0.01	<0.01	0.58	0.02	0.59
Valine	78.8	78.7	81.8	75.7	75.1	82.3	1.76	0.98	0.02	0.01	0.16	0.22

[1] SEM = average standard error of the means.

Duroc pigs showed higher AID for all the AAs studied (see Table 4). Histidine, leucine, phenylalanine, threonine, cysteine, proline and serine AID was lower for F2 pigs in the growing phase (Interaction effect PP × PT, $P < 0.05$, Supplementary Figures S3 and S4). The higher CP content of SP diets also elevated the AID on most of the studied AAs, except for lysine, methionine, threonine, proline and glycine.

Viscera and muscle protein synthesis

All pigs fully recovered 1 day after catheterization surgery and their DM intake and average daily gain remained within the range proposed for pigs of their age. Nevertheless, the jugular catheter of one of the Duroc pigs in fattening period was (accidentally) removed from its place the day before L−phenylalanine infusion; therefore, no FSR measurements were obtained from that animal. Fractional and absolute synthesis rates of protein are presented in Table 5.

Overall, FSR was numerically higher in viscera tissues than in muscles and decreased with age in most of target tissues, except for *biceps femoris*, where the opposite trend was observed ($P < 0.01$; Table 5). Hybrid F2 pigs showed higher FSR in liver and in *longissimus dorsi* ($P < 0.01$) compared with Duroc. On the contrary, Duroc pigs tended ($P = 0.09$) to synthesize more protein in duodenum compared with F2 animals, becoming significant in LP diets ($P = 0.01$). Moreover, in liver and *longissimus dorsi*, FSR differences registered between Duroc and F2 at growing phase ($P < 0.01$) did disappear in fattening pigs (Interaction effect PP × PT, $P < 0.05$, Figure 2, b) and c). Dietary CP content did not impact FSR except for *biceps femoris*, where SP diet tended to lead to higher synthesis rates ($P = 0.07$). Regarding ASR, PP influence was significant ($P < 0.01$) on all the studied tissues, showing as expected a superior body mass in the finishing phase. As happened with the FSR, F2 pigs showed higher ASRs ($P < 0.05$) compared with Duroc pigs. Absolute synthesis rate was not estimated for duodenum due to lack of exact tissue weight.

Table 5. Fractional (FSR) and absolute protein synthesis rate (ASR) in duodenum and liver viscera tissues, and in biceps femoris and longissimus dorsi skeletal muscles in F2 crossbred pigs (n = 8) and purebred Duroc pigs (n = 7), fed with two dietary CP levels (low, LP v. standard, SP).

Tissue	Phase (PP)		Producing type (PT)		Diet (DI)			*P*-value				
	Growing	Fattening	Duroc	F2	LP	SP	SEM[1]	PP	PT	DI	PP × PT	PT × DI
FSR (%/day)												
Duodenum	53.3	44.9	52.0	46.2	48.8	49.4	2.23	0.02	0.09	0.84	0.89	0.01
Liver	46.7	39.0	36.1	49.6	42.3	43.4	2.02	0.01	<0.01	0.71	0.01	0.50
Biceps femoris	6.6	8.1	7.2	7.6	7.0	7.7	0.25	<0.01	0.23	0.07	0.82	0.26
Longissimus dorsi	10.7	9.0	7.6	12.1	9.8	9.9	0.49	0.02	<0.01	0.86	<0.01	0.40
ASR (g/day)												
Liver	58.1	123.8	82.3	99.6	96.3	85.6	4.99	<0.01	0.02	0.15	0.15	0.28
Biceps femoris	4.1	18.3	9.6	12.8	10.9	11.6	0.43	<0.01	<0.01	0.27	0.01	0.99
Longissimus dorsi	13.9	38.3	20.3	32.0	26.4	25.8	1.84	<0.01	<0.01	0.81	0.39	0.72

[1] SEM = average standard error of the means.

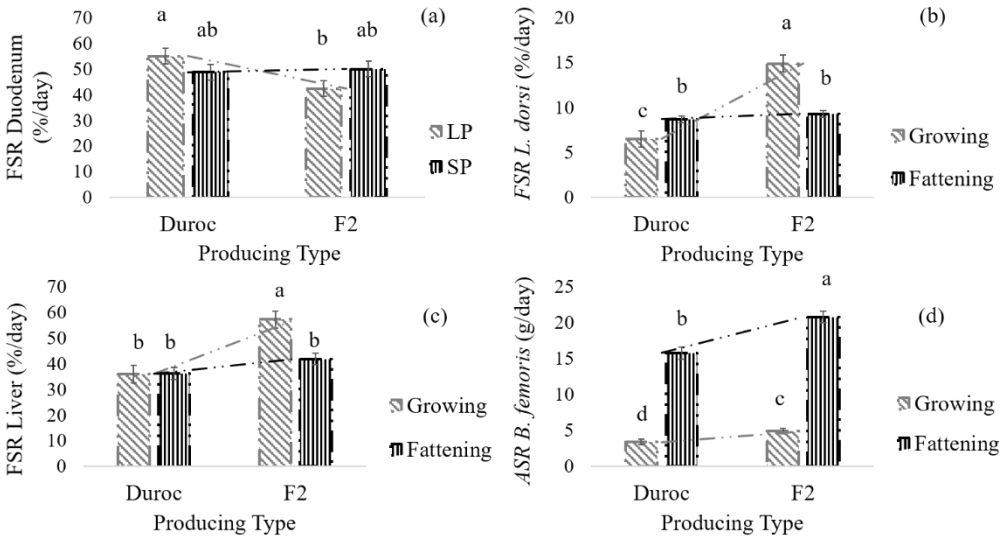

Figure 2. Fractional synthesis rate (FSR) of duodenum affected by the interaction of producing type (PT; Duroc v. F2) and dietary CP (standard, SP v. low, LP) (Figure 2a). FSR of longissimus dorsi (Figure 2b) and liver (Figure 2c) affected by the interaction of production phase (PP; Growing v. Fattening) and pig PT. Absolute synthesis rate (ASR) of biceps femoris affected by the interaction of PP and PT (Figure 2d). Above each bar, different letters (^{a,b}) indicate significant differences ($P < 0.05$). Error bars = SEM.

Nitrogen balance

Daily nitrogen intake and excretion (feces and urine) together with the nitrogen balance, as the difference between input and output, are presented in Supplementary Table S2. As expected, animals during fattening period ingested, excreted, and retained more nitrogen ($P < 0.01$) than growing animals. At fattening, more nitrogen was excreted via urine in Duroc pigs compared with F2 ($P < 0.01$, Supplementary Figure S5), reflecting also in less retention in the former ($P < 0.01$). Feeding SP diets to animals significantly increased nitrogen intake ($P < 0.01$) and tended to increase its retention ($P = 0.08$).

Discussion

Methodological approach

The main objective of this trial was to refine protein requirements in pigs by analyzing the specific impact of genotype (i.e. PT), dietary CP restriction (2% reduction) and their potential interactions. The experiment was conducted in two different stages of development (phases), a) when animal retains mostly protein (20–30 kg BW) and b) when fat becomes the principal component of the daily body gain (80–90 kg BW). To detect potential differences in protein metabolism and to refine requirements, FSR [in muscle (*longissimus dorsi*, *biceps femoris*) and viscera (liver and duodenum)] together with ileum AA-digestibility and nitrogen balance were determined.

Our experimental approach involved both surgery and confinement in metabolic cages to the pigs under study to get digestibility, nitrogen balance and FSR results; such a complex experimental procedure limited the number of available animals for the experiment. Authors are aware that having 32 animals for a 2 × 2 × 2 factorial design might have been a limiting factor to reach consistency in both results and conclusions; however, the absence (or scarce impact) of interaction effects among main factors would indicate that, in this scenario, experimental underpowering was not a relevant issue, and hence the number of animals used was considered enough to achieve the proposed objectives.

In relation to the genotypes employed it is necessary to point out that some Duroc-crossbred lines have been selected to improve lean deposition, although pure Duroc breed remains the most common line exploited as a fatty pig to produce high-quality products with high intramuscular fat infiltration (Latorre et al., 2003b). To commercially promote such features, Duroc males are castrated early and slaughtered at higher ages, while lean breed males, destined for leaner pork cuts, are not. Since one of the main objectives of the present assay was to study protein metabolism under commercial conditions, Duroc males were surgically castrated. Moreover, castration may involve maximizing potential differences between PTs. The anabolic effects of testicular hormones seem to impact feed efficiency, nitrogen retention and protein deposition in entire males (Ruiz-Ascacibar et al., 2019b). Hence, definition of breed

throughout the text has been replaced by PT, which includes the effect of genotype plus castration in the case of Duroc males.

Effect of production phase

As expected, fattening pigs (88.6 ± 6.26 kg BW) showed higher level of feed intake and average daily gain (Table 2), along with higher AID of essential AAs in ileum; however, growing pigs (29.5 ± 3.19 kg BW) showed an improved FSR in almost all tissues analyzed. Only ATTD for EE was significantly higher in fattening pigs, which is consistent with the existing literature (Noblet et al., 2013). Several studies have reported that nutrient digestibility increases linearly with the animal's development, mainly due to a lessening in the digesta transit (Noblet et al., 2013) and/or the development of hindgut microbiota (Knecht et al., 2020). In the present study, fattening pigs showed better AID for DM as well as for most of essential AAs (histidine, leucine, lysine, phenylalanine and threonine). Digestibility coefficients for AAs were variable but did agree with previous studies (Liu et al., 2016). Such variability could be explained by differential affinity either for molecular transport through the brush border membrane (Bröer, 2008), or the variation in AA richness in the endogenous fraction (Mosenthin et al., 2000).

Despite the variability in FSR data in the existing literature due to differences in animal age and analytical procedures, our FSR (%/day) findings were within the range proposed by previous authors (Rivera-Ferre et al., 2005; Wang et al., 2007). Differences between viscera and skeletal muscle tissues have also been reported, being superior the former tissues mainly due to their higher metabolic activity and protein turnover. Increased protein metabolism, fast growth and highly efficient protein synthesis in young mammals has been widely documented, peaking at birth and decreasing rapidly afterwards (Davis et al., 2008). In our study, FSR in *biceps femoris* was an exception, being significantly higher in the fattening period. Authors are not aware of previous data to confirm the reported exception, although muscles from the hind limb show a later maturation than those from the rest of the body (Darinskii, 1975).

Effect of producing type

Previous experimental evidence (Morales et al., 2002) has been suggested that fatty pigs (i.e. Iberian or Duroc) have higher voluntary feed intake than modern lean lines (i.e. Landrace or Pietrain). This is consistent with our findings during the fattening phase, although the differences recorded did not reach statistical significance; however, the opposite trend was detected during the growing phase. It has traditionally been accepted that Duroc is a fatter breed, due to its high intramuscular fat content. However, genetic selection exercised on Duroc pigs to ameliorate leanness and reduce fat content has improved lean gain in Duroc lines. Several studies have evidenced that Duroc or Duroc-crossbred pigs grow at a similar rate (Čandek-Potokar et al., 1998) or even faster than lean breeds (Latorre et al., 2003).

Authors are not aware of data showing differences in nutrient digestibility among PTs, but in our case Duroc pigs showed higher ATTD (for EE and NDF) and AID (for DM, CP and all the AAs), although it was not translated to better CP retention rates or lower nitrogen excretion. Intestine is a very active metabolic organ responsible of releasing AAs into the peripheral blood for the rest of the organism, but it has been detected that the quantity and proportion of AAs in the portal system differs from that absorbed by the intestine (Baracos, 2004). Considering the high tendency for Duroc pigs to have higher duodenum FSR, an important part of the AAs absorbed by the intestine may have been used for its own protein metabolism.

Fractional synthesis rate values obtained from both liver and *longissimus dorsi* muscle in F2 pigs were higher than those of Duroc pigs, suggesting that F2 pigs synthesized a higher proportion of protein on a daily basis (%/day: 37% and 59%, for liver and *longissimus dorsi*, respectively). Moreover, differences were more pronounced in young pigs, and diminished with age and/or development (PP × PT interaction). In *biceps femoris* muscle, FSR differences did not reach statistical significance, although ASR also differ between PTs (on average 33%, see figure 2d) because the greater protein pool in F2.

The higher FSR and ASR (just ASR in the case of *biceps femoris*) shown by lean pigs should explain their tendency to grow faster. In this sense, Rivera-Ferre et al. (2006) also reported greater protein deposition in Landrace than Iberian gilts, but in

contradiction with the present results, no breed differences were reported in FSR, when nitrogen flows were calculated by cumulative urinary isotope excretion after an oral dose of [^{15}N]-glycine. Likewise, Rivera-Ferre et al. (2005) using an identical $^{2}H_5$−phenylalanine protocol than the current study, concluded that Iberian pigs showed higher synthesis rates in muscles and viscera than Landrace pigs. According to the existing literature, the type of muscular fiber could affect FSR; thus those animals with predominantly slow-twitch oxidative fibers (type I) showed greater FSR than those with fast-twitch glycolytic (type II) ones (Goodman et al., 2012). Nonetheless, Duroc pigs seem to have a higher proportion of slow-twitch oxidative fibers than Pietrain boars (Werner et al., 2010); however, the possibility that crossbred F2 [Pietrain × (Duroc × Landrace)] have higher proportion of slow-twitch oxidative fibers than purebred Duroc pigs seems to be negligible. Therefore, the mechanism underlying the differences in FSR between Duroc and F2 should be related with additional factors, such as the effect of gender or castration on protein metabolism that needs to be further elucidated.

Nevertheless, the predominant type of muscle fiber in the animal may not be the only factor conditioning the rate of protein synthesis. In this sense, Liu et al. (2015) found that some Landrace lines showed higher abundance of protein precursors related to protein deposition and muscle growth in their tissues, such as p-AKT, mTOR, p70S6K, than a Chinese indigenous fatty pig (Bama mini-pig). The abundance of such positive regulators also decreases with age, and thus reduces the protein synthesis in skeletal muscle. In fact, mTOR (serine/threonine protein kinase) is an important mechanism for the regulation of protein synthesis in cells (Deng et al., 2014), and when it phosphorylates other proteins such as ribosomal protein p70S6K coordinates gene transcription and protein translation, involved in the regulation of growth, proliferation and differentiation of cells.

Effect of dietary CP

Experimental diets were formulated to meet the nutrition requirements for growing/fattening pigs (FEDNA, 2013). Although CP concentration in LP diets was reduced by 2%, requirements of essential AAs were covered in both diets by using crystalline AAs. The CP reduction improved both average daily gain and feed

conversion ratio (see Table 2). A reduction to certain levels (2 to 3%) with optimal adjustment of AAs is widely accepted, and allows to maintain animals growth and performance, promote nitrogen accretion (Wang et al., 2019), and in consequence reduce both cost of feeding and nitrogen waste. An interaction in feed conversion ratio between PP and dietary CP content was seen, being differences between diets more pronounced in the growing period. Better feed efficiencies were provided by LP diets, which is consistent with Wang et al. (2019) who showed that pigs in growing phase were more sensitive to CP diet changes than in fattening phase.

The effect of dietary CP on AID of DM, CP, and AAs was investigated. When dietary CP was reduced by 2 percentage units, the AID of DM was maintained, as was the ATTD of DM and the rest of nutrients, except for CP. Pigs fed LP diet presented lower AID for most AAs, including histidine, isoleucine, leucine, phenylalanine, and valine.

Although the precise relationship between dietary CP supply and AID is open to discussion, several studies agree with our findings by describing how protein AID increase linearly with dietary CP until reaching a plateau (Furuya and Kaji, 1989; Li et al., 1993; Fan et al., 1994). Moreover, Furuya and Kaji (1989) stated that influence of endogenous AAs on AID becomes negligible at CP levels like those proposed in our study. In this sense, Fan et al. (1994) described that both CP and AAs AID tend to reach a plateau, but not simultaneously: CP, methionine, threonine and tyrosine ileal digestibility leveled off when dietary CP reached values of 15.36, 13.70, 15.53 and 16.29 %, respectively; anyway, this plateau is over the CP level reached in LP diets (14.7 and 12.9 % for growing and fattening animals respectively). On the other hand, the opposite effect was also described by Li et al. (1993), although the study is based on early-weaned pigs and they applied higher levels of CP inclusion (19.5 to 25.5% of CP), obtaining a reduction in AID when dietary CP increased. In their study, authors claim that the total supply of AAs from the high protein diets may have exceeded the maximum capacity of efficiency of AAs transport throughout the intestinal mucosa. Our study never reached those CP levels, and our animals exhibited a more developed digestive tract.

Incorporation of free AAs from crystalline form varied among experimental diets (1.4 and 1.2 in growing and 0.57 and 0.29 % in fattening pigs, for LP and SP, respectively).

This fact might have influenced AID since crystalline AAs are rapidly and completely absorbed (Otto et al., 2003). However, incorporation of free synthetic AAs may imply less secretion of proteolytic enzymes, due to a lower intact dietary protein present into the small intestine, and that fact might have influenced AID (Yen et al., 2004). Therefore, a long-term effect of crystalline AAs inclusion on AID is unpredictable (Y. M. Wang et al., 2019).

Experimental diets were formulated to cover all essential AA requirements, however a slight but significant diet effect on FSR was observed. Protein restriction tended to decrease FSR in *biceps femoris* (Table 5); moreover, in lean animals, duodenal-FSR was also restricted when giving LP diet (PT × dietary CP content, Figure 2a). In that sense, the observed restriction cannot be explained by essential AAs metabolism (such fraction did not differ among diets) although it can be hypothesized that the observed reduction in FSR could be a consequence of a possible restriction of non-essential AAs supply in lean (F2) pigs to attain the maximum potential of protein accretion. In relation to the existing literature, no effect of CP or lysine restriction on FSR was reported by Rivera-Ferre et al. (2005), although, Sève et al. (1986) observed that by decreasing CP supply in young pigs (17 days old), duodenal protein metabolism experimented a FSR depression, but with increases in protein accretion due to a concomitant decrease in fractional degradation rate. In agreement with the former author, Li et al. (2016) demonstrated that diet may be relevant at longer experimental periods since skeletal muscle can be stimulated by the availability of AAs (i.e., arginine, lysine or leucine). Such stimulation would disappear with animal's maturity (Bandt, 2016).

Conclusions

Most of the parameters studied were influenced by the PP, where fattening pigs had higher growth, apparent digestibility coefficients and tissue protein accretion, whereas growing pigs showed more active protein metabolism. Although Duroc pigs showed higher AID for all the AAs, growing F2 pigs got higher FSRs in liver and *longissimus dorsi* and higher nitrogen retention rates with lower nitrogen waste. This information may reveal differences in AA requirements between both PT, with Duroc pigs receiving excess of dietary AAs. A 2% reduction of dietary CP concentration in the

LP diets resulted in improved average daily gain and feed conversion ratio. Although this dietary CP reduction was detrimental for AA apparent digestibility and nitrogen retention, hardly affected the protein synthesis rates.

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Supplementary material

Table S1. Apparent ileal digestibility (AID, %) of conditionally essential amino acids (AA), and non-essential (AA) of purebred Durocs (n = 15) and F2 pigs (n = 16) in growing and fattening phases, and fed 2 dietary levels of CP (low, LP v. standard, SP).

Amino Acids	Phase (PP)		Producing type (PT)		Diet (DI)			*P*-value				
	Growing	Fattening	Duroc	F2	LP	SP	SEM [1]	PP	PT	DI	PP × PT	PT × DI
Conditionally essential AA												
Arginine	86.6	84.9	88.1	83.4	82.3	89.2	1.32	0.40	0.02	<0.01	0.09	0.16
Cysteine	77.1	81.6	82.8	75.9	73.5	85.2	2.06	0.14	0.03	<0.01	0.01	0.85
Proline	82.3	82.7	85.6	79.3	80.8	84.1	1.40	0.84	0.01	0.12	0.02	0.84
Tyrosine	76.8	76.8	82.6	71.0	72.6	80.9	2.10	0.99	<0.01	0.01	0.12	0.39
Non-essential AA												
Alanine	76.9	71.9	78.5	70.3	67.9	80.9	2.16	0.11	0.01	<0.01	0.19	0.85
Aspartic acid	80.3	81.0	83.8	77.6	76.5	84.8	1.92	0.80	0.03	0.01	0.34	0.87
Glutamic acid	91.5	87.2	90.9	87.9	87.9	90.8	0.95	<0.01	0.04	0.04	0.59	0.81
Glycine	69.3	72.7	77.9	64.1	65.9	76.1	4.98	0.64	0.07	0.17	0.79	0.22
Serine	76.0	82.8	84.0	74.8	75.7	83.1	1.81	0.01	<0.01	0.01	0.02	0.69

[1] SEM = average standard error of the means.

Table S2. Nitrogen balance, including intake and excretion of nitrogen through feces and urine (g/day), nitrogen retention (g/day) and nitrogen retained of intake in F2 males and castrated pure Duroc pigs, in two production phases (PP, growing and fattening) and fed diets with different CP (low, LP v. standard, SP).

Parameters	Phase (PP)		Producing type (PT)		Diet (DI)			*P*-value				
	Growing	Fattening	Duroc	F2	LP	SP	SEM[1]	PP	PT	DI	PP × PT	PT × DI
Intake	25.2	51.3	38.7	37.8	35.5	41.0	1.37	<0.01	0.65	0.01	0.10	0.15
Faecal excretion	11.9	16.4	15.8	12.5	14.1	14.2	1.19	0.01	0.06	0.97	0.24	0.97
Urinary excretion	3.9	15.1	11.5	7.4	8.3	10.6	0.87	<0.01	0.01	0.10	<0.01	0.15
Retention	9.4	19.7	11.3	17.8	13.0	16.2	1.24	<0.01	<0.01	0.08	0.11	0.83
Nitrogen retained, % of intake	37.8	38.9	30.4	46.3	37.6	39.1	3.45	0.83	<0.01	0.77	0.18	0.96

[1] SEM = average standard error of the means.

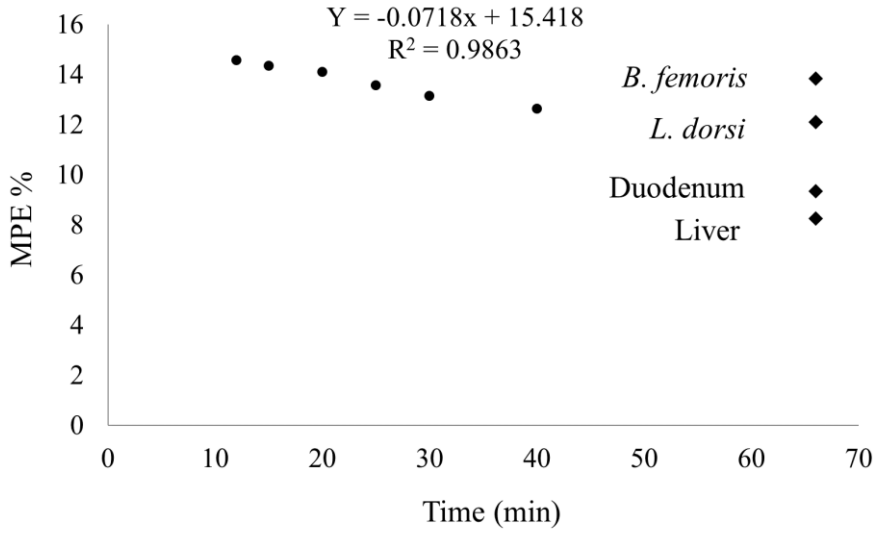

Figure S1. Example of a plasma enrichment curve (MPE) in free phenylalanine at 12, 15, 20, 25, 30 and 40 min after a flooding dose of this amino acid, and MPE in liver, duodenum, longissimus dorsi (*L. dorsi*) and biceps femoris (*B. femoris*) muscles after slaughter of a lean F2 pig fed a standard diet (SP) in the growing phase. Values are presented as mole percent excess (MPE, %).

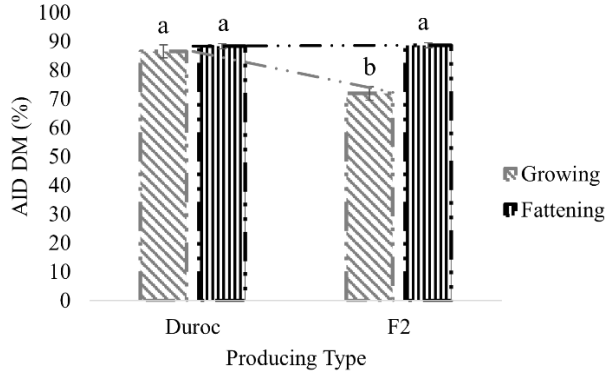

Figure S2. Interaction effect between production phase (growing v. fattening) and pig producing type (Duroc v. F2) on dry matter (DM) apparent ileal digestibility (AID, %). Above each bar, different letters (^{a,b}) indicate significant differences ($P < 0.05$). Error bars = SEM.

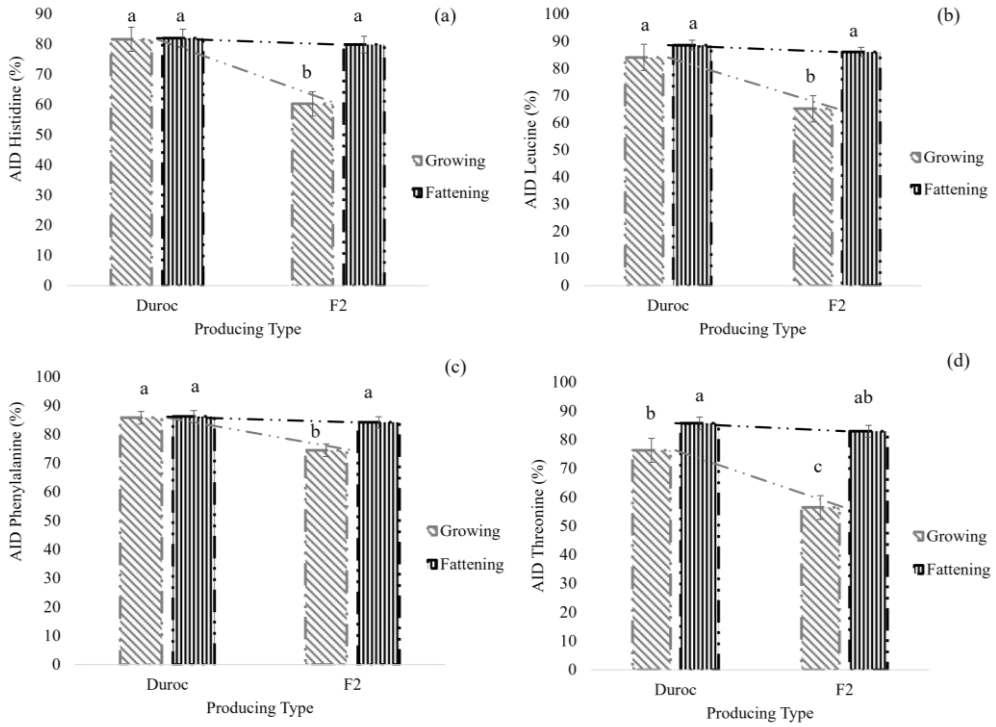

Figure S3. Apparent ileal digestibility (AID, %) of histidine, leucine, phenylalanine, and threonine essential amino acids affected by production phase (growing v. fattening) and pig producing type (Duroc v. F2). Above each bar, letters (^{a,b}) indicate significant differences ($P < 0.05$). Error bars = SEM.

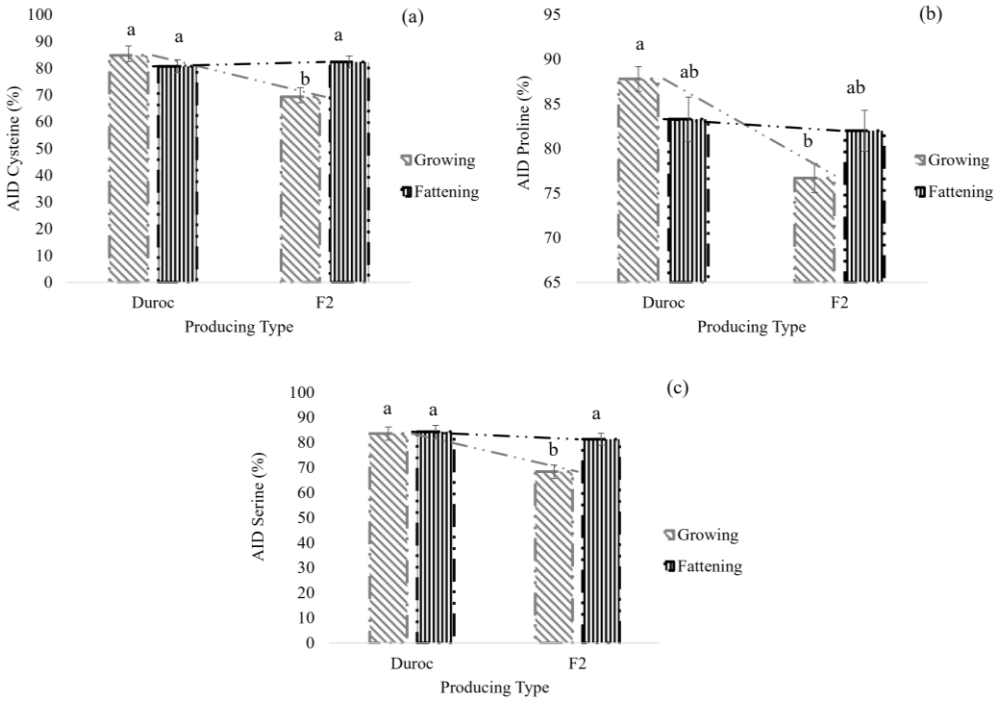

Figure S4. Apparent ileal digestibility (AID, %) of cystine, proline and serine affected by production phase (growing v. fattening) and by pig producing type (Duroc v. F2). Above each bar, letters (^{a,b}) indicate significant differences ($P < 0.05$). Error bars = SEM.

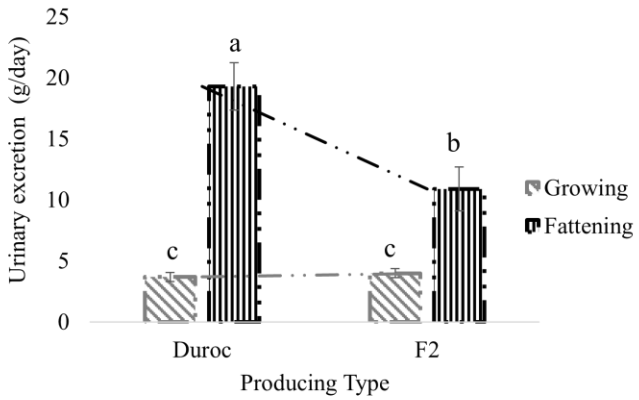

Figure S5. Urinary nitrogen excretion affected by the interaction between production phase (growing v. fattening) and pig producing type (Duroc v. F2). Above each bar, letters (^{a,b}) indicate significant differences ($P < 0.05$). Error barrs = SEM.

CHAPTER III

Age evolution of lipid accretion rate in boars selected for lean meat and Duroc barrows

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Abstract

Fatty acid (FA) deposition in growing–fattening pigs is mainly based on endogenous lipid synthesis, but also direct FA incorporation from the diet. To evaluate the direct fat incorporation rates and the endogenous desaturation action of the stearoyl-CoA desaturase (SCD) enzyme, a deuterium (D)-labeled saturated FA (d_{35}-C18:0) was added to the diet. Sixteen three-way (3W) crossbred boars, and thirty-two purebred Duroc barrows homozygous for the *SCD* single nucleotide polymorphism rs80912566 (16 CC/16 TT), were used. Half of the animals of each genotype belonged to the growing and fattening phases. The fractional incorporation rate (FIR) of dietary fat in growing pigs was generally higher in adipose tissues, whereas in fattening pigs it was higher in the liver. Duroc pigs exhibited lower FIRs than 3W pigs, suggesting lower rates of endogenous synthesis by 3W pigs. Real fractional unsaturation rates (FURs) increased with age by the higher FIRs in 3W pigs and the de novo synthesis pathway in Duroc genotypes. Moreover, pigs carrying the *SCD*_T allele showed more enhanced oleic acid biosynthesis than Duroc CC pigs. In conclusion, suitable feeding protocols should be designed for each pig type to optimize production traits, considering that the metabolic pathway of FA for its deposition may differ.

Keywords: apparent digestibility; incorporation rate; pigs; producing type; stearoyl-CoA desaturase; unsaturation rate

1. Introduction

Swine selection has traditionally focused on lean productive efficiency; however, consumer demand for high-quality products has considerably increased over the last years (García-Gudiño et al., 2021); therefore, traditional fatty breeds such as Iberian or specific Duroc lines have been selected for relevant quality traits such as higher levels of intramuscular fat (IMF) (Estany et al., 2017; Gol et al., 2019). To enhance these quality traits, pigs are raised to a heavier slaughter weight and males are usually castrated to avoid boar taint, as they reach sexual maturity (Latorre et al., 2003b; Pérez-Ciria et al., 2022). The Duroc breed has long been selected for lean growth, although certain lines have preserved and expressed both high lipogenic activity and lipid deposition capacity (Wood et al., 2008). In these fattier producing types (PTs), the most sought-after effects are increased IMF content and improved fatty acid (FA) composition (Alonso et al., 2015; Ramírez and Cava, 2007), by the promotion of monounsaturated FA in detriment of saturated FA, bearing in mind the reported adverse effects of saturated FA on human health (Hammad et al., 2016; Wu et al., 2020).

Despite the use of suitable genetic lines to obtain premium meat products, the management of fat incorporation throughout the growing–fattening phases is complex, as it involves the deposition of FA into adipocytes from both sources, diet and endogenous origins. In pigs, lipid synthesis occurs mostly in adipose tissue (O'Hea and Leveille, 1969), and it is generally accepted that deposited lipids from lipid synthesis exceed those from direct dietary incorporation, with endogenous oleic (C18:1 *c*9), palmitic (C16:0), and stearic (C18:0) acids accounting for 80% of total FA deposited (Dunshea and D'Souza, 2003; Kloareg et al., 2007). Thus, FA composition in tissues is related to specific rates of deposition, synthesis, and desaturation (Poklukar et al., 2020). Transformation of saturated FA to monounsaturated FA is enhanced by a single nucleotide polymorphism of the stearoyl CoA desaturase gene (*SCD*; rs80912566), which regulates a rate-limiting enzyme (SCD) that has a main function of maintaining proper fluidity of cellular lipids without affecting IMF content or backfat thickness. The main substrates of SCD are palmitoyl and stearoyl-CoA, which are desaturated at the Δ9 position, transforming them into de novo palmitoleoyl and oleoyl-CoA, respectively. The *SCD*_T allele enhances this

process compared with the alternative *SCD*_C allele (Estany et al., 2014), thus promoting the synthesis of monounsaturated FA.

To optimize fat deposition and enable a comprehensive understanding, it is necessary to build a dynamic model of lipid development that combines endogenous FA de novo synthesis and exogenous dietary deposition (Juárez et al., 2017; Kloareg et al., 2005). In this context, the biochemical and genetic mechanisms involved in fat deposition between fatty and lean PTs needs to be clarified (Poklukar et al., 2020). Therefore, the objective of the present study is to evaluate lipid metabolism in different pig PTs by analyzing both de novo synthesis and dietary FA incorporation into several tissues.

2. Materials and Methods

Protocols and experimental procedures were approved by the Ethics Committee for Animal Experiments of the University of Lleida (Ref: CEEA 09–05/16). The care and use of animals were in accordance with the Spanish Policy for Animal Protection RD 53/2013, which meets the European Union Directive 2010/63 on the protection of animals used for experimental purposes.

2.1. Animals, Diets, and Experimental Design

The experiment was carried out in the Swine Research Centre (CEP; Torrelameu, Lleida, Spain) using 48 male pigs at two growth phases (GP), 24 growing pigs (28.42 ± 0.861 kg of body weight (BW); μ ± SE) and 24 fattening pigs (87.40 ± 1.256 kg BW). Each GP was composed of three PTs of eight pigs each. The lean type consisted of (i) 8 three-way (3W) crossbred boars [Pietrain sires × (Duroc × Landrace) dams], while the fatty type consisted of 16 purebred Duroc barrows, of which (ii) 8 were homozygous for the *SCD*_C allele, and the remaining (iii) 8 were homozygous for the *SCD*_T allele for the *SCD* rs80912566 genotype. Pigs were genotyped by allelic discrimination assay with primers and probes following Estany et al. (2014). Upon arrival at the experimental facility, pigs within the same GP and PT were randomly assigned to one of the two dietary treatments and placed in groups of four in 55% concrete slatted-floor pens (2.10 × 2 m^2), where they remained the first 5 days as a dietary adaptation period. During the last 6 days, pigs were housed individually in metabolic cages (2 × 1.04 m2), weighting the pigs just before housing them in the metabolic cages. For each GP, two experimental diets [standard (SP) and low (LP)

protein] were formulated as previously described (Sarri et al., 2021). Titanium dioxide (TiO_2; 5 g/kg dry matter (DM)) was included as an inert flow marker, and 290 mg/kg DM of deuterium (D)-labeled stearic acid (d_{35}-C18:0; Tracer, Madrid, Spain) was also included in each experimental diet on the last 6 days of each GP. Diets were supplied ad libitum, measuring the daily feed supply and waste, and pigs had free access to drinking water. The ingredients and chemical composition of the experimental diets can be reviewed in a previous study (Sarri et al., 2021), while the dietary FA composition is shown in Table 1. Pigs were culled on day 11.

Table 1. Fatty acid composition (% of total fatty acids) of the two-phase experimental diets (growing and fattening), differing in crude protein content (standard, SP vs. low, LP).

Fatty Acids, %	**Growing**		**Fattening**	
	LP	**SP**	**LP**	**SP**
Myristic (C14:0)	0.14	0.14	0.26	0.27
Palmitic (C16:0)	14.27	14.17	17.07	17.44
Palmitoleic (C16:1)	0.12	0.12	0.31	0.30
Margaric (C17:0)	0.14	0.15	0.16	0.16
Stearic (C18:0)	2.62	3.06	2.91	3.27
Oleic (C18:1 *c*9)	18.76	18.04	20.14	19.77
Vaccenic (C18:1 *c*11)	0.96	1.00	1.19	1.21
Linoleic (C18:2 *c*9, *c*12)	56.22	55.33	51.73	50.72
Linolenic (C18:3 *c*9, *c*12, *c*15)	5.73	6.96	4.99	5.54
Arachidic (C20:0)	0.33	0.34	0.38	0.39
Eicosenoic (C20:1 *c*11)	0.28	0.27	0.36	0.37
Behenic (C22:0)	0.25	0.28	0.31	0.36
Lignoceric (C24:0)	0.18	0.17	0.21	0.20

2.2. Sample Collection and Dissection Process

Blood samples were collected daily (between 0.900 and 1000 h) without fasting in ethylenediamine tetraacetic acid tubes from the jugular vein for five consecutive days

before culling. Plasma was obtained by centrifugation (2500 rpm for 10 min) and stored at 80 °C for further analysis. On the last day, animals were euthanized by an intravenous infusion of sodium thiopental (Esteve S.A., Oudewater, The Netherlands), and immediately weighted and eviscerated. Age at slaughter was 79.43 ± 1.875 and 159.38 ± 3.359 days of age for 3W pigs, and 86.25 ± 1.356 and 150.69 ± 2.323 days of age for Duroc pigs for the growing and fattening phases, respectively. Digesta from the last part of the upper intestine (15–20 cm) was taken to determine apparent ileal digestibility (AID); and subcutaneous adipose tissue (SC), liver, and longissimus dorsi and semimembranosus skeletal muscles were sampled from the left half carcass. Samples of SC and longissimus dorsi muscle were taken between the third and fourth last ribs, while those of semimembranosus muscle were taken from the third most proximal to the spine. Samples were stored immediately at −80 °C until required for fat and FA determinations. The loin and leg from the right half carcass were cut based on Walstra and Merkus (1996) standards, and reserved for relative allometric coefficients (k) determination by dissecting tissues. The liver and the right half carcass loin were weighted, as well as the right leg, which was dissected into muscles, bones, skin and SC, intermuscular tissue, and remainder (blood vessels, ligaments, and tendons) in a controlled-environment dissection room. Measurements of IMF were performed in gluteus medius and semimembranosus muscles by the Sohxlet method according to the Association of Official Analytical Chemists (AOAC, 2006) (ref.920.39).

2.3. Analytical Procedures

2.3.1. Apparent Ileal Digestibility

Samples of ileum content were freeze-dried and homogenized and, along with experimental diet samples, were analyzed for DM content (AOAC, 2006) (ref. 934.01). Crude protein (CP) content (nitrogen × 6.25) was analyzed by Dumas combustion (Tru Spec CN; Leco Corporation, St. Joseph, MI, USA) (ISO, 2008). Fatty acid methyl esters were obtained in duplicate by transesterification of 75 mg-samples (Tor et al., 2021). Titanium dioxide was analyzed in ileum and dietary ashes using inductively coupled plasma mass spectroscopy (7700x, Agilent Technologies, Tokyo, Japan) following the Darambazar (2019) procedure with some modifications,

including the digestion with 6.5 mL H_2SO_4 (7.4 M) for 1.5 h at 200 °C and the addition of 5 mL H_2O_2 (30%, *v*/*v*) after cooling.

2.3.2. Tissue Fatty Acids Analysis

Total lipids from plasma, liver, and adipose tissues (including SC and IMF of longissimus dorsi and semimembranosus muscles) were extracted (Folch et al., 1957) using a chloroform-methanol solution (2:1, *v*/*v*). Solvents were evaporated under vacuum (at 40 °C), and FA were subsequently obtained by saponification (Aldai et al., 2006) by adding 1.2 mL of saponification solution [KOH (5M) in methanol/water (50:50, *v*/*v*)], and placing the sealed tubes, flushed with nitrogen, in a water bath during 60 min at 60 °C. Then, glacial acetic acid was added to neutralize the KOH fraction and FA were extracted using petroleum spirit. The solvent was evaporated under vacuum and samples were re-diluted in isopropanol, vortexed, and filtered through a 0.2 µm hydrophilic polytetrafluoroethylene membrane.

Measurements of D-labeled FA were performed in duplicate by ultra-high-performance liquid chromatography coupled to a Xevo triple quadrupole mass spectrometer (TQD; Waters, Milford, MA, USA). The system was equipped with an electrospray ionization source and an ACQUITY HSS-T3 column (2.1 × 150 mm; 1.8 µm). A multiple reaction monitoring method was designed and optimized to include the following FA: C18:0, d_{35}-C18:0, C18:1 *c*9, and d_{33}-C18:1 *c*9. Cone voltage and collision energy were optimized for all transitions, and the absence of cross-signaling between D-labeled and unlabeled FA channels was checked. Results were processed using QuanLynx V4.1 software (MassLynx, Waters Corporation, Milford, MA, USA).

2.4. Calculations

Nutrient AID was calculated as described in Sarri et al. (2021) as follows:

$$y = 1 - \left(\frac{\text{marker}_{\text{feed}}}{\text{marker}_{\text{ds}}} \times \frac{Z_{\text{ds}}}{Z_{\text{feed}}} \right)$$

where y is the coefficient of AID of a nutrient; Z_{ds} and Z_{feed} are the nutrient concentration in ileum and in the diet, respectively; and marker_{feed} and marker_{ds} represent marker (TiO_2) concentrations in diet and ileum, respectively.

$$\text{FIR} = \left(\frac{\text{MPE } d_{35}\text{– C18:0 }_{\text{tissue}}}{\text{aveMPE } d_{35}\text{–C18:0 }_{\text{plasma}}} \times \frac{100}{t} \right)$$

Plasma and tissue d_{35}-C18:0 enrichment was expressed as molar percent excess (MPE) (Sarri et al., 2021). The percentage of plasma d_{35}-C18:0 incorporated per day (fractional incorporation rate: FIR, %/day) into fat depots was calculated from the equation:

where MPE d_{35}-C18:0 $_{\text{tissue}}$ is the enrichment in d_{35}-C18:0 of stearic acid in tissues; aveMPE d_{35}-C18:0 $_{\text{plasma}}$ is the average d_{35}-C18:0 enrichment of stearic acid in the plasma pool during the labeled diet consumption; and t is the labeling time in days.

Apparent and real fractional unsaturation rates (FURs) in SC were calculated as the percentage of C18:0 and d_{35}-C18:0 unsaturated per day (%/day) to C18:1 *c*9 and d_{33}-C18:1 *c*9, respectively.

Both were calculated as follows:

$$\text{Apparent FUR} = \left(\frac{\text{C18:1 } c9 \text{ }_{\text{tissue}}}{\text{C18:0 }_{\text{tissue}}} \times \frac{100}{t} \right)$$

$$\text{Real FUR} = \left(\frac{d_{33}\text{–C18:1 } c9_{\text{tissue}}}{d_{35}\text{–C18:0}_{\text{tissue}}} \times \frac{100}{t} \right)$$

where C18:1 *c*9 $_{\text{tissue}}$ and C18:0 $_{\text{tissue}}$ of apparent FUR are the enrichment in C18:1 *c*9 and C18:0 FA in the same tissue, respectively; both FA in this index can come from endogenous and exogenous sources; while in the real FUR, d_{33}-C18:1 *c*9 $_{\text{tissue}}$ and d_{35}-C18:0 $_{\text{tissue}}$ are the enrichment in d_{33}-C18:1 *c*9 of oleic acid and d_{35}-C18:0 of stearic acid in the same tissue, respectively, and should reflect SCD activity; and t is the labeling time in days.

The relative growth coefficients (*k*) of each dissection component of the right leg were studied in relation with body growth. They were obtained as the slope of the regression of each component weight on body weight from the allometric equation ($y = ax^k$) (Huxley, 1932), with both logs transformed to linearize the equation as follows:

$$\log(y) = k \log(x) – \log(a)$$

where log *y* is the weight of each leg component, log *x* is the body weight, log *a* is the intercept, and *k* is the allometric growth coefficient.

2.5. Statistical Analysis

The statistical analysis of AID, FIR, and apparent and real FUR was performed by applying a MIXED model using SAS statistical software (v9.4; SAS Institute Inc., Cary, NC, USA), including the GP (growing and fattening) and PT (Duroc CC, Duroc TT, and 3W) and their two-way interaction as fixed effects, where each pig was considered an experimental unit. Because moderate restriction of dietary CP over such a short period of time did not imply significant differences or interactions in any of the parameters studied, the effect was dropped from the model for these traits. To consider the repeated measures (GP), the residual was modeled using an unstructured covariance matrix. Differences between least square means were assessed using the Student's *t*-test. Results were reported as least square means and their SE. Significant differences and tendencies were declared at $p \leq 0.05$ and $0.05 < p < 0.10$, respectively.

The allometric coefficients for the liver, loin, and different leg components and their standard errors were estimated using overall data (growing and fattening phases) and, within PTs, using generalized linear model (GLM) procedures. The Student's *t*-test was used to test if each coefficient was statistically different from 1.

3. Results

3.1. Production Data and Allometric Tissue Growth

A summary of intakes and performance parameters is provided in Table 2. Pigs significantly increased voluntary feed intake with age ($p < 0.001$). Although no differences were found between PTs in the growing phase, in the fattening phase Duroc CC pigs showed significantly higher feed intake than Duroc TT pigs (GP × PT interaction effect; $p = 0.032$). In terms of production performance, fattening pigs also showed higher average daily gain (ADG) than growing pigs ($p < 0.001$). No differences between PTs were found in growing pigs, whereas in the fattening phase 3W pigs had higher ADG than Duroc TT pigs, and Duroc CC pigs were in between 3W and Duroc TT pigs (GP × PT interaction effect; $p = 0.045$). The same was observed with the feed:gain ratio, although no significant interaction between GP and PT was detected.

Table 2. Least square means (± SE) for average daily feed intake (ADFI), average daily gain (ADG), and feed:gain ratio in growing and fattening three-way (3W) crossbred pigs, and purebred Duroc pigs of TT/CC SCD genotype.

Items	**Producing Type (PT)**	**Growth Phase (GP)**		***p*-Value**		
		Growing	**Fattening**	**GP**	**PT**	**GP × PT**
Animals (*n*)		24	24			
	3W	966.84 ± 37.673 ^{c}	2131.38 ± 110.492 ^{ab}			
ADFI, g/day	Duroc CC	867.25 ± 37.673 ^{c}	2441.48 ± 127.578 ^{a}	<0.001	0.159	0.032
	Duroc TT	916.78 ± 37.673 ^{c}	2045.23 ± 110.492 ^{b}			
Performance data						
	3W	418.63 ± 31.640 ^{cd}	1053.53 ± 99.324 ^{a}			
ADG, g/day	Duroc CC	440.67 ± 31.640 ^{cd}	816.41 ± 106.182 ^{ab}	<0.001	0.013	0.045
	Duroc TT	374.37 ± 29.596 ^{d}	625.69 ± 106.182 ^{bc}			
	3W	2.27 ± 0.215 ^{ab}	1.97 ± 0.294 ^{b}			
Feed:gain	Duroc CC	1.94 ± 0.201 ^{b}	2.64 ± 0.314 ^{ab}	0.090	0.228	0.095
	Duroc TT	2.22 ± 0.201 ^{ab}	2.96 ± 0.340 ^{a}			

$^{a, b, c}$ Within each variable, means with different superscripts differ significantly ($p \leq 0.05$).

To assess the maturity kinetics between PTs in various organs and carcass components, average allometry coefficients at 80 and 150 days of age (59 kg difference in BW) were calculated and are shown in Table 3.

Table 3. Allometric coefficients (*k*) of liver, loin, ham, and ham components, in two producing types (PTs) of pigs (three-way (3W) crossbred pigs and purebred Duroc pigs) between 80 and 150 days of age with 28 and 87 kg body weight, respectively.

Items	**Producing Type (PT)**		***p*-Value**
	Duroc (*k* ± SE)	**3W (*k* ± SE)**	**PT**
Animals (*n*)	20	12	
Whole parts			
Liver	0.73 ± 0.051 *	0.77 ± 0.069 *	0.689
Loin	1.01 ± 0.033	1.03 ± 0.048	0.705
Ham	1.08 ± 0.024 *	1.05 ± 0.027	0.455
Ham components			
Skin and subcutaneous fat	1.37 ± 0.072 *	1.27 ± 0.079 *	0.332
Intermuscular fat	1.02 ± 0.098	1.40 ± 0.111 *	0.015
Intramuscular fat (GM [1])	1.42 ± 0.118 *	1.38 ± 0.144 *	0.848
Intramuscular fat (SM [1])	0.56 ± 0.062 *	0.76 ± 0.069 *	0.058
Ham muscles	1.08 ± 0.041 *	1.03 ± 0.051	0.345
Biceps femoris	1.11 ± 0.032 *	1.05 ± 0.047	0.884
Gluteus medius	1.09 ± 0.068	1.07 ± 0.081	0.825
Semimembranous	0.81 ± 0.049 *	0.80 ± 0.058 *	0.917
Bones	0.80 ± 0.035 *	0.77 ± 0.047 *	0.666
Sacrum	0.52 ± 0.133 *	0.51 ± 0.150 *	0.955
Coxae	0.85 ± 0.057 *	0.87 ± 0.074	0.828
Femur	0.87 ± 0.041 *	0.82 ± 0.050 *	0.379

[1] Abbreviations: GM, gluteus medius; SM, semimembranosus.

* Allometric coefficients different from 1 ($p \leq 0.05$).

Liver and bones showed allometry coefficients below 1, indicating an advanced degree of maturity as corresponds to vital organs and the skeletal system. However, the opposite was seen in SC, intermuscular fat, and IMF of gluteus medius, showing allometry coefficients up to 1, indicating a high rate of relative growth. The intermuscular adipose tissue of Duroc pigs was an exception, as its allometric coefficient was not dissimilar to 1, and was lower than that of the leaner type 3W pigs ($p = 0.015$).

It should be noted that IMF of semimembranosus, as well as the semimembranosus muscle itself, showed a relative growth coefficient lower than 1 in both PTs, suggesting a higher degree of maturity of this muscle. Moreover, Duroc pigs presented a ham muscle allometry coefficient higher than 1, whereas in 3W pigs the ham allometry coefficient did not differ from 1.

3.2. Apparent Ileal Digestibility

The AID coefficients of DM, CP, and EE are shown in Table 4. The effect of PT on AID interacted with GP (GP × PT interaction effect). In growing phase, 3W pigs showed lower AID than Duroc pigs for DM, CP, and EE, while AID did not differ between Duroc genotypes (CC and TT). However, in the fattening phase there were no differences in AID among PTs. Only in 3W pigs did AID of CP and EE increase with age ($p < 0.05$).

The AID of saturated, monounsaturated, and polyunsaturated FA is reported for each GP and PT in Figure 1. Polyunsaturated FA showed the highest AID, reaching values around 80%, with no significant differences between the three PT within each GP. Monounsaturated FA were less digested than polyunsaturated FA in the growing phase, but differences disappeared with animal development. In the growing phase, the AID of monounsaturated FA differed among PT, being better digested in Duroc CC, followed by Duroc TT and finally by 3W pigs. Saturated FA showed the lowest AID in both GP, although it increased significantly in the fattening phase. While growing 3W pigs had the lowest AID of saturated FA, reaching close to 40%, in the fattening phase 3W pigs reached both Duroc genotypes, and Duroc CC showed significantly higher AID than Duroc TT pigs.

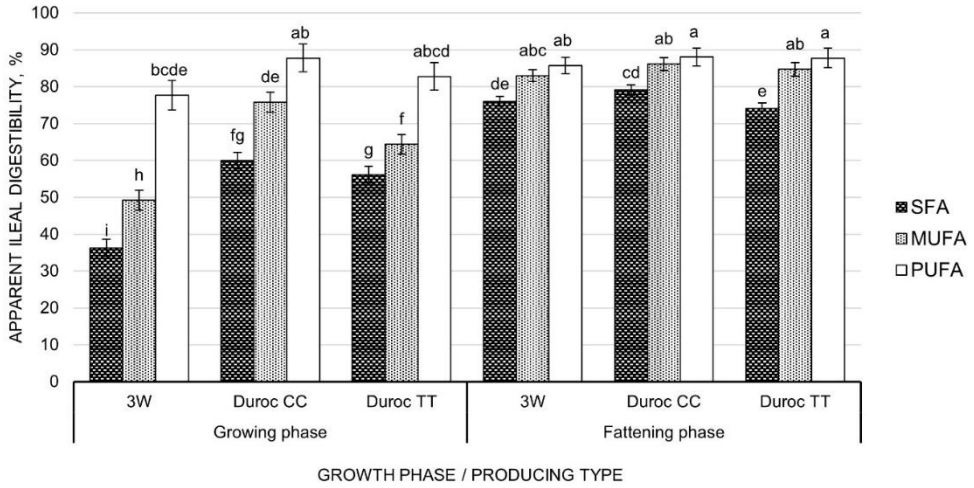

Figure 1. Apparent ileal digestibility of fatty acids, by three-way (3W) crossbred pigs and Duroc purebred pigs of TT/CC SCD genotype, at two growth phases (growing vs. fattening). [a, b, c, d, e, f] means with different superscripts differ significantly ($p \leq 0.05$). Saturated fatty acids (SFA): C14:0, C16:0, C17:0, C18:0, C20:0, and C22:0; Monounsaturated fatty acids (MUFA): C16:1, C18:1 c9, C18:1 c11, and C20:1 c11; Polyunsaturated fatty acids (PUFA): C18:2 c9, c12 and C18:3 c9, c12, and c15.

Table 4. Least square means (±SE) for apparent ileal digestibility (AID) of dry matter (DM), crude protein (CP), and ether extract (EE), between three-way (3W) crossbred pigs and purebred Duroc pigs of TT/CC SCD genotype, belonging to two growth phases (growing vs. fattening).

AID	Producing Type (PT)	Growth Phase (GP)		*p*-Value		
		Growing	Fattening	GP	PT	GP × PT
	Animals (*n*)	24	24			
	3W	71.95 ± 2.101 ^{c}	78.38 ± 2.667 ^{bc}			
DM	Duroc CC	86.52 ± 2.101 ^{a}	82.04 ± 2.851 ^{ab}	0.724	0.002	0.053
	Duroc TT	83.82± 2.246 ^{ab}	79.66 ± 3.080 ^{ab}			
	3W	77.59 ± 1.625 ^{c}	83.75 ± 2.099 ^{ab}			
CP	Duroc CC	88.40 ± 1.737 ^{a}	84.56 ± 2.099 ^{ab}	0.547	0.012	0.010
	Duroc TT	84.57 ± 1.737 ^{ab}	79.34 ± 2.267 ^{bc}			
	3W	61.08 ± 3.936 ^{c}	82.25 ± 2.778 ^{ab}			
EE	Duroc CC	80.59 ± 3.936 ^{ab}	84.24 ± 2.970 ^{a}	< 0.001	0.011	0.045
	Duroc TT	74.10 ± 3.936 ^{b}	83.49 ± 3.208 ^{ab}			

$^{a, b, c}$ Within each nutrient, means with different superscripts differ significantly ($p \leq 0.05$).

3.3. Fractional Incorporation Rate (FIR)

The FIR of dietary stearic acid (d_{35}-C18:0) is an index of daily incorporation of FA relative to its plasma availability. Estimated values of FIR in the liver, SC, longissimus dorsi, and semimembranosus IMF are shown in Table 5. The FIR differed significantly among the tissues studied; in the liver, fattening pigs showed a 3.4-fold higher FIR than growing pigs ($p < 0.001$), with no interaction with PT. However, in the SC the GP and PT did interact ($p = 0.037$), so that significant differences were observed among PT in fattening but not in growing pigs. The 3W pigs significantly increased their SC-FIR over time, whereas in Duroc TT and Duroc CC it remained stable or numerically decreased in Duroc CC pigs. Moreover, the FIR of the longissimus dorsi muscle was impacted by both PT and GP. Regarding GP, the FIR of the longissimus dorsi muscle was much higher in the growing than in the fattening phase ($p < 0.001$); this effect was true in all PT, although it was more pronounced in 3W animals (GP × PT interaction effect; $p = 0.051$). In semimembranosus muscle, FIR decreased in Duroc CC and 3W pigs in the fattening phase, whereas it remained stable in Duroc TT pigs. In 3W pigs, the dietary d_{35}-C18:0 FA in semimembranosus muscle was under the limit of quantification.

3.4. Oleic Acid De Novo Synthesis: Δ9-Desaturase Activity

Oleic acid in fat depot comes from two sources: oleic acid synthesis as a product of Δ9-desaturase activity and direct incorporation of dietary oleic acid. Apparent Δ9-desaturase activity (Table 6) is commonly determined through the FA profile of adipose tissue. Indeed, this value represents the result of both anabolic and catabolic lipid processes occurring throughout the lifespan of the animal and does not reflect necessarily the activity at a specific time-interval. Apparent Δ9-desaturase activity is also masked by oleic acid deposited directly from the diet.

To evaluate the real Δ9-desaturase activity at a specific time-interval, the appearance of d_{33}-C18:1 *c*9 FA in SC originated from the administered form of D-labeled stearic acid (d_{35}-C18:0) was quantified. Table 6 shows the estimated apparent and real FUR as a measure of the oleic acid synthesis. Thus, the apparent activity corresponds to the entire lifespan of the pig, whereas the real activity corresponds to the specific oleic acid synthesis (or FUR) that happened during the specific 6 day-period of the study.

Table 5. Least square means (±SE) for fractional incorporation rate (FIR, %) of deuterated stearic acid (d35-C18:0) in the liver, subcutaneous adipose tissue (SC), longissimus dorsi (LD) and semimembranosus (SM) muscles, between three-way (3W) crossbred pigs and purebred Duroc TT/CC for the *SCD* genotype, belonging to two growth phases (growing vs. fattening).

FIR	Producing Type (PT)	Growth Phase (GP)		*p*-Value		
		Growing	Fattening	GP	PT	GP × PT
Animals (*n*)		24	24			
LIVER	3W	11.87 ± 1.746 [b]	52.50 ± 6.005 [a]	<0.001	0.242	0.139
	Duroc CC	13.61 ± 1.746 [b]	37.95 ± 6.487 [a]			
	Duroc TT	12.69 ± 1.746 [b]	37.98 ± 6.005 [a]			
SC	3W	18.90 ± 2.717 [b]	31.76 ± 3.876 [a]	0.415	<0.001	0.037
	Duroc CC	11.78 ± 2.717 [bc]	7.38 ± 4.475 [c]			
	Duroc TT	13.62 ± 2.905 [bc]	12.22 ± 3.876 [bc]			
LD	3W	14.45 ± 1.618 [a]	4.31 ± 0.464 [b]	<0.001	<0.001	0.051
	Duroc CC	7.54 ± 1.618 [b]	1.57 ± 0.536 [c]			
	Duroc TT	6.62 ± 1.618 [b]	2.33 ± 0.464 [c]			
SM	3W	1.39 ± 0.176 [a]	Under LOQ *	0.019	0.187	0.040
	Duroc CC	1.31 ± 0.176 [a]	0.63 ± 0.120 [b]			
	Duroc TT	1.26 ± 0.176 [a]	1.22 ± 0.111 [a]			

[a, b, c] Within each tissue, means with different superscripts differ significantly ($p \leq 0.05$). * Value under the limit of quantification.

Table 6. Least square means (± SE) for apparent and real fractional unsaturated rate (FUR; %/day) in subcutaneous adipose tissue between three-way (3W) crossbred pigs and purebred Duroc of TT/CC *SCD* genotype, belonging to two growth phases (growing vs. fattening).

FUR	Producing Type (PT)	Phase (GP)		*p*-Value		
		Growing	Fattening	GP	PT	GP × PT
Animals (*n*)		24	24			
Apparent	3W	5.47 ± 0.231 [a]	2.03 ± 0.121 [d]			
	Duroc CC	2.84 ± 0.216 [c]	1.60 ± 0.121 [e]	<0.001	<0.001	<0.001
	Duroc TT	4.25 ± 0.216 [b]	1.99 ± 0.121 [d]			
Real	3W	2.16 ±0.300 [c]	7.23 ± 0.617 [a]			
	Duroc CC	2.14 ± 0.300 [c]	5.28 ± 0.660 [b]	<0.001	0.107	0.016
	Duroc TT	1.56 ± 0.300 [c]	7.68 ± 0.617 [a]			

[a, b, c] Within each rate, means with different superscripts differ significantly ($p \leq 0.05$).

The PT and GP affected apparent FUR and showed a significant interaction between them ($p < 0.001$). Apparent FUR was higher in growing than in fattening phase ($p < 0.001$). Growing 3W pigs showed the highest FUR values, followed by Duroc TT and Duroc CC pigs. However, in the fattening phase, apparent FUR values in 3W and Duroc TT pigs were similar, while Duroc CC pigs kept a significantly lower FUR value. Regarding real FUR activity, it behaved opposite to the apparent FUR as pigs aged; thus, fattening animals presented significantly greater FUR values ($p < 0.001$) than growing animals. In addition, significant differences between PTs were also detected but only in the fattening phase (GP × PT interaction effect; $p = 0.016$), where 3W and Duroc TT pigs had higher FUR values than Duroc CC pigs.

4. Discussion

The authors are aware that the number of individuals per group in the present trial may limit the accuracy of the data; however, the same individuals were simultaneously used to analyze the kinetics of protein synthesis to obtain a complete picture of nutrient metabolism in pigs (Sarri et al., 2021). The whole process required surgical catheterization and confinement of pigs in metabolic cages; therefore, the experimental design restricted the available number of experimental animals.

This study aimed to compare specific aspects of the fat metabolism of two types of pigs generally used in southern Europe for different productive purposes. The 3W crossbred pigs come from intensive selection to enhance lean carcass and feed conversion; these leaner pigs are slaughtered at lighter weights to take advantage of their high growth potential. However, heavy pigs intended for high-quality pork (e.g., specific Duroc lines or Iberian pigs) are slaughtered at heavier weights and males are usually castrated to avoid boar taint and improve fat deposition (Huber et al., 2018). Thus, the term PT used throughout the manuscript is employed because it includes both genotype and castration traits, as Duroc barrows were surgically castrated shortly after birth to meet commercial conditions. In addition, within the heavy Duroc line, pigs were genotyped and selected to be homozygous for the *SCD*_T and *SCD*_C alleles to exploit their differential Δ9-desaturase activity and oleic acid synthesis capability (Estany et al., 2014). This study was performed in two GP (28.42 ± 0.861 kg BW and 87.40 ± 1.256 kg BW for growing and fattening phases, respectively), in

which growing pigs showed differential rates of protein synthesis (Sarri et al., 2021) and also of fat metabolism, with a subsequent accretion that may be altered by selection strategies exerted on the genotypes used.

Fatty acids are deposited in adipose tissues through two mechanisms: direct FA incorporation, mostly from the diet (Kouba et al., 2003), but also from mobilization from other fat depots; and through de novo synthesis processes, using different precursors at different rates. The biological diversity of FA in adipose tissues and the variety of precursors make fat accretion a complex process. To address this topic, the authors administered D-labeled stearic acid (d_{35}-C18:0), with molecular hydrogens of the hydrocarbon chain that were labeled with deuterium, to monitor direct FA incorporation. Likewise, labeled oleic acid (d_{33}-C18:1 *c*9), with all hydrogens of the hydrocarbon chain D-labeled, was considered a valid index of endogenous FA de novo synthesis, since it derives directly from the desaturation of dietary d_{35}-C18:0 by the action of SCD.

The validity of the FIR relies on the rate between d_{35}-C18:0 incorporation into adipose tissues and available d_{35}-C18:0 in plasma. Thus, the robustness of the FIR depends on (i) the stability of plasma d_{35}-C18:0 enrichment and (ii) the absence of d_{35}-C18:0 recycling. Regarding the former point (i), plasma d_{35}-C18:0 enrichment leveled off after 72 h of its dietary administration, and consistent detection was obtained in adipose tissues 72 h thereafter. Considering that pigs were fed ad libitum and that digestion and intestinal absorption may buffer the discontinuity of discrete meals, plasma d_{35}-C18:0 enrichment was arguably constant. In relation to the last point (ii), the analytical protocol allowed the identification of d_{35}-C18:0 in plasma and tissues, so the possibility of background contamination or endogenous return in plasma was negligible. Following the same principle, the appearance of d_{33}-C18:1 *c*9 in tissues should be associated with d_{35}-C18:0 availability, without any possible interference with exogenous sources. Nevertheless, d_{33}-C18:1 *c*9 was only consistently detected in SC with our analytical approach, since SCD activity is significantly higher in this tissue (Guillevic et al., 2009; Kouba et al., 1997).

4.1. Production Data and Differential Growth Intensity

In line with previous findings (Solé et al., 2021), Duroc pigs homozygous for the *SCD*_T allele presented lower feed consumption than Durocs carrying the *SCD*_C allele, and tended to show lower performance in the fattening phase.

Regarding allometric coefficients, no abnormalities were observed in the growth profile during the experimental period (Kouba and Sellier, 2011). Considering that *SCD* genotype variation only affects FA composition but not fat content (Estany et al., 2014; Henriquez-Rodriguez et al., 2016), datasets from both Duroc genotypes (TT and CC) were pooled and used as a single group. As previously described (Landgraf et al., 2006), pigs showed a high degree of maturity in the liver and a proximal-distal gradient in the hindlimb bones, all in negative allometry. Adipose tissue accretion was in positive allometry, whereas intermuscular fat differed between PTs. These differences in fat metabolism could be attributed to divergence maturity of adipose tissue between fatty and lean pigs (Landgraf et al., 2006; Poklukar et al., 2020). The 3W pigs had a higher allometry coefficient in intermuscular fat, and a high tendency in semimembranosus muscle IMF. These results are consistent with an elevated ratio of intermuscular to SC fat, particularly in the Pietrain breed and to a lesser extent in purebred or crossbred Duroc, as well as a higher ratio in boars than in barrows (Kouba and Sellier, 2011).

For skeletal muscles, the semimembranosus was in negative allometry, indicating a higher degree of maturity than other muscles in the ham. Likewise, the allometric growth rate of this muscle indicates how a greater degree of maturity may advance IMF metabolism, which is the last fat depot to develop (Bosch et al., 2012).

4.2. Apparent Ileal Digestibility

The results showed a clear effect of GP on the mechanisms regulating fat absorption and deposition; moreover, the effect of GP was not homogeneous and interacted with PT. The AID of EE was higher and homogenous in fattening pigs, whereas in growing pigs EE-AID was impacted by PT, being significantly reduced in 3W pigs. Such interaction also was observed in several FA fractions.

The link between animal development and the rate of FA digestibility was previously suggested by Powles et al. (1995), who revealed that the physicochemical structure of FA effectively alters the digestibility process. Digestibility is favored by the increase of the unsaturation rate and the reduction of the hydrocarbon chain length through micelle formation (Duran-Montgé et al., 2007; Ndou et al., 2019), although such effect (saturated/unsaturated FA ratio) was found more pronounced in young than in old pigs (Powles et al., 1995). Our findings also confirmed that saturated FA were the least digested, followed by monounsaturated and polyunsaturated FA, with mean AID coefficients of 63.59%, 73.87%, and 84.95%, respectively.

It should also be pointed out that dietary EE content and FA profiles differed between diets, with fattening diets containing a higher proportion of EE (60%) than the growing ones. In this regard, previous studies suggested that fat AID may be increased with fat inclusion level (Wang et al., 2020) or may differ with FA composition (Duran-Montgé et al., 2007). In any case, a differential dietary composition in EE may have masked the effect of age on fat digestibility.

Regarding pig PT, growing 3W pigs showed lower AID for DM, CP, and EE, coinciding with lower rates of AID in saturated and monounsaturated FA, although these coefficients converged in the fattening phase. These findings agree with previous studies (Freire et al., 1998; Len et al., 2009) in which fatty genotypes appeared to use dietary nutrients more efficiently at earlier ages than leaner ones, due to both earlier development of their digestive tract and higher enzyme activity.

4.3. Fractional Incorporation Rate of Stearic Acid

Incorporation of dietary fat into tissues differed between liver and adipose tissues, and semimembranosus IMF was the depot with the lowest FIR, as corresponds to a mature organ with a low allometry coefficient. The liver was much more active during the fattening phase in all PTs, whereas IMF of skeletal muscles had higher FIR in the growing phase, showing PT-dependence for each GP. Distinct tissues play different roles in fat metabolism (Duran-Montgé et al., 2009; O'Hea and Leveille, 1969); while in pigs adipose tissue is the most active for FA synthesis (O'Hea and Leveille, 1969), the liver is mainly involved in lipid oxidation and long-chain PUFA synthesis. Age also affects lipid metabolism on a large scale. Duran-Montgé et al. (2009) established

that lipogenic gene expression was higher in adipose tissue at 60 kg BW, whereas at 100 kg BW it was greater in the liver. In this regard, it was reported that adipose tissue reached the highest lipogenic activity at 120 days of age, decreasing gradually thereafter (Scott et al., 1981). Therefore, both liver and adipose tissue may change their role in fat metabolism over time.

Differences between PTs in SC-FIR showed up mainly in the fattening phase, when fat deposition is predominant (Kouba and Sellier, 2011). While Duroc pigs maintained their FIR stable throughout age, 3W pigs significantly increased it, along with an improvement in their FA-AID, which suggests an increase of dietary fat incorporation with age. However, in IMF, the FIR of 3W pigs showed a different development than in SC. The longissimus dorsi FIR was higher in 3W than in Duroc pigs at both GP, but experienced the greatest decrease in the fattening phase. This decrease in FIR could explain the lower IMF content in this leaner genotype compared to Duroc (Alonso et al., 2015; Wood et al., 2008). Dietary fat incorporation in 3W pigs may be more centered on other fat depots, such as SC or intermuscular adipose tissue, since this latter showed a higher relative growth rate than Duroc.

The lower rates of d_{35}-C18:0 incorporation recorded in Duroc pigs may be explained by the dietary FA dilution with saturated and monounsaturated FA coming from endogenous synthesis and/or desaturation. Several authors have described the increased lipolytic activity of Duroc pigs during their growth (Bosch et al., 2012), and the higher adipogenic and lipogenic gene expression in adipose tissues of fatty pigs (Palma-Granados et al., 2019) in comparison with leaner breeds. Consequently, fat incorporation in Duroc pigs appears to be less dependent on the availability of dietary FA in contrast to the leaner 3W genotype. However, processes associated with lipid mobilization, lipolysis, and extracellular matrix formation are upregulated in lean pigs (Tao et al., 2017; Tor et al., 2021; G. H. Zhang et al., 2014).

4.4. Endogenous Oleic Acid Synthesis: Oleic/Stearic Ratio

Since stearic acid is the main substrate of the SCD enzyme (Kloareg et al., 2005), the C18:1 *c*9/C18:0 ratio has been largely used as a measure of apparent SCD activity. In this regard, significant increases in apparent SCD activity with adipose tissue maturity have been reported (Bosch et al., 2012; Henriquez-Rodriguez et al., 2016). However,

in the present study, apparent FUR was significantly lower in the fattening than in the growing phase; possible reasons will be discussed further below.

Moreover, the authors are unaware of any previous in vivo experimental models using labeled FA as an index of real SCD activity in pigs; the existent bibliography comprises only in vitro models (Guillevic et al., 2009; Kouba et al., 1997). In this approach, (^{14}C) oleic acid is obtained through the incubation of tissues with (^{14}C) stearic acid, and in line with our results, significant increases in real SCD activity were reported with age in animals between 51 and 95 kg BW (Kouba et al., 2003), and despite this, activity remained unchanged or even decreased up to 128 kg BW. Conversely, previous expression studies described decreases in *SCD* gene expression in adipose tissues as animals fattened, between 60 and 100 kg BW (Duran-Montgé et al., 2009). However, some authors have found no direct correlation between gene expression level and FA profile (Bartz et al., 2013), and these differences could rely on the poorly predicted post-transcriptional regulation of *SCD* among tissues by their mRNA levels (Franks et al., 2017).

Concerning PT, it is worth mentioning that animals were genotyped for the rs80912566 *SCD* polymorphism, and in the 3W group, five growing and seven fattening pigs were homozygous for the *SCD*_T allele, with the rest (three growing pigs and one fattening pig) being heterozygous (CT). The *SCD*_T allele is almost fixed in some pig breeds, including Landrace and Pietrain, although in Duroc it segregates at intermediate frequencies (Estany et al., 2014).

Following the present results, both Duroc TT and 3W pigs showed higher apparent and real FUR than Duroc CC pigs, although these differences in real FUR were only found in the fattening phase. Such differences mostly agree with patterns previously reported (Estany et al., 2014; Henriquez-Rodriguez et al., 2016), demonstrating that the *SCD*_T allele enhances FA desaturation, with Duroc TT and Duroc CT pigs having 2% and 1% more monounsaturated FA, and 2% and 1% less saturated FA than Duroc CC pigs, respectively (Estany et al., 2017). This favorable effect of the *SCD*_T allele has also been confirmed in other Duroc crossbreds such as Duroc × Iberian (Estany et al., 2014). Although their pigs were between 95 and 130 kg BW, the authors

suggested that the variation in SCD activity was maintained throughout the growing–fattening period.

In our work, the significant decrease of apparent FUR related to animal maturity may be due to a stagnation in the C18:1 *c*9/C18:0 ratio with age, which makes the rate of desaturation per day lower, through (i) lower oleic acid incorporation or (ii) greater stearic acid incorporation into SC. The high availability (8.27 g/kg DM), high digestibility (85.76%), and improved de novo synthesis (245.02%; real FUR) of oleic acid in the diet during the fattening phase suggests that the first possibility (i) should be neglected. In relation to the second possibility (ii), 3W and Duroc genotypes appeared to behave differentially. The increase in the concentration of stearic acid in the SC of fattening 3W pigs is explained by the increase of FIR in this depot during the fattening phase, which also increases the substrate of the SCD enzyme, promoting de novo synthesis (Skiba et al., 2011). However, the same explanation cannot be applied to the Duroc pigs since FIR remained stable in Duroc TT pigs or slightly decreased in Duroc CC pigs during the growing–fattening period. If exogenous FA incorporation cannot explain the increase in stearic acid pool, then de novo synthesis may account for such increase, as about 80% of total FA deposition comes from biosynthesis (Kloareg et al., 2007). This assumption is also supported by the fact that the SC allometry coefficient in Duroc pigs increased with the same intensity as that of 3W pigs, despite the lower FIR and similar FUR of Duroc pigs compared to 3W pigs in the fattening phase. In that sense, the increased content of unsaturated FA in SC with animal maturity is consistent with previous studies using similar animals (Bosch et al., 2012). In that case, the reduction in apparent FUR in Duroc pigs is compatible with the increased de novo synthesis of oleic acid, since real FUR accounts for the increase in d_{33}-C18:1 *c*9 per unit of d_{35}-C18:0; therefore, the incorporation of de novo synthesized unlabeled stearic acid in SC would not alter the d_{33}-C18:1 *c*9/d_{35}-C18:0 ratio.

5. Conclusions

Considering the experimental limitations, stearic and oleic acids have been used to obtain valid indexes of FA incorporation and de novo synthesis, although this latter could only be analyzed in SC. Our findings suggest that fat synthesis in growing pigs is low, and their FA incorporation relies more on direct fat incorporation than on

biosynthesis. However, the synthetic FA activity increases significantly with animal maturity, particularly in the fatty Duroc genotypes in which FA accretion relied mostly on the lipogenesis, while the leaner 3W pigs depended more on direct FA incorporation. Therefore, during feed formulation, leaner pigs should have higher dietary FA requirements than fatty ones, while the opposite would be true for FA precursors (i.e., glucose, starch, etc.). In addition, we could find higher biosynthetic action of the *SCD*_T allele of the rs80912566 *SCD* polymorphism.

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CHAPTER IV

The impact of genetics on gut microbiota of growing and fattening pigs under moderate N restriction

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Abstract

Characterization of intestinal microbiota is of great interest due to its relevant impact on growth, feed efficiency and pig carcass quality. Microbial composition shifts along the gut, but it also depends on host (i.e., age, genetic background), diet composition and environmental conditions. To simultaneously study the effects of producing type (PT), production phase (PP) and dietary crude protein (CP) content on microbial populations, 20 Duroc pigs and 16 crossbred pigs (F2), belonging to growing and fattening phases, were used. Half of the pigs of each PT were fed a moderate CP restriction (2%). After sacrifice, contents of ileum, cecum and distal colon were collected for sequencing procedure. Fattening pigs presented higher microbial richness than growing pigs because of higher maturity and stability of the community. The F2 pigs showed higher bacterial alpha diversity and microbial network complexity (cecum and colon), especially in the fattening phase, while Duroc pigs tended to have higher Firmicutes/Bacteroidetes ratio in cecum segment. *Lactobacillus* was the predominant genus, and along with *Streptococcus* and *Clostridium*, their relative abundance decreased throughout the intestine. Although low CP diet did not alter the microbial diversity, it increased interaction network complexity. These results have revealed that the moderate CP restriction had lower impact on intestinal microbiota than PP and PT of pigs.

Keywords: Microbiota; swine; intestinal tract; protein restriction; pig producing type.

1. Introduction

Optimizing feed efficiency is one of the current challenges for the swine industry that also promotes the reduction of both feeding costs and environmental impact (Gardiner et al., 2020; Pomar and Remus, 2019). Within this framework, reducing crude protein (CP) level in diets balanced with synthetic amino acids is a widely used strategy which allows the improvement of nitrogen utilization and the reduction of the nitrogen load from manure (Yuming Wang et al., 2018). This approach complies with the European Union Council Directive 1991/676/EEC (1991) concerning the protection of waters against nitrates. In addition, the growing demand for pork products of improved palatability and nutritional value has led to the development of alternative production systems of great economic importance (Díaz-Caro et al., 2019) based on breeds with specific fatness traits to produce dry-cured ham or premium pieces, such as Duroc or Iberian pigs.

Together with management (nutrition, biosecurity, vaccination) and genetic plans, implementation of new production strategies should also consider animal's physiology (Gardiner et al., 2020; Yang et al., 2018). In this regard, it has been evidenced that gut microbiota plays important roles in promoting immune system development (Niederwerder et al., 2016), regulating host nutrient metabolism (Quan et al., 2019; Wu et al., 2021), modulating phenotypic traits (Guo et al., 2008; Wu et al., 2021) and producing beneficial substances (Richards et al., 2005). Gut microbiota consists of a complex ecosystem that is established through a sequence of dynamic successions of the dominant microbial groups. These changes occur throughout the entire intestinal tract and over time (Zhao et al., 2015) in order to adapt to endogenous and exogenous factors to which the individual is subjected. Substantial progress in high-throughput sequencing techniques have enabled the characterization of gut microbial communities and their interaction with the host, which has received increasing interest in recent decades due to its potential contribution to production traits.

Since there is high potential to modulate gut microbiome in different managing scenarios (Trevisi et al., 2021), it would be possible to reshape the community structure to achieve different productive goals (Wu et al., 2021). For this purpose, a

further understanding of the factors affecting gut microbiota and their interactions seems essential. Thus, the objective of the present study was to evaluate the effects of a moderate CP restriction and the producing type (PT) of pigs (Duroc pigs as heavy and F2 crossbreed as lean pigs) on gut microbiome structure across the intestinal tract (ileum, cecum and distal colon) throughout the animal's development (growing and fattening phases).

2. Materials and Methods

Protocols and experimental procedures were approved by the Ethics Committee for Animal Experimentation of the University of Lleida, under Project License CEEA 09-05/16. Care and use of animals were in accordance with the Spanish Policy for Animal Protection RD 53/2013, which meets the European Union Directive 2010/63 on the protection of animals used for experimental and other scientific purposes.

2.1. Animals, Diets and Sampling Procedure

A total of 36 male pigs belonging to two production phases (PPs; growing and fattening), were housed in the same environmentally controlled room at the Swine Research Center located in Torrelameu (CEP; Lleida, Spain). Twenty of these 36 pigs were surgically castrated purebred Duroc pigs, of which 12 were in growing phase (mean ± standard error: 26.4 ± 3.11 kg of body weight (BW) at sacrifice) and eight in fattening phase (86.1 ± 2.74 kg BW), while the remaining 16 pigs were entire crossbreds (F2) (Pietrain sires × [F1: Duroc × Landrace] dams), eight in growing phase (30.5 ± 1.36 kg BW) and eight in fattening phase (91.1 ± 1.23 kg BW). Moreover, two experimental diets were formulated for each PP, which were provided *ad libitum* for the 15 days (4 days of dietary changeover plus 11 days of adaptation) prior to slaughter. Both diets were isoenergetic and identical in covering the nutritional requirements but differing in 2% of CP concentration. Half of the pigs of each PT were allotted to one of the two dietary treatments, standard protein (SP) diet and low protein (LP) diet, formulated with 17% and 15% CP in growing phase and 15% and 13% CP in fattening phase, respectively. All diets were supplemented with synthetic amino acids to meet the nutrient requirements recommended by FEDNA (de Blas et al., 2013). The ingredients and chemical composition of the diets are provided in Table 1. The ambient temperature during the entire experimental period was

maintained between 23.5 and 25 ºC, under a mechanical ventilation system and natural light. Moreover, animals of the same PP and PT were placed in groups of four in 55% concrete slatted-floor pens (2.1 × 2 m^2) the first 9 days, and then placed individually in metabolic cages during the last 6 days, as was previously described in (Sarri et al., 2021).

Table 1. Ingredients and chemical composition of the two-phase experimental diets.

Items [1]	Growing phase		Fattening phase	
	Low protein	**Standard protein**	**Low protein**	**Standard protein**
Ingredients (g/kg DM)				
Corn	294.8	246.5	190.3	99.2
Barley	290.0	287.8	466.0	512.8
Wheat	200.0	200.0	200.0	200.0
Soybean meal	137.6	195.0	65.8	113.6
Beet pulp dehydrated	30.0	30.0	30.0	30.0
Calcium carbonate	13.4	13.2	10.0	10.0
Mono Calcium phosphate	9.4	8.8	7.0	6.4
Soybean oil	9.0	6.3	17.0	17.1
L-Valine	6.8	8.0	0.0	0.0
Sodium chloride	4.6	4.6	4.1	4.1
L-Lysine HCL	4.2	2.4	3.7	2.1
Vitamin- Mineral mix [2]	4.0	4.0	4.0	4.0
L-Threonine	1.6	0.8	1.2	0.5
DL- Methionine	1.0	0.5	0.6	0.3
L-Tryptophan	0.3	0.2	0.2	0.0
Chemical composition (g/kg DM)				
Dry matter (g/kg FM)	900.1	897.1	909.9	908.4
Crude Protein	147.2	166.9	129.27	154.95
Ether Extract	24.9	26.9	39.7	43.2
Ash	65.8	70.3	58.1	52.8
NDF	173.3	179.9	211.8	211.6
ME (kcal/kg)	3108	3094	3185	3185

[1] Complete diets differed in 2% crude protein concentration (standard protein vs low protein) for pigs in growing and fattening production phases. DM: dry matter; FM: fresh matter; ME: metabolizable energy, NDF: neutral detergent fiber.

[2] The vitamin and mineral mixture composition is available in Sarri et al. (2021).

At the last day of the trial, pigs were sacrificed, and the contents of ileum, cecum and distal colon were collected simultaneously for microbial characterization. Samples were immediately frozen in dry ice and stored at –40 ºC until further analysis.

2.2. Genomic DNA Extraction, 16S rRNA Amplicon Sequencing and Bioinformatics

Microbial genomic DNA was extracted using DNeasy PowerLyzer PowerSoil Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Mock community DNA was included as positive control for library preparation (Zymobiomics Microbial Community DNA, ZymoResearch, Irvine, CA, USA).

Samples were amplified using primers 341F and 805R, which target the V3-V4 region of the bacterial and archaeal 16S rRNA. The PCR was performed in 10 μL final volume with 0.2 μM primer concentration. The PCR included: 3 min at 95 °C (initial denaturation) followed by 25 cycles of 30 s at 95 °C, 30 s at 55 °C, and 30 s at 72 °C, and a final elongation step of 5 min at 72 °C. PCR products were purified using AMPure XP beads (Beckman Coulter, Nyon, Switzerland) with a 0.9 × ratio according to the manufacturer's instructions.

The paired-end sequencing was conducted following an Illumina Miseq sequencing 300x2 approach. Quality control filtering and OTU binning of the resulting sequences were executed using DADA2 software (Callahan et al., 2016). Finally, taxonomic assignment of phylotypes was performed using a Bayesian Classifier trained with Silva database (Q. Wang et al., 2007). Extraction and sequencing of DNA and bioinformatic procedures were carried out by Microomics Systems, S.L. (Barcelona, Spain).

2.3. Statistical Analysis

Analysis described below were performed in duplicate aiming to assess: (i) differences in ileal, cecal and colonic microbiota between PTs (Duroc vs F2) in both PPs, and (ii) differences in ileal, cecal and colonic microbiota between experimental diets (SP vs LP) in both PPs.

Sequence data were normalized to the same mean and alpha diversity indices were calculated (R Core Team, 2020; *vegan* package) to measure the variability of species

within a sample. Then, data were analyzed with a linear model including PT × PP and Diet × PP as fixed effects (R Core Team, 2020; *stats* package). Data from the three intestinal segments (ileum, cecum and distal colon) were analyzed separately to decrease datasets sparsity (certain OTUs were not present in each one of the three locations and, therefore, working on a single dataset would highly increase the number of zeros). Contrasts between either PT or Diets were performed by Tukey's test (R Core Team, 2020; *emmeans* package). Individual samples out of three standard deviations of the mean were discarded and not included to the statistical analysis. Significant effects were declared at $p < 0.05$ and tendency to difference at p between 0.05 and 0.10.

To circumvent the compositional bias problem (Calle, 2019; Gloor et al., 2017), we applied the Aitchison's centered log ratio (clr) transformation to carry the data to a Euclidean space, after replacing zeros by adding 1 to each value. To measure differences in microbiome composition between samples, beta diversity was approached through performing a partial least squares-discriminant analysis (PLS-DA) based on clr (R Core Team, 2020; *mixOmics* package). To test whether differences in microbiota composition between treatments were statistically significant, a permutational multivariate analysis of variance (PERMANOVA) was conducted based on the clr Euclidean distance, including PT x PP and Diet x PP interactions and calculating statistical significance after 10 000 random permutations (R Core Team, 2020; *vegan* package). To decipher which genera abundance were responsible for the differences between treatments, an ANOVA-like differential expression (ALDEx) analysis was conducted over those genera present at least at 50% of the individuals (R Core Team, 2020; *Aldex2* package) (Fernandes et al., 2013). Finally, to describe the interactions within ileum, cecum and colon microbial community, we performed a network analysis through Sparse Correlations for Compositional data (SparCC) technique (R Core Team, 2020; *SpiecEasi* package) (Friedman and Alm, 2012) over those genera present at least at 50% of the individuals. Microbial networks were graphically represented (R Core Team, 2020; *igraph* package) and their complexity was described in terms of number of nodes (genera), number of edges (significant positive or negative correlations), node degree (number of connections that any node establishes with other nodes) and betweenness centrality

(measure of centrality in a graph based on shortest paths). Differences in microbial composition between intestinal segments were described by (i) graphical representation of the relative abundance of major genera (> 1% analyzed sequences), (ii) graphical representation of core microbial community in each intestinal segment, (iii) alpha diversity indices, statistically assessed using a linear model including Segment x PP as fixed effects and (iv) beta diversity, approached by the graphical representation of PLS-DA and PERMANOVA.

3. Results

3.1. Dataset Features

Sequencing generated a total of 3 709 916 high-quality sequences obtained from the 108 digestive content samples from the three intestinal regions. The mean number of sequences per sample was 34 251 for the ileum, 31 167 for the cecum, and 37 635 for the distal colon. These sequences resulted in a total of 181 OTUs in ileum (33 OTUs per sample), 395 OTUs in cecum (107 OTUs per sample) and 453 OTUs in colon (136 OTUs per sample). The unclassified rate of OTUs at genera level increased along the gut segments, with 8.9%, 11.3% and 14.2% for ileum, cecum, and colon samples, respectively.

3.2. Alpha Diversity

Microbial diversity indices along with the Firmicutes/Bacteroidetes ratio were analyzed in both PTs of pigs in each PP (Table 2). The Firmicutes/Bacteroidetes ratio is not presented for the ileum segment due to the low abundance of Bacteroidetes in that intestinal segment. This was also analyzed for the dietary CP content in growing and fattening phases (Table S1), although diet effect was negligible.

The PT had a significant influence on microbial diversity (Table 2). F2 pigs harbored a more diverse bacterial community compared to Duroc pigs along the intestinal tract. Significant differences were detected in ileum ($p = 0.014$ and 0.009 for Shannon and Simpson indices, respectively), cecum (Shannon index, $p = 0.033$), and colon (Simpson index, $p = 0.047$). Moreover, F2 pigs showed higher microbial richness in the cecum ($p < 0.010$), and higher evenness in both ileum and distal colon segments ($p = 0.011$) compared to Duroc.

Table 2. Microbial alpha diversity indices (based on OTUs), and Firmicutes/Bacteroidetes ratio (Ratio F/B) in ileum, cecum, and distal colon segments.

Item [1]	Growing phase		Fattening phase		s.e.m.	*p*-value	
	Duroc	F2	Duroc	F2		PT	PP
n	12	8	8	8			
Ileum [2]							
Shannon index	1.53b	1.64ab	1.47b	2.18a	0.160	0.014	0.186
Simpson index	0.61b	0.69ab	0.66ab	0.81a	0.049	0.009	0.102
Richness [3]	31.48ab	24.38b	31.50ab	43.88a	3.854	0.446	0.020
Evenness	0.45	0.52	0.43	0.58	0.040	0.011	0.785
Cecum							
Shannon index	3.18	3.35	3.22	3.76	0.177	0.033	0.189
Simpson index	0.92	0.92	0.91	0.95	0.018	0.422	0.618
Richness	90.23b	104.88ab	106.50ab	134.50a	8.973	0.010	0.013
Evenness	0.71	0.72	0.70	0.77	0.034	0.200	0.457
Ratio F/B	47.57	24.40	35.68	2.37	18.409	0.089	0.309
Distal colon							
Shannon index	3.53	3.83	3.67	3.77	0.128	0.076	0.650
Simpson index	0.94	0.96	0.94	0.95	0.009	0.047	0.742
Richness	123.64	140.00	146.13	153.72	9.595	0.138	0.045
Evenness	0.74	0.78	0.74	0.78	0.017	0.011	0.820
Ratio F/B	28.73	10.92	7.65	11.49	8.110	0.185	0.112

[1] Obtained in pigs differing in their producing type (PT): purebred Duroc vs crossbred F2 (Pietrain × F1: Duroc × Landrace) and production phase (PP): growing (28.5 kg) vs fattening (88.1 kg). Standard error of the mean (s.e.m.) and significance of PT and PP effects are shown. Mean values within a row followed by different letters differ significantly at $p = 0.05$.

[2] Ratio F/B could not be calculated in ileum samples due to the low abundance of Bacteroidetes phylum in that intestinal tract (0.28% of analyzed sequences).

[3] Only one PT by PP interaction was significant (Ileum-Richness: $p = 0.014$) and the rest of interactions were not included in the table.

In all three intestinal segments, fattening pigs presented a significantly higher microbial richness than growing ones ($p < 0.05$). A significant interaction was also found in ileum between PT and PP effects ($p = 0.014$), where fattening F2 pigs had significantly higher microbial richness than growing F2 pigs, although no differences were detected between growing and fattening Duroc pigs.

The Firmicutes/Bacteroidetes ratio between both PTs in growing and fattening phases was only calculated in cecum and distal colon. In the cecum, Duroc pigs showed a slight tendency to have higher ratio than F2 pigs ($p = 0.089$) with no variation with PP. Although no significant differences between PTs nor PPs were obtained in the distal colon, the Firmicutes/Bacteroidetes ratio in Duroc pigs numerically decreased from growing to fattening phases, whereas in F2 pigs it remained constant.

3.3. Microbial Composition Throughout the Intestinal Tract

Alpha diversity indices were also obtained between intestinal segments in the two PPs (Table S2), in which the ileum segment had lower Shannon and Simpson indices, microbial richness and evenness than the lower intestine segments (cecum and distal colon) in both growing and fattening phases ($p < 0.001$). In addition, no significant differences between the cecum and distal colon were detected in each PP, except for microbial richness in the growing phase that was significantly higher in the distal colon. This is graphically evidenced in Figure 1 where the relative abundance of the main bacterial genera is presented. The lower intestinal regions exhibited a higher number of genera with higher evenness in their relative abundance than those in the upper intestine. In relation to the PP (Table S2), when all intestinal segments were

considered, fattening pigs showed significantly higher Shannon index and microbial richness than growing pigs ($p < 0.05$), as well as a slight tendency to have higher Simpson index ($p = 0.084$). However, only Simpson index in ileum and microbial richness in the cecum presented significant differences.

In both PPs, microbial community displayed similar dynamics between Duroc and F2 pigs (Figure 1). *Lactobacillus* was the predominant genus in the ileum of growing and fattening pigs, and it decreased progressively from ileum to distal colon. The same dynamics were observed in *Streptococcus* and *Bifidobacterium* (growing phase, Figure 1A,B), and *Clostridium sensu stricto 1* and *Terrisporobacter* (fattening phase, Figure 1C,D), which seemed to colonize almost exclusively the upper intestinal regions. Many other genera with similar relative abundance were found in the cecum, of which *Megasphaera*, *Eubacterium*, *Dialister* and *Mitsuokella* stood out in the growing phase, maintaining or even increasing their abundance in the distal colon (Figure 1A,B). However, in the fattening phase, *Alloprevotella* was abundant in the cecum of both PTs (Figure 1C,D), and *Methanobrevibacter*, *Lachnospiraceae XPB1014* group, *Ruminococcaceae UCG-02* and *Ruminococcaceae UCG-05* were enriched in the colon of fattening Duroc pigs (Figure 1C).

The core microbiota was identified along the ileum (Figure S1), cecum (Figure S2), and distal colon (Figure S3) as the shared OTUs by all individuals at each PP. The upper intestine showed higher number of shared sequences than the lower gut segments, with 57% and 48% of the sequences in the ileum of growing and fattening pigs, respectively (Figure S1). *Unclassified Lactobacillus* was the predominant shared OTU throughout the intestine. It accounted for more than 71% of the shared sequences in the ileum, and progressively decreased to represent 29.6% in the colon of fattening pigs (Figure S3B). The colon of growing pigs was the exception, in which the most shared OTU was *unclassified Mitsuokella* with 13.8% of the shared sequences (Figure S3A).

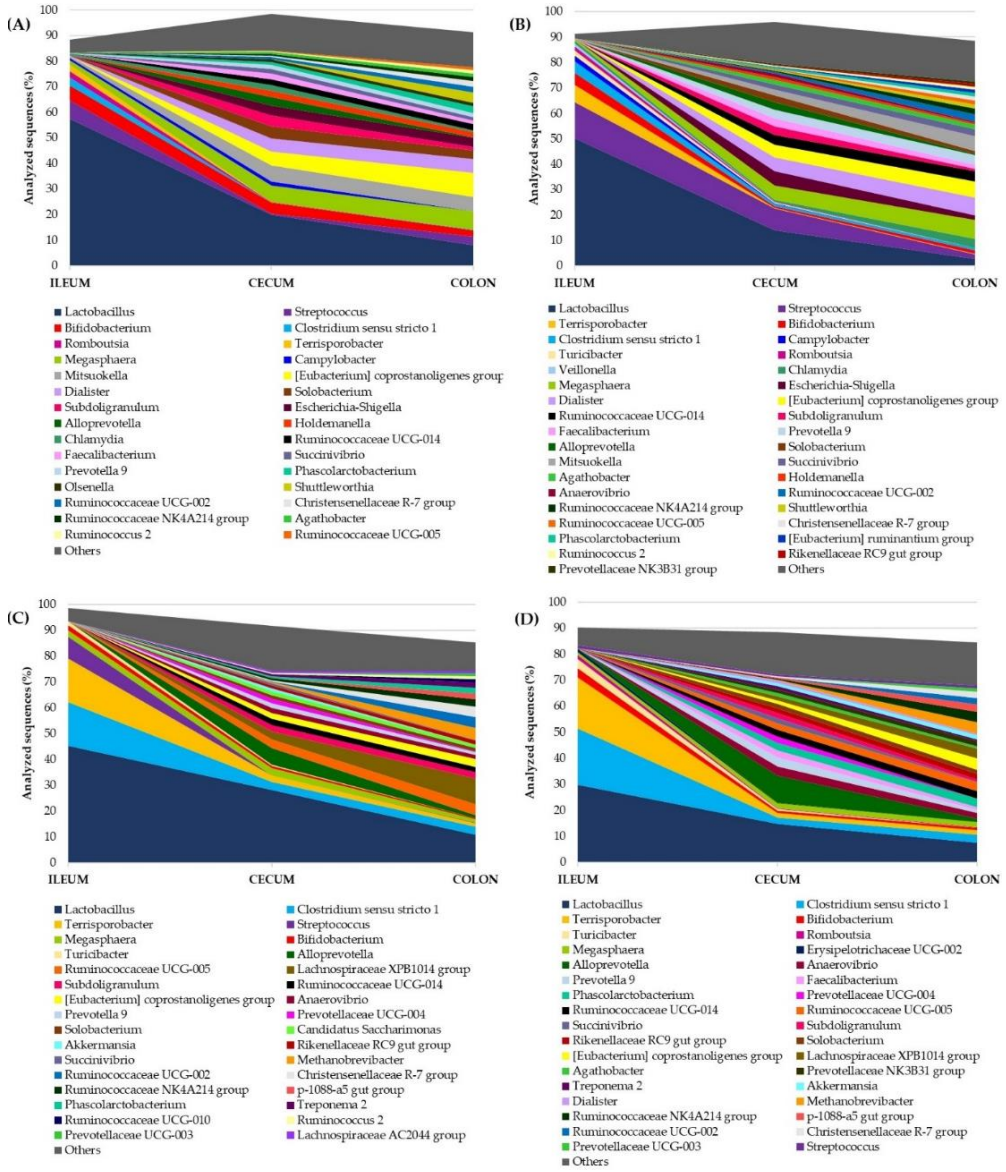

Figure 1. Genera abundance throughout the intestinal tract in (A) growing Duroc pigs (26.4 kg), (B) growing F2 pigs (31.6 kg), (C) fattening Duroc pigs (85.1 kg), and (D) fattening F2 pigs (91.1 kg). Low-abundance genera (< 1% analyzed sequences) are represented as "Others".

3.4. Beta Diversity

Figure 2 graphically represents the beta diversity of the microbial community inhabiting the three intestinal segments studied in the animals fed the two dietary treatments in growing and fattening phases. The effect of PP was important in all intestinal segments ($p < 0.001$), while no significant differences were found for the dietary CP content ($p > 0.433$), although it seemed to be more evident in the growing phase than in the fattening phase. The beta diversity of the two PTs belonging to both PPs is represented in Figure 3. The overall clustering of microbial community samples suggested that microbiota in the ileum (Figure 3A), cecum (Figure 3B) and distal colon (Figure 3C) was different when growing and fattening animals were compared ($p < 0.001$). In a similar manner, differences in microbial community composition between the two PTs were found in ileum and cecum segments ($p < 0.05$, Figure 3A,B) but disappeared in colon ($p = 0.171$, Figure 3C); nevertheless, statistical differences in genera abundance between PTs could not be detected by ALDEx analysis, regardless of intestinal segment.

The beta diversity analysis of microbial OTUs in ileum, cecum and distal colon is graphically represented in Figure S4, in which the clustering is clearly differentiated among the three intestinal segments ($p < 0.001$), although the microbial communities of the ileum appeared to be more distinct than those of the more distal segments.

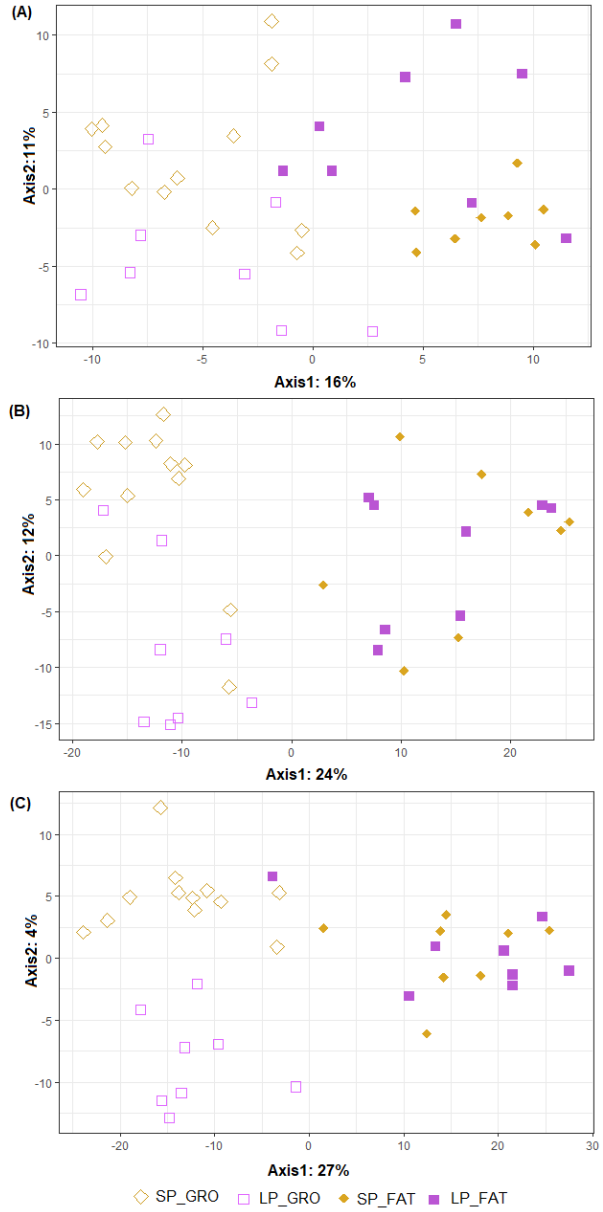

Figure 2. Graphical representation of partial least squares-discriminant analysis (PLS-DA) on microbial OTUs in ileum (A), cecum (B) and distal colon (C). Obtained in pigs differing in their protein intake: standard protein (SP) vs low protein (LP) and in their production phase: growing (GRO: 28.5 kg) vs fattening (FAT: 88.1 kg). Each point represents a different sample and a greater distance between two points infers a higher dissimilarity between them. Statistical comparisons were made between PP ($p < 0.001$ for all intestinal segments), between Diets ($p = 0.916$, 0.433 and 0.573 for

ileum, cecum and distal colon, respectively) and to test PP by Diet interaction (p = 0.239, 0.489 and 0.638 for ileum, cecum and distal colon, respectively).

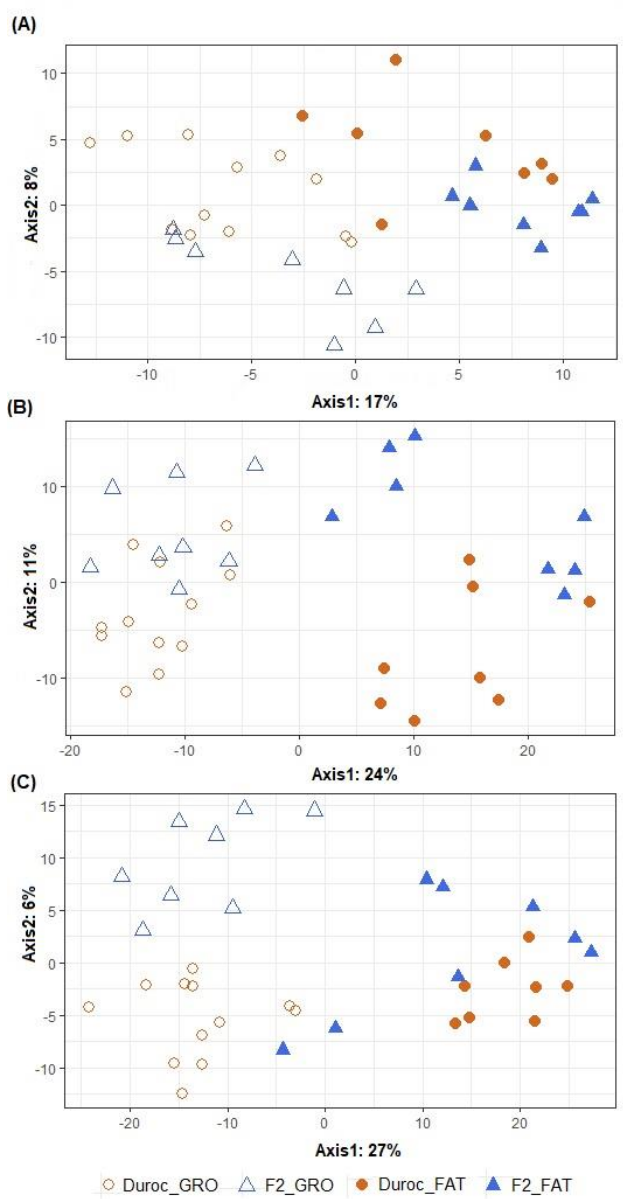

Figure 3. Graphical representation of partial least squares-discriminant analysis (PLS-DA) on microbial OTUs in ileum (A), cecum (B) and distal colon (C). Obtained in pigs differing in their producing type (PT): Duroc vs F2 (Pietrain × F1: Duroc × Landrace) and production phase (PP): growing (GRO: 28.5 kg) vs fattening (FAT: 88.1 kg). Each point represents a different sample and a greater distance between two

points infers a higher dissimilarity between them. Statistical comparisons were made between PP ($p < 0.001$ for all intestinal segments), between PT ($p = 0.044$, 0.032 and 0.171 for ileum, cecum and distal colon, respectively) and to test PP by PT interaction ($p = 0.667$, 0.542 and 0.232 for ileum, cecum and distal colon, respectively).

3.5. Microbial Networks

Microbial networks were built to test interactions among microbial genera in each intestinal segment. Degree of interaction was studied through the number of genera (nodes) that established significant interactions (edges) with other genera, as well as the number of interactions established per node (node degree).

The overall microbial network complexity increased across the intestinal segments: the mean number of nodes and edges in each intestinal segment were 12 and 13 in ileum, 50 and 155 in cecum, and 68 and 230 in distal colon (Table S3). In the ileum segment, the most notable differences in microbial networks were observed between PPs (Figure S5 and Figure S8): growing pigs showed more complexity in their microbial networks than fattening pigs, in terms of number of nodes, edges and node degree (Table S3). Regarding dietary CP content, pigs fed the LP diet showed more complex networks through the intestine than those fed the SP diet, especially in ileum (Figure S5) and colon segments (Figure S7), while in the cecum (Figure S6) it was only detected in the growing phase. In turn, F2 pigs exhibited more complex microbial networks than Duroc pigs in cecum (Figure S9) and distal colon (Figure S10), in both growing and fattening phases. However, a different evolution in microbial network complexity in cecum and colon segments was detected between the two PTs tested: while microbial network complexity increased considerably as animals aged in the case of F2 pigs, in Duroc pigs remained constant in both PPs.

Betweenness centrality, which measures the possible influence of an individual node on other nodes, was also calculated (Table S3). This measure was higher in growing LP-fed pigs, especially in the colon and the cecum segments, and in the cecum of fattening SP-fed pigs. Moreover, it was higher in the microbial network of F2 pigs compared to Duroc ones and tended to increase from growing to fattening phases in both PTs, with the sole exception of the ileum of Duroc pigs. The increase of betweenness centrality with age was more pronounced in F2 pigs.

4. Discussion

Authors are aware of the limiting number of animals per group with the 2 × 2 × 2 factorial design to reach consistency in both results and conclusions. The present study was part of a complex trial in which animal's protein and lipid metabolism were also studied (Sarri et al., 2021). In addition, the collection of samples from ileum, cecum and distal colon involved the slaughter of the animals. The complexity of experimental procedures and the ethics committee indications in minimizing the experimental animals limited the number of pigs. Moreover, the scarce interaction effects among the main factors would indicate that, in this scenario, experimental underpowering was not a relevant issue, and hence the number of animals used was considered enough to achieve the proposed objectives.

4.1. Effect of Production Phase

Differences in intestinal bacterial composition between the two PPs were studied since animal age regulates changes in nutrient digestibility and metabolism, immunity and hormone status, and tissue development with a direct impact on microbiome. Intestinal tract samples from growing and fattening phases were obtained from pigs of two PTs, at about 83 and 154 days of age, respectively. Among all alpha diversity indices analyzed, microbial richness was the unique that showed significant differences between growing and fattening phases. In all intestinal segments, fattening pigs achieved higher values than growing pigs. This is in agreement with previous reports (Ke et al., 2019; X. Wang et al., 2019), which described increased microbial richness as animal matured. Microbial diversity also increases substantially after weaning as a sign of overall development and stability of microbiome composition (X. Wang et al., 2019). However, several studies indicated that diversity indices reach their highest rates before 150 days of age (De Rodas et al., 2018; Han et al., 2018; Ke et al., 2019).

In the present study, the ileum microbiota presented a significant interaction (PP × PT) in their richness. While microbial richness increased with age in F2 pigs, it remained constant between growing and fattening phases in Duroc pigs. In a previous report, ileum microbiota showed high variability in diversity indices across ages (De Rodas et al., 2018), suggesting that the upper intestine displays less stability in

microbial communities compared to the lower intestine because of the reduced abundance of microorganisms (Crespo-Piazuelo et al., 2018). Authors are not aware whether this variability has a genetic component.

Gut microbiota of both PPs clustered separately in the three intestinal segments in the PLS-DA graphical representations, evidencing a differential microbial composition throughout the productive period, as was previously described (Han et al., 2018). In addition, microbial network complexity in cecum and distal colon increased considerably from growing to fattening phases, especially in the F2 pigs. Similar results were obtained by Ke et al. (2019), who found increased interaction network in pigs from 25 to 120 days of age, which may be associated with greater microbial diversity and stability of matured pigs. Higher diversity and microbial network complexity may also lead to improved capacity for digestion and metabolism (e.g., complex carbohydrates, protein) (Ke et al., 2019). However, in Duroc pigs the architecture of microbial network remained constant in both PPs. This difference between PTs may be associated with the earlier maturity of castrated Duroc pigs compared to commercial crossbreds (Hutchens et al., 1982), along with the earlier development of digestive organs and higher digestive enzyme activity reported in fatty breeds (Freire et al., 1998; Len et al., 2009).

At the phylum level, although no significant differences were detected in Firmicutes/Bacteroidetes ratio throughout the intestinal tract, it numerically decreased with age. The lower ratio correlates with the increasing proportion of Bacteroidetes phylum as pigs aged found in previous studies (Kostic et al., 2013; Zhao et al., 2015).

4.2. Effect of Producing Type of Pig

The study of gut microbiota between the two extreme PTs of pig aimed to elucidate certain microbiota traits responsible for the phenotypic differences. Duroc pig is a preferred breed for producing premium dry-cured products due to its high intramuscular fat content, and excellent fatty acid composition (Wood et al., 2004). To promote such features, pigs are sacrificed at high weights and, therefore, males are castrated to avoid boar taint. On the other hand, F2 pigs have been selected to improve productive parameters in order to obtain low-priced lean meat, thus, pigs are slaughtered earlier and are not castrated. Since the effect of sex and breed are not

separated, the definition of breed has been replaced by the term "producing type" that also includes the effect of castration in the case of Duroc males.

PT also impacted the alpha diversity, with F2 pigs having significantly higher microbial diversity than Duroc pigs across the three intestinal segments, according to Shannon and Simpson indices. Moreover, F2 pigs also presented higher microbial richness (in cecum) and evenness (in ileum and colon). This implies that F2 pigs host a higher number of different taxa with more similar abundances. Considering that the rearing conditions throughout the experiment were controlled, these differences may be correlated with physiological traits. These results are in agreement with those obtained by several studies (Bergamaschi et al., 2020; Pajarillo et al., 2014; Seradj et al., 2020) in which the leaner pig breeds presented higher diversity indices than fattier or unimproved ones. For instance, Duroc showed lower diversity than Landrace pure breed (Bergamaschi et al., 2020; Pajarillo et al., 2014), being Landrace the typical genetically lean pig. However, the opposite was also reported, with Duroc and Jinhua breeds having higher diversity than Landrace, Hampshire and Yorkshire (Xiao et al., 2018, 2017). Discrepancies between these studies may have resulted from different diet compositions, environmental conditions, or the use of distinct intestinal segment contents. Higher bacterial diversity is generally considered favorable in terms of stability and resilience to dysbiosis and potential pathogens threat (Ju et al., 2008). In addition, F2 pigs presented a more complex microbial network architecture in terms of both node degree and betweenness centrality which may also contribute to their higher robustness (Dunne et al., 2002), that is the microbial community's ability to cope with disturbances (Costa-Roura, 2021). These traits may be responsible to their improved indices of producing performance and apparent CP digestibility obtained in a previous trial (Seradj et al., 2020). Authors are not aware of the interaction effect of breed and sex on microbial populations. Contrary to our results, X. Wang et al. (2020) reported that castrated Hainan special wild boars had significantly higher diversity than entire ones, caused by their decreased androgen secretion (Harada et al., 2016). Our results may suggest that genetic background has a more important influence on the microbial composition than sex, as was previously described (Kovacs et al., 2011).

Regarding Firmicutes/Bacteroidetes ratio, the balance between these two dominant phylogenetic types in the gut microbiota has been closely related to adiposity and fat

metabolism (Yang et al., 2018). However, this ratio varies across intestinal segments and over time due to dynamic compositional changes. In the present study, Duroc pigs tended to have a higher ratio in the cecum than F2 pigs, which is consistent with the results obtained by Xiulan Guo et al. (2008) who evidenced that the percentage of Bacteroidetes in the cecum segment had a negative correlation with backfat thickness ($R^2 = 0.63$). Although no significant differences were found in their study, Firmicutes phylum was numerically higher in obese pigs (Xiulan Guo et al., 2008), suggesting the potential implication of such phylum in carbohydrates degradation and subsequent fat deposition. In the case of distal colon, no significant differences were detected between PTs and PPs. However, the Firmicutes/Bacteroidetes ratio of growing Duroc pigs was numerically higher than fattening Duroc pigs and F2 pigs in both PPs. Despite the limitations of comparing different studies, the increased proportion of Bacteroidetes with age in Duroc pigs may be in accordance with Crespo-Piazuelo et al., (2018) who found that Iberian pigs of almost 50 kg BW had a considerable proportion of Bacteroidetes in the colon segment. In addition, feces from 240-day-old Jinhua fatty pigs also showed higher relative abundance of Bacteroidetes than Landrace pigs (Yang et al., 2018).

Microbial community in ileum and cecum segments clustered separately between both PTs of pig through beta diversity analyses, although no genera with significantly different abundance could be identified. However, numerical differences in genera abundance between fatty and lean pigs are in accordance with previous studies. Other authors have already found slightly higher levels of *Lactobacillus* genus in high quality meat breeds (Park et al., 2014; Xiao et al., 2017). Moreover, several studies have also observed that *Clostridium* is found in higher proportion in fattier pigs than in their lean counterparts (Bergamaschi et al., 2020; Wu et al., 2021). Duroc pigs also harbored higher abundance of *Streptococcus* and *Bifidobacterium* genera, which were also higher in Jinhua pigs (Xiao et al., 2018) than in Landrace.

4.3. Effect of Dietary CP Content

Although breeding selection is generating highly efficient animals, there is still some questions over their gut microbiota adaptation to nitrogen-restricted diets. Two percentage units were reduced in the experimental LP diets with respect to SP. All diets were supplemented with essential amino acids to meet the nutritional requirements for both growing and fattening phases (de Blas et al., 2013). This approach is an established strategy in the precision feeding systems. The objective is to reduce the nitrogen load from manure, which has demonstrated to improve nitrogen utilization without compromising pig performance indices (Gloaguen et al., 2014; Seradj et al., 2020), and to protect animals against intestinal disorders (Qiu et al., 2017; Yuming Wang et al., 2018). In addition, the 2% CP difference between SP and LP diets has resulted in similar differences in ether extract content, which derives from maintaining a comparable metabolizable energy value (Table 1). Although the latter difference may have some degree of impact on hindgut microbiota (Yan et al., 2013), priority was given to maintaining metabolizable energy levels.

Although gut microbiota diversity was not affected during the 15-day experiment, LP-fed pigs presented more complex microbial networks than SP-fed pigs along the three intestinal segments, especially in ileum and colon segments. In the cecum, this effect was registered only during the growing period. These results suggested that, although the limited impact of a 2% CP restriction, microbiota underwent a controlled adaptation process, emerging new relationships between microbes that may result in new metabolic pathways. A similar phenomenon has been previously described in ruminants (Costa-Roura et al., 2020) and malnourished children (Ghosh et al., 2014), leading to improved nutrient utilization and maintenance of normal physiology. In accordance with our results, neither Zhou et al. (2016) nor Seradj et al. (2020) found variation in microbial diversity with the use of moderate change in CP (2-3%) level, whereas a slight reduction in the abundance of certain genera was found in their low protein diet. These previous experiments justified their results by a long-term adaptation of the gut microbiota to nutrient availability. However, the present study lasted only for 15 days, suggesting rapid adaptation.

4.4. Microbiota Composition Throughout the Intestinal Tract

Several studies have described the variation in microbial populations across the intestinal segments, which are anatomically and functionally distinct. In addition, microbial populations are modified by inherent variations in the environmental conditions (i.e. pH, molecular oxygen and oxidation/reduction potential), transit time, substrate and the presence of gut receptors throughout the intestinal tract (Zhao et al., 2015). The more neutral pH, slower time of transit, and higher substrate availability in the more distal intestinal segments (Gresse et al., 2019; Zhao et al., 2015) allow a higher microbial growth and diversity (Crespo-Piazuelo et al., 2018; J. Wang et al., 2019). Therefore, the highest number of OTUs in this study was found in the distal colon (453 OTUs), followed by the cecum (395 OTUs) and the ileum (181 OTUs). Similarly, diversity indices followed the same progression, being higher in the lower intestinal segments than in the ileum. In addition, the increased microbial interactions reported in the present study in the lower gut segments may be coincident with the improved microbial communities stability (Gresse et al., 2019).

In addition, microbial composition is subjected to other factors such as genetics, age, diet and environmental conditions, therefore, it is difficult to compare the ratio of species abundance between different studies (Zhao et al., 2015). Firmicutes/Bacteroidetes ratio in ileum could not be calculated due to the low proportion of Bacteroidetes phylum in this gut segment, which is consistent with the existing literature (Niu et al., 2015). Recent studies (De Rodas et al., 2018; J. Wang et al., 2019) have showed that microbial composition of the upper and lower intestine are different. While the upper intestine (including ileum) harbors a higher abundance of Firmicutes and Proteobacteria phyla, the lower intestine (including cecum and distal colon) contains a higher proportion of Bacteroidetes, although Firmicutes remains the most abundant phylum in this segment too (Crespo-Piazuelo et al., 2018; J. Wang et al., 2019). The lower Firmicutes/Bacteroidetes ratio of the distal colon compared to the cecum may be due to the increased proportion of Bacteroidetes and the decreased proportion of Firmicutes from proximal to distal parts (J. Wang et al., 2019; Zhao et al., 2015). In contrast, the opposite relationship has also been reported by previous authors (Niu et al., 2015).

As was previously defined (De Rodas et al., 2018), the most abundant OTUs found throughout the intestinal tract belonged to *Lactobacillus* genus. *Lactobacillus* was also the most predominant genus of the core microbiota of all intestinal segments (Gresse et al., 2019; Ke et al., 2019), with the exception of distal colon of growing pigs. This genus has been related with immunological response of the host. Clostridia class (*Terrisporobacter* and *Clostridium*) was also abundant, especially in the fattening phase, and showed similar pattern of change across intestinal segments (Gresse et al., 2019; Zhou et al., 2016). *Bifidobacterium* was more abundant in the upper intestine (J. Wang et al., 2019), while Ruminococcaceae and Lachnospiraceae families were more abundant in the lower intestine (Trevisi et al., 2021; J. Wang et al., 2019). The later has been associated with degradation and fermentation of carbohydrates and subsequent production of short-chain fatty acids, such as butyric acid (Gresse et al., 2019).

5. Conclusions

Microbial community was mainly affected by the PP and PT of pig. Fattening pigs showed higher microbial richness than growing pigs, which is attributable to the higher maturity and stability of their microbial community. In addition, F2 pigs presented higher bacterial diversity and microbial network complexity especially in the fattening phase, while Duroc pigs tended to have higher Firmicutes/Bacteroidetes ratio in cecum. The moderate restriction in dietary CP content increased the complexity of microbial interaction network, whereas it had limited impact on microbial community composition.

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Supplementary material

Table S1. Microbial alpha diversity indices (based on OTUs), and Firmicutes/Bacteroidetes ratio (Ratio F/B) in ileum, cecum, and distal colon segments.

Item [1]	Growing phase		Fattening phase		SEM	*p*-value	
	SP	LP	SP	LP		Diet	PP
n	12	8	8	8			
Ileum [2]							
Shannon index	1.60	1.57	1.86	1.79	0.160	0.640	0.186
Simpson index	0.66	0.65	0.73	0.74	0.049	0.855	0.102
Richness	26.98	28.88	37.13	38.25	3.854	0.845	0.020
Evenness	0.50	0.47	0.52	0.49	0.040	0.381	0.785
Cecum							
Shannon index	3.18	3.35	3.56	3.42	0.177	0.943	0.189
Simpson index	0.92	0.92	0.92	0.93	0.018	0.776	0.618
Richness	95.88	99.25	127.25	113.75	8.973	0.572	0.013
Evenness	0.70	0.73	0.74	0.74	0.034	0.629	0.457
Ratio F/B	48.16	23.81	7.76	30.30	18.409	0.904	0.309
Distal colon							
Shannon index	3.65	3.72	3.66	3.79	0.128	0.394	0.650
Simpson index	0.94	0.95	0.94	0.95	0.009	0.546	0.742
Richness	134.14	129.50	151.97	147.88	9.595	0.634	0.045
Evenness	0.75	0.76	0.76	0.76	0.017	0.597	0.820
Ratio F/B	27.09	12.55	10.06	9.08	8.710	0.254	0.112

[1] Obtained in pigs differing in their protein intake (Diet): standard protein (SP) vs low protein (LP) and in their production phase (PP): growing (28.5 kg) vs fattening (88.1 kg). Standard error of the mean (SEM) and significance of Diet and PP effects are shown. Mean values within a row followed by different letters differ significantly at $p = 0.05$. No significant Diet by PP interaction was found and their values were not included in the table.

[2] Ratio F/B could not be calculated in ileum samples due to the low abundance of Bacteroidetes phylum in that intestinal tract (0.28% of analyzed sequences).

Table S2. Microbial alpha diversity indices (based on OTUs).

Item[1]	Growing phase			Fattening phase			SEM	*p*-value	
	Ileum	Cecum	Distal colon	Ileum	Cecum	Distal colon		Segment	PP
n	20	20	20	16	16	16			
Shannon index	1.58c	3.23b	3.65ab	1.82c	3.49ab	3.72a	0.116	<0.001	0.032
Simpson index	0.65c	0.91a	0.95a	0.74b	0.91a	0.95a	0.023	<0.001	0.084
Richness	28.45c	95.75b	130.65a	37.69c	120.50a	142.38a	6.119	<0.001	0.002
Evenness	0.49b	0.71a	0.75a	0.52b	0.74a	0.76a	0.023	<0.001	0.124

[1] Samples were obtained from three intestinal segments (ileum, cecum and distal colon) in pigs differing in their production phase (PP): growing (28.5 kg) vs fattening (88.1 kg). Standard error of the mean (SEM) and significance of intestinal Segment and PP effects are shown. Mean values within a row followed by different letters differ significantly at $p = 0.05$. No significant Segment by PP interaction was found and their values were not included in the table.

Table S3. Network metrics in the ileum, cecum and distal colon of Duroc and F2 pigs, fed diets of standard (SP) and low (LP) crude protein contents in the growing and fattening phases.

Item	Growing phase				Fattening phase			
	Duroc	**F2**	**SP**	**LP**	**Duroc**	**F2**	**SP**	**LP**
Ileum								
Nodes	14	16	12	14	9	9	8	11
Edges	19	21	14	19	11	9	5	9
Node degree	2.71	2.63	2.33	2.71	2.44	2.00	1.25	1.64
Betweenness	0.79	0.38	0.67	0.64	0.22	4.00	0.13	0.64
Cecum								
Nodes	43	56	43	53	50	53	52	52
Edges	117	157	81	144	106	270	185	180
Node degree	5.44	5.61	3.77	5.43	4.24	10.19	7.12	6.92
Betweenness	4.88	6.91	1.65	3.79	6.16	11.19	8.19	6.62
Colon								
Nodes	66	70	66	71	64	71	67	72
Edges	146	312	176	204	137	417	177	270
Node degree	4.42	8.91	5.33	5.75	4.35	11.75	5.28	7.50
Betweenness	4.42	15.69	5.38	9.59	4.90	20.89	4.82	16.15

Network interactions analysis were performed through Sparse Correlations for Compositional data (SparCC) technique (R Core Team, 2020; SpiecEasi package) over those genera present at least at 50% of the individuals. Their complexity was described in terms of number of nodes (genera) and edges (significant positive or negative correlations), node degree (number of connections that any node establishes with other nodes) and betweenness centrality (measure of centrality in a graph based on shortest paths).

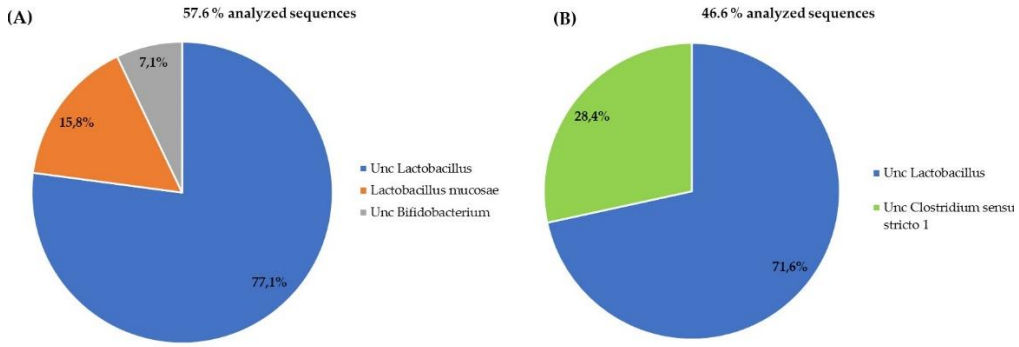

Figure S1. Microbial core community composition (OTUs) in ileum of Duroc and F2 (Pietrain × F1: Duroc × Landrace) pigs in either growing (A, 28.5 kg) or fattening (B, 88.1 kg) production phases. Unc: unclassified.

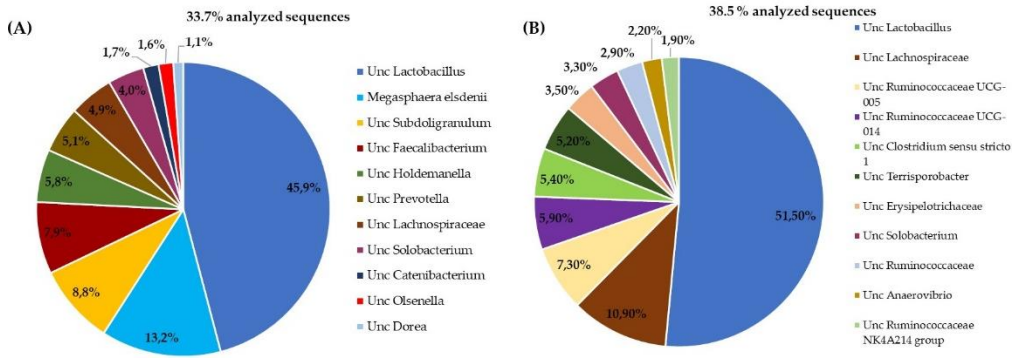

Figure S2. Microbial core community composition (OTUs) in cecum of Duroc and F2 (Pietrain × F1: Duroc × Landrace) pigs in either growing (A, 28.5 kg) or fattening (B, 88.1 kg) production phases. Unc: unclassified.

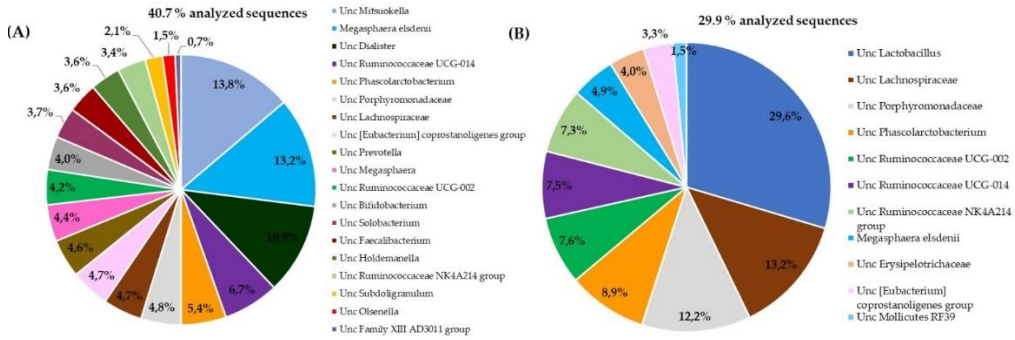

Figure S3. Microbial core community composition (OTUs) in distal colon of Duroc and F2 (Pietrain × F1: Duroc × Landrace) pigs in either growing (A, 28.5 kg) or fattening (B, 88.1 kg) production phases. Unc: unclassified.

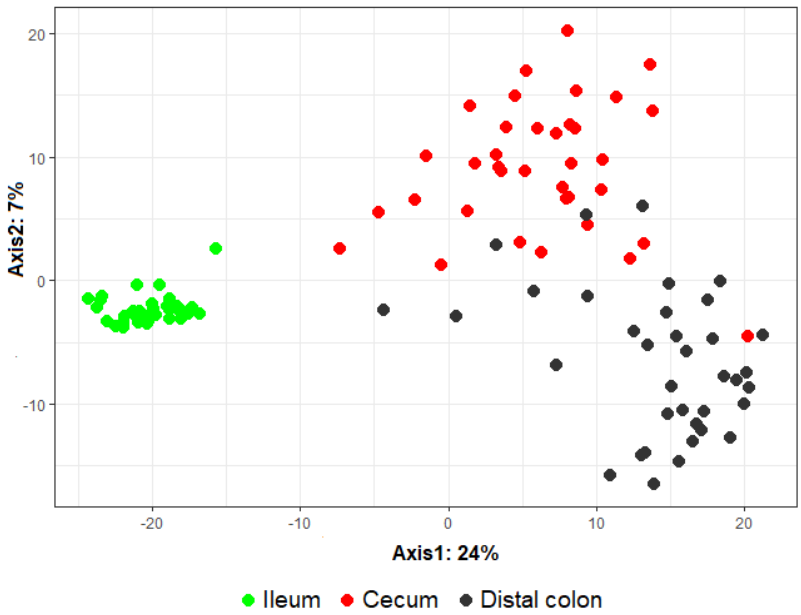

Figure S4. Graphical representation of partial least squares-discriminant analysis (PLS–DA) on microbial OTUs in ileum, cecum and distal colon. Each point represents a different sample and a greater distance between two points infers a higher dissimilarity between them. Samples clearly clustered by intestinal segment, indicating that ileum, cecum and distal colon harbored different microbial communities (PERMANOVA $p < 0.001$).

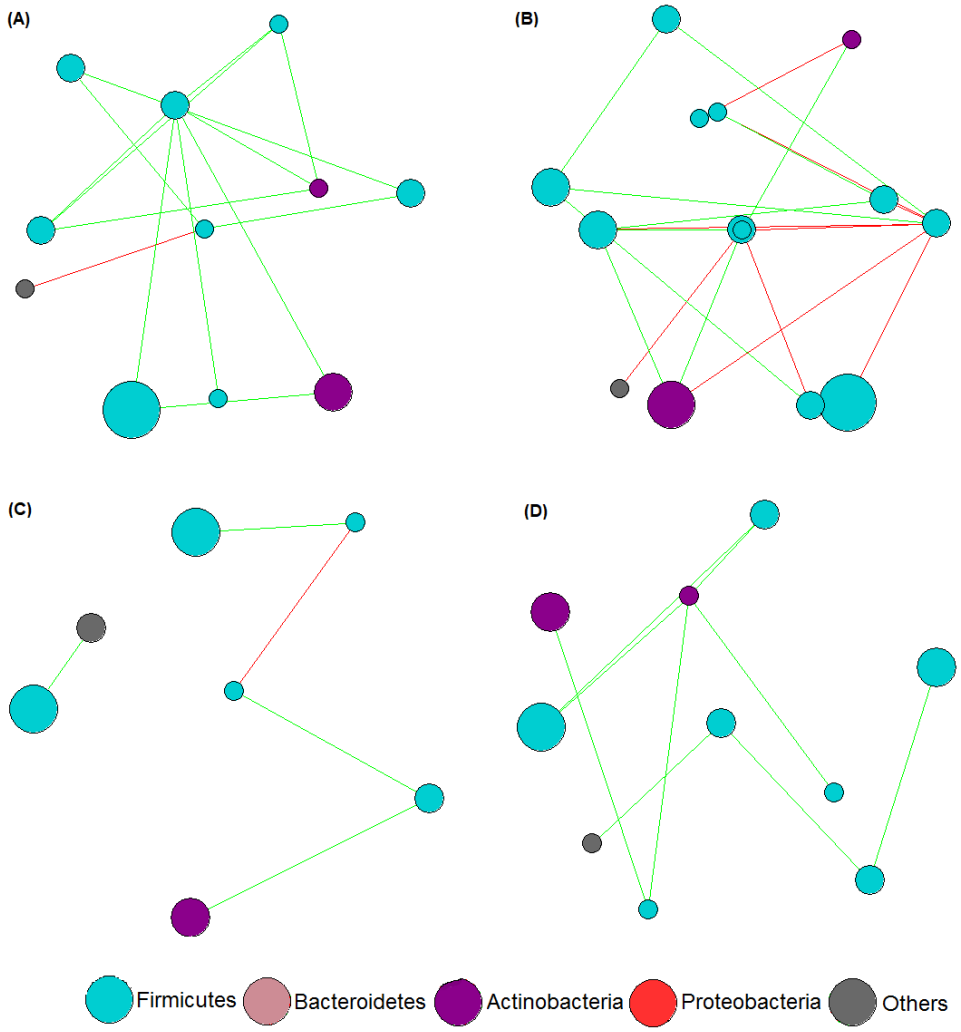

Figure S5. Microbial genera network in ileum. Obtained in pigs differing in their protein intake: (A–C) standard protein vs (B–D) low protein and production phase: (A–B) growing (28.5 kg) vs (C–D) fattening (88.1 kg). Green and red edges indicate positive and negative correlations, respectively. Node size is proportional to genera abundance and node color indicates phyla affiliation. Minor phyla are represented as "Others".

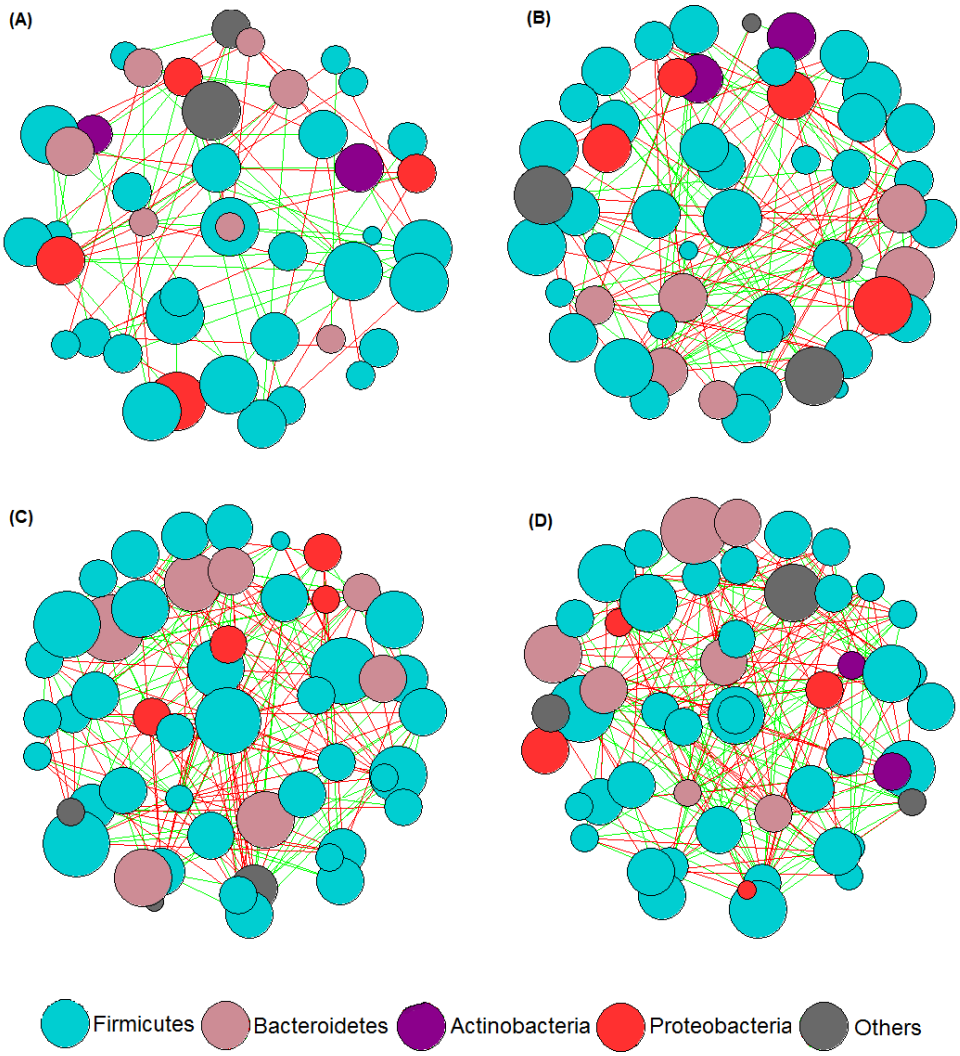

Figure S6. Microbial genera network in cecum. Obtained in pigs differing in their protein intake: (A–C) standard protein vs (B–D) low protein and production phase: (A–B) growing (28.5 kg) vs (C–D) fattening (88.1 kg). Green and red edges indicate positive and negative correlations, respectively. Node size is proportional to genera abundance and node color indicates phyla affiliation. Minor phyla are represented as "Others".

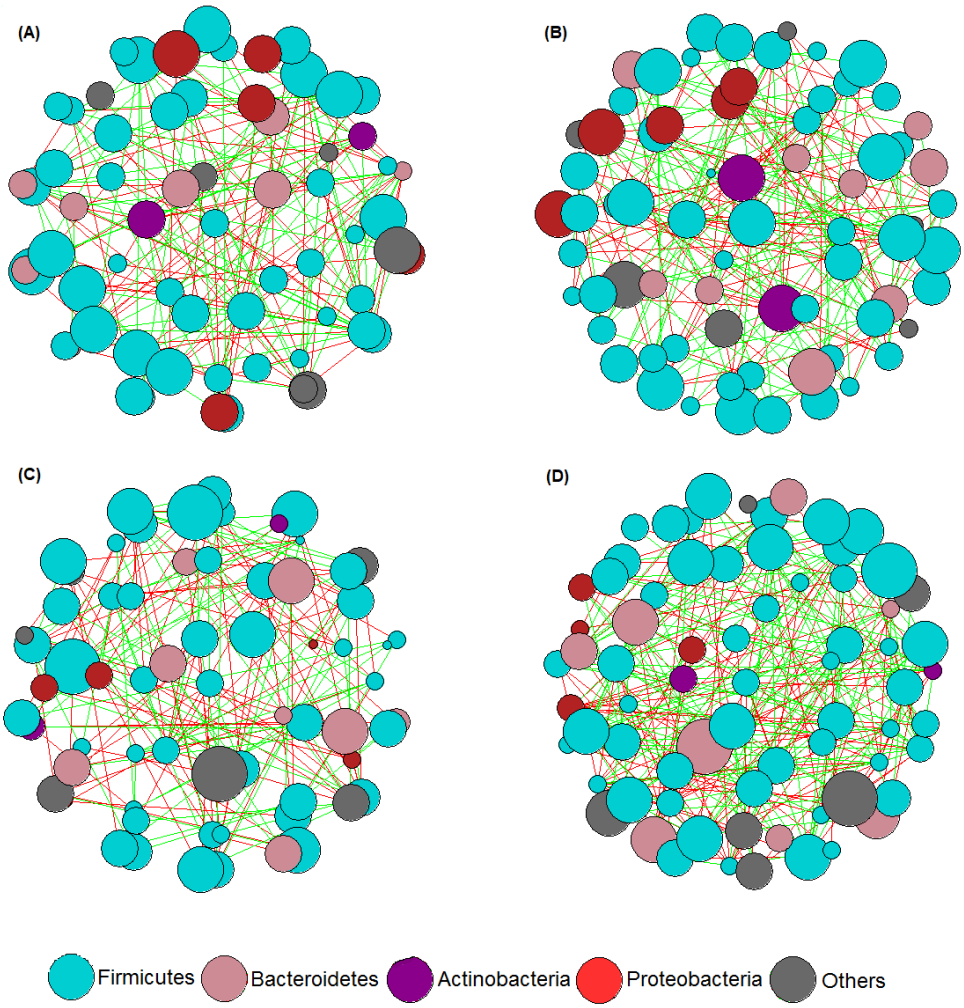

Figure S7. Microbial genera network in distal colon. Obtained in pigs differing in their protein intake: (A–C) standard protein vs (B–D) low protein and production phase: (A–B) growing (28.5 kg) vs (C–D) fattening (88.1 kg). Green and red edges indicate positive and negative correlations, respectively. Node size is proportional to genera abundance and node color indicates phyla affiliation. Minor phyla are represented as "Others".

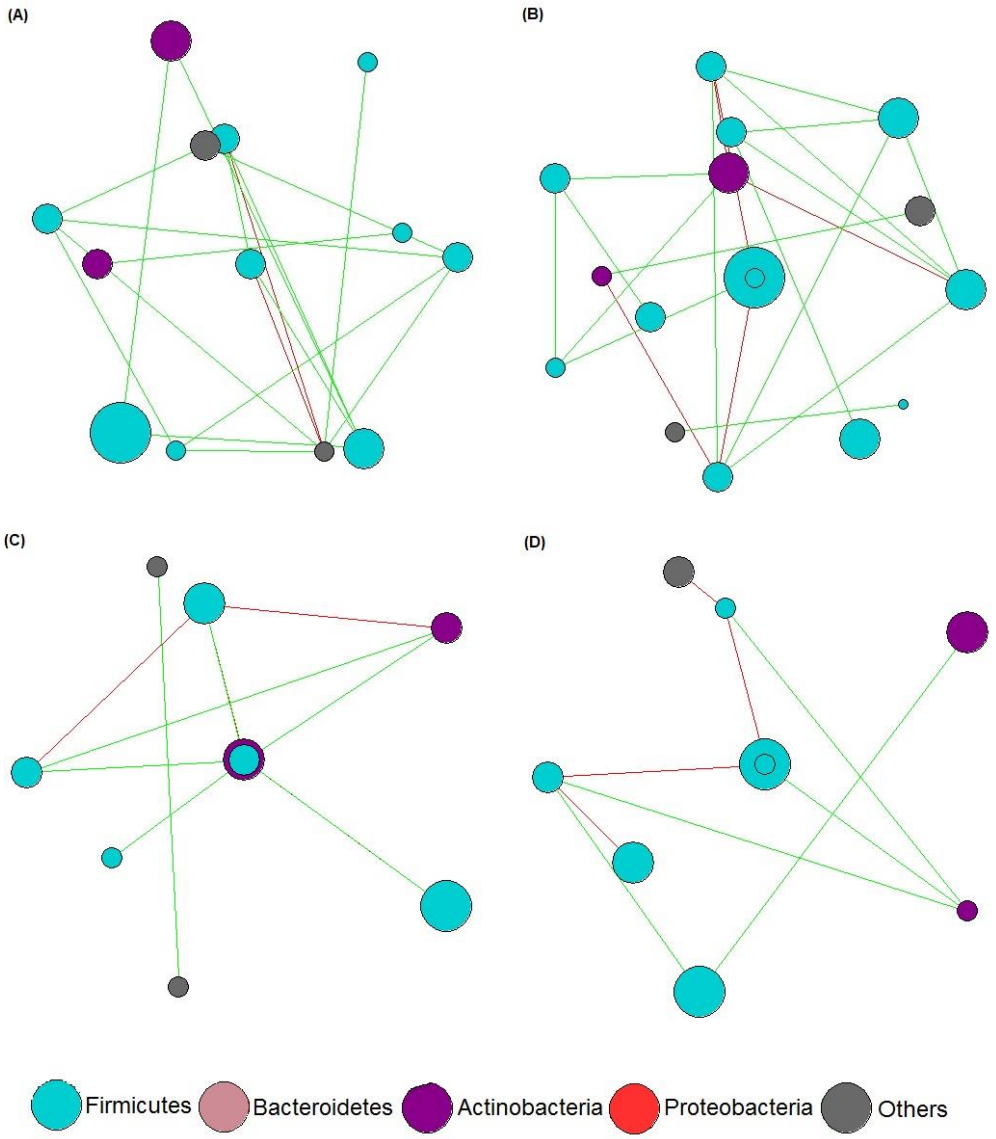

Figure S8. Microbial genera network in ileum. Obtained in pigs differing in their production type: (A–C) Duroc vs (B–D) F2 (Pietrain × F1: Duroc × Landrace) and production phase (A–B) growing (28.5 kg) vs (C–D) fattening (88.1 kg). Green and red edges indicate positive and negative correlations, respectively. Node size is proportional to genera abundance and node color indicates phyla affiliation. Minor phyla are represented as "Others".

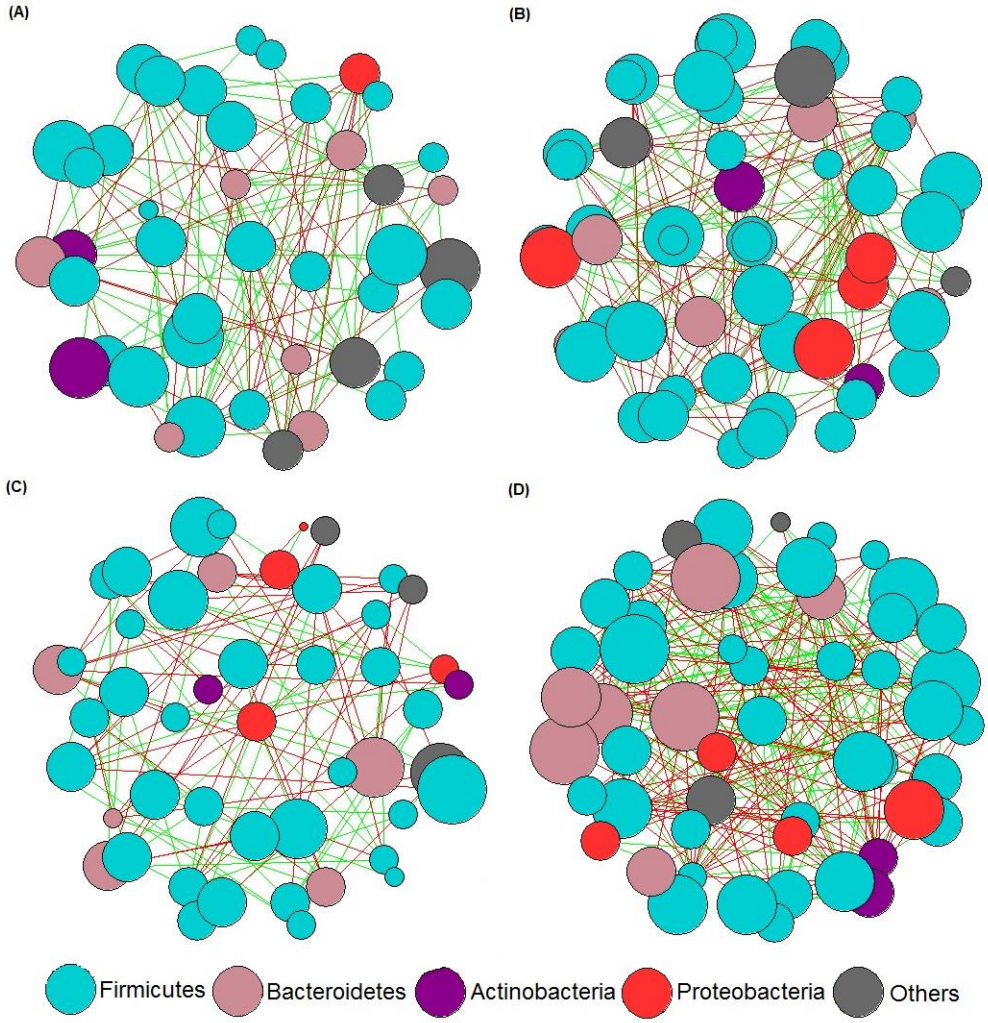

Figure S9. Microbial genera network in cecum. Obtained in pigs differing in their production type: (A–C) Duroc vs (B–D) F2 (Pietrain × F1: Duroc × Landrace) and production phase (A–B) growing (28.5 kg) vs (C–D) fattening (88.1 kg). Green and red edges indicate positive and negative correlations, respectively. Node size is proportional to genera abundance and node color indicates phyla affiliation. Minor phyla are represented as "Others".

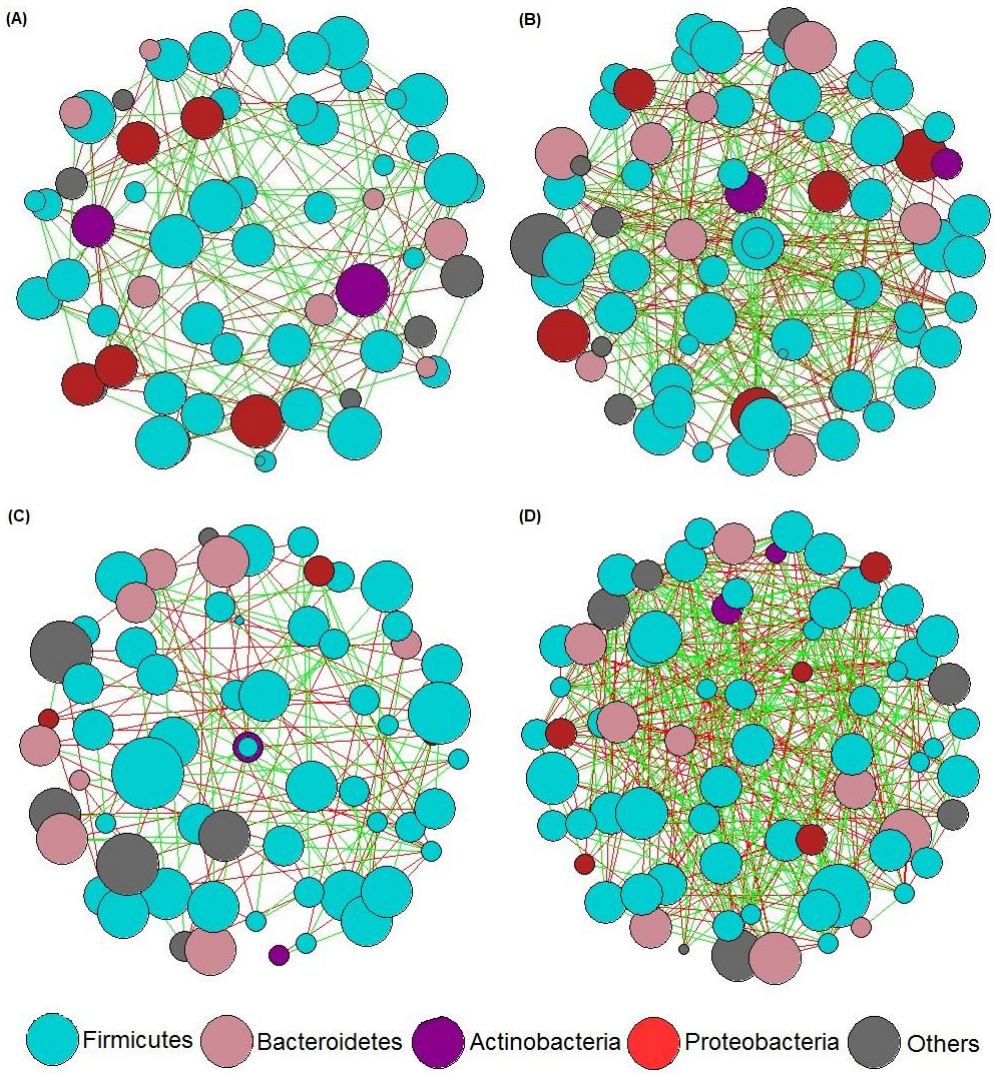

Figure S10. Microbial genera network in distal colon. Obtained in pigs differing in their production type: (A–C) Duroc vs (B–D) F2 (Pietrain × F1: Duroc × Landrace) and production phase (A–B) growing (28.5 kg) vs (C–D) fattening (88.1 kg). Green and red edges indicate positive and negative correlations, respectively. Node size is proportional to genera abundance and node color indicates phyla affiliation. Minor phyla are represented as "Others".

GENERAL DISCUSSION

Modern swine production relies mostly on long-term breeding programs, where selected breeds and their crosses are used to increase reproductive traits and efficiency and ultimately obtain lean meat at a competitive price (Knap and Rauw, 2008). These commercial genotypes coincide with a differentiated pig production in which selection criteria are based on improved meat eating quality, where specific traits such as fat deposition and FA profile are prioritized (Fernandez et al., 1999; Wood et al., 2008). Pigs belonging to these two market niches have been used in the present thesis to analyze their variation in different aspects of protein and fat metabolism, intestinal microbiota and GHG emissions, which will be discussed below. These two types of pigs differed not only in their genotype, but also in their sex condition as will be exposed in the following section "Experimental Approach", so the term producing type has been used throughout the work to include both endogenous effects. In addition, all these aspects were examined at two different ages or GP, the growing phase (28.0 ± 0.76 Kg), in which protein deposition theoretically predominates, and the fattening phase (87.4 ± 1.26), in which fat deposition takes prominence. Finally, the pigs were exposed to two experimental diets in each GP, differing only by 2 percentage units in CP within the commercially managed range (FEDNA, 2013), in the framework of nitrogen waste reduction through manure.

1. Experimental approach

Two PT of pigs were selected to perform all the experimental procedures for this thesis, so that one was representative of the Spanish intensive swine production (lean PT), and the other of the high-quality pork market (fatty PT). For the lean PT, a three-way (3W) crossbreed of Pietrain sires crossed with Duroc × Landrace (F1) dams was used. Pietrain breed has been commonly employed as a terminal sire due to its enhanced conformation, production performance and lower backfat thickness (Oliver et al., 1994). On the other hand, Landrace breed has been the pillar for F1 hybrids for the maternal line, due to its well adaptability, maternal behavior, reproductive performance and lean growth (MAPA, 2019). Moreover, Duroc breed has been also included into the genetic program by its high prolificacy and contribution to improve meat quality without compromising pig growth (Alonso et al., 2015; Oliver et al., 1994).

By contrast, purebred Duroc males were chosen as a fatty PT and considered as a model of this specific and differentiated market niche. Duroc breed was introduced to Europe from the United States and was widely distributed, gaining great popularity due to its adaptability, growth rates and fat characteristics (Latorre et al., 2009; MAPA, 2019). Although this breed also underwent intensive selection for faster growth and higher lean deposition, meat from Duroc or Duroc-sired offspring generally exhibits improved texture, juiciness, flavor scores (Dunshea and D'Souza, 2003; Ngapo and Gariépy, 2008), and higher IMF content than European white breeds (Latorre et al., 2009, 2003a; Wood et al., 2008). For this reason, Duroc is commonly used as a terminal sire in fatty crossbreeds or used as purebred (Cameron, 1990; Latorre et al., 2009); for instance, Duroc is used as a sire line for the production of Spanish premium Iberian meat products (BOE, 2014), and for the protected designation of origin (PDO) "Teruel ham and shoulder" (BOA, 2021).

Moreover, the use of the Duroc as a terminal sire in breeding programs with autochthonous breeds aims to improve prolificacy, growth performance, carcass conformation, and reduce the production period without lessening meat quality (Lopez-Bote, 1998; Morcuende et al., 2007). A clear example of these crosses is the one made with Iberian, an autochthonous breed in the Mediterranean area highly appreciated for its quality attributes for dry-cured products. On the other hand, the wide distribution of Duroc has resulted in a heterogeneous breed, with a high variability in quality traits among genetic lines (Cilla et al., 2006; Lonergan et al., 2001) due to the disparity of selection criteria established in different regions. Although some of these lines preserve and express high lipogenic activity and lipid deposition capacity (Cilla et al., 2006; Wood et al., 2008), Duroc lines highly selected for lean conversion efficiency might have decrease quality traits. In these sense, some authors have recognized decreases in meat quality when crossing some Duroc lines with Iberian pigs (Ayuso et al., 2016; Benítez et al., 2018; Ramírez and Cava, 2007; Ventanas et al., 2006).

Besides genotype, age contributes to greater carcass pieces weight and adiposity, which in turn include higher IMF content (Čandek-Potokar et al., 1998; Latorre et al., 2019) and a positive correlation with oleic acid proportion (Benítez et al., 2021; Cilla et al., 2006). Furthermore, higher adiposity decreases salt diffusion speed and

seasoning losses during processing (Virgili et al., 2003), and is also related to a higher overall proportion of saturated FA, which contribute to the sensory traits of dry-cured ham. Conversely, lower adiposity is associated with a higher proportion of polyunsaturated FA, which are more prone to oxidation and soften the adipose tissue, impairing its technological processing and suitability for dry-cured meat products (Čandek-Potokar et al., 2017).

The clear advantages of using heavier pigs to obtain high-quality pork products require castration of the males to avoid boar taint, as these pigs reach sexual maturity during their production period, in addition to preventing aggressive and sexual behavior (Prunier et al., 2006). Castration, however, is not used for pigs intended for fresh lean meat, because their production period is shorter in order to maximize leanness and feed efficiency (Bonneau and Weiler, 2019). This sex difference, moreover, may maximize their potential divergence in protein and fat metabolism. Therefore, was necessary to define the term 'producing type' to include both, genotype and sex variation among pigs, since Duroc males were surgically castrated soon after birth, whereas the 3W pigs were not, to meet commercial conditions.

2. Protein synthesis

The metabolic rate of protein turnover, including protein synthesis and degradation, has been the subject of several studies to explain the underlying physiological and biochemical mechanisms responsible for the potential for lean tissue deposition. These mechanisms are modified by the effect of endogenous and exogenous factors, which are exposed in *Chapter I* of this thesis. In *Chapter II*, protein FSR were examined in two visceral tissues, the liver and the duodenal intestinal section, being organs with a relevant role in the digestion, absorption, and metabolism of nutrients; as well as in two skeletal muscles of great physiological and economic relevance, the *longissimus dorsi* and the *biceps femoris*, as they represent the large part of the loin and ham cuts, respectively.

As reviewed in *Chapter I*, most visceral tissues, especially liver and upper intestinal tract, showed higher FSR than skeletal muscles (Bregendahl et al., 2008; Cross et al., 2020; Garlick et al., 1976; Ten Have et al., 2019) due to their higher metabolic activity. Furthermore, the evolution of FSR of most tissues between the two GP was

in agreement with the available literature, which supports that the highest FSR peak occurs during the early postnatal period and this progressively decreases over time (Davis et al., 2008, 1996). It is explained by the elevated content and activity of ribosomes in neonatal pigs (Skjaerlund et al., 1994), along with a higher abundance of positive regulators of the mechanistic target of rapamycin signaling pathway (Davis et al., 2008). Surprisingly, the *biceps femoris* showed the opposite trend between GP, with greater FSR in the fattening than in the growing phase in both PT. This could be due either to a later development or to a more intense protein turnover in production stages close to slaughter. The individual is constantly subjected to positive (feed intake, exercise, or injury) and negative stimuli (fasting states or inactivity) throughout growth and adulthood, which could have an impact on overall muscle mass if the protein balance is altered for a sufficient period of time due to the high adaptability of skeletal muscle (Wallace et al., 2016).

According to a recent study evaluating the transcriptional regulation of *biceps femoris* between two developmental stages (Benítez et al., 2021), genes upregulated in pigs of 24 kg BW were more associated with the earlier processes of myogenesis (i.e., proliferation and initial stages of differentiation), while upregulated genes in older pigs (50 kg BW) were more involved in the later processes of myogenesis (i.e., advanced differentiation and hypertrophy). In addition, functions related to extracellular matrix organization were also activated in these latter pigs, which regulates development, growth and repair of skeletal muscle. These results would explain the greater FSR in this muscle. On the other hand, when Ayuso et al. (2016) assessed *longissimus dorsi* gene expression, they described that regulation of genes related to muscle growth and metabolism in distinct developmental stages differed between the two pig genotypes utilized. This may coincide with the results obtained in our study, in which the *longissimus dorsi* presented a positive FSR-evolution in Duroc pigs between growing and fattening phases, while in 3W pigs it decreased.

Few studies have compared *in vivo* protein metabolism between different pig genotypes (Rivera-Ferre et al., 2006, 2005), and current studies have focused on comparing extreme pig breeds with a clear difference in their lean tissue deposition capacity (Nieto et al., 2002), using autochthonous breeds (e.g., Iberian breed) versus highly selected breeds for lean deposition (e.g., Landrace breed). According to Rivera-

Ferre et al. (2005), who conducted a similar experimental design, reported higher muscle FSR in purebred Iberian gilts than in leaner purebred Landrace. These results were associated with higher fractional rates of protein degradation (FDR) in Iberian gilts, given their lower ASR as they had smaller muscles, explaining their lower growth efficiency. However, in our study, the leaner PT exhibited higher FSR than the fatty PT in both liver and *longissimus dorsi* muscle in the growing phase. A recent study performed in broiler (Maharjan et al., 2020), also found higher FSR in the broiler line that exhibited higher lean mass deposition, even though the animals were submitted to hot and cool seasons.

The higher FSR in 3W pigs did not match with a greater AA availability (Table 4, *Chapter II*), and had low impact on growth performance parameters, although average daily gain (ADG) tended to be favorable to 3W pigs. This trend corresponds to what was seen in our previous work (see *Annex*), where 3W pigs presented a higher overall ADG than Duroc pigs, especially in the feeding phase II, when pigs had an intermediate weight between growing and fattening phases. However, when total protein content of each tissue was considered to measure ASR, 3W pigs exhibited higher lean deposition, which is also explained by their higher nitrogen retention capacity (Table S2, *Chapter II*). These results are in agreement with the findings of Edwards et al. (2003) and Werner et al. (2010), who reported a higher proportion of lean and dressing percentage in Pietrain-sired crossbred offspring than those from Duroc.

Since muscle fiber type composition seems to not explain the FSR-differences between PT (*Chapter I*; Guo et al., 2011; Werner et al., 2010), other underlying mechanisms may be more responsible for that; for example, sex condition, because Duroc pigs were surgically castrated whereas 3W pigs were entire boars. Differences in carcass characteristics between boars and barrows have been previously reported, with higher lean deposition in boars but significantly lower fat deposition than surgically castrated males (Boler et al., 2014; Kress et al., 2020; Pauly et al., 2008; Suster et al., 2006). Differences in lean content and muscle growth have frequently been attributed to the effects of androgen hormones, mostly testosterone (Bonneau, 1998; Jinliang Xue et al., 1997). As discussed in *Chapter I* of this thesis, to our knowledge, only studies performed by Mulvaney (1984) and Skjaerlund et al. (1994)

compared protein turnover between boars and barrows. While Skjaerlund et al. (1994) found no differences in FSR or FDR between both sexes by *in vivo* and *in vitro* procedures, which would correspond to the earlier age of the pigs (1– to 4–weeks-old pigs); Mulvaney (1984) reported lower FSR and FDR for barrows at either prepubertal (40 kg BW) or pubertal (75 kg BW) stage. These latter results would support, in part, those obtained in our study, although we are not aware if their results obtained by *in vitro* techniques are fully comparable with *in vivo* trials (Wendowski et al., 2017).

The anabolic effects of testosterone has been suggested in numerous studies (Basualto-Alarcón et al., 2013; Rosa-Caldwell and Greene, 2019; Steiner et al., 2017; Wendowski et al., 2017; White et al., 2013), as was also found that androgens reduce protein degradation (Rossetti et al., 2017) through the ubiquitin proteasome pathway (Kruse et al., 2020) or through hepatic urea cycle (Birzniece et al., 2011). Testosterone inhibits the action of certain enzymes present in the urea cycle, decreasing the hepatic urea synthesis and thus, reducing protein degradation and loss of AA through urine, while increasing the amount of AA susceptible for muscle anabolism (Lam et al., 2017). This is consistent with the results obtained in *Chapter II*, where 3W pigs showed a significantly lower nitrogen loss through urine than Duroc pigs, which lead to a higher protein retention.

Regarding the effect of dietary CP level, 3W pigs showed lower duodenal FSR compared to Duroc pigs when fed the low-CP diet (LP). Considering that 3W pigs have a lower AID than Duroc pigs, the drop in CP percentage could have resulted in a lower availability of AA for synthesis in that tissue (*Chapter I*), even if the LP diet was supplemented with synthetic AA to meet the nutrient requirements (FEDNA, 2013). In this sense, Duroc pigs show a better adaptability to a lower CP content in the diet.

3. Fat metabolism

In addition to optimizing diets to improve animal efficiency and growth performance, improving the quality of meat products has become a key objective to satisfy consumer demand. Meat quality is a complex concept that includes organoleptic, nutritional and technological characteristics, in which the content and composition of fat tissue play a relevant role (Wood et al., 2008). Considering that animal nutrition

can influence both fat deposition and synthesis (Lu et al., 2020; Madeira et al., 2013), it is required to understand and build dynamic models of lipid incorporation throughout the production cycle of the animal to define feeding strategies that would optimize fat profile.

In pigs, FA deposited in adipose tissues derive from direct incorporation, mainly from diet but also fat mobilization (Kouba et al., 2003), and to a large extent from biosynthesis processes (Dunshea and D'Souza, 2003; Kloareg et al., 2007). However, knowledge about the biochemical and genetic mechanisms of their development over time are scarcely defined, as well as lacks information about possible differences between breeds and/or pig PT. In this case, we are dealing with two PT generally used in southern Europe for distinct production purposes and fat profiles. Therefore, in *Chapter III*, pigs were exposed to a D-labeled stearic FA (d_{35}–C18:0) in the diet to characterize its direct FIR into different adipose tissues. In addition, we investigated the rates at which d_{35}–C18:0 desaturated to monounsaturated FA (oleic acid, d_{33}–C18:1 *c*9), designated as real FUR. For the latter objective, all pigs were genotyped for the single nucleotide polymorphism of the *SCD* gene (rs80912566). This polymorphism modulates the activity of the rate-limiting enzyme SCD, which is responsible for the desaturation at the Δ9 position of certain saturated FA, where C18:0 is one of the preferred ones, converting them into their monounsaturated FA (Guillou et al., 2010). The *SCD*_T allele potentiates Δ9-desaturase activity in comparison with the alternative *SCD*_C allele (Estany et al., 2014; Henriquez-Rodriguez et al., 2016; Solé et al., 2022), being almost fixed in some breeds such as Pietrain, Landrace, Iberian or wild boars, whereas *SCD*_T allele in Duroc pigs segregates at intermediate frequencies (Estany et al., 2014). Therefore, to maximize differential oleic acid synthesis, Duroc pigs were selected to be homozygous for the *SCD*_T and *SCD*_C alleles. The authors are not aware that such analyses have been performed previously on pigs.

It has been stablished that different tissues perform different functions in fat metabolism, and it can be modified throughout the animal productive cycle. Although most of the FA biosynthesis in pigs occurs in the adipose tissue itself (Dunshea and D'Souza, 2003), Duran-Montgé et al. (2009) reported differences in lipogenic gene expression, between tissues, at distinct BW; being higher in adipose tissue at 60 kg

BW, but higher in liver at 100 kg BW. Our results showed that the direct incorporation of dietary d_{35}–C18:0 (FIR) among tissues as well as the degree of its unsaturation changed between age or GP. While the IMF had a much more active FIR in the growing phase, in the liver it was much higher in the fattening phase. The IMF of the *semimembranosus* presented the lowest FIR, which coincided with the negative allometric coefficient of the same fat depot and of the muscle itself, showing a higher degree of maturity, which would lead to advanced metabolic activity. In the case of the SC, the FIR remained stable or even slightly decreased in Duroc pigs, while in 3W pigs it increased with age.

Regarding pig PT, although no differences were detected in the liver, 3W pigs showed a higher FIR in the SC and the IMF of *longissimus dorsi* compared to Duroc genotypes. Moreover, in the *longissimus dorsi*, 3W pigs experienced the greatest decrease in FIR with age, which could be explained by a redirection of dietary FA to other fat depots, such as SC tissue, whose FIR increased significantly in the fattening phase, or intermuscular fat, whose allometric coefficient was positive and much higher than that of Duroc genotypes. The authors suggest that the higher FIR in 3W pigs compared to Duroc pigs was due to distinct fat incorporation pathways. While 3W pigs seem to rely more on direct incorporation of dietary FA, in Duroc pigs FA biosynthesis could play a more relevant role. This hypothesis is reinforced by the FUR results.

Although with our analytical technique we could only detect consistent measures of FUR in SC tissue, we were able to detect SCD enzyme activity *in vivo* by measuring the appearance of d_{33}–C18:1 *c*9 in tissue, which could only come from unsaturation of d_{35}–C18:0, directly incorporated through diet. Although previous expression studies described a reduction of *SCD* gene expression with age in adipose tissues (Duran-Montgé et al., 2009), we found much higher real FUR in the fattening phase than in the growing phase. These results agree with those of Kouba et al. (2003), who performed an *in vitro* study in which they incubated tissues with [^{14}C] stearic acid and analyzed the appearance of [^{14}C] oleic acid. They also described an increase in SCD activity in pigs between 51 and 95 kg BW, although it stagnated or decreased thereafter.

Furthermore, the apparent activity of the SCD enzyme has been measured in previous studies as the C18:1 *c*9/C18:0 ratio, referred to as apparent FUR in *Chapter III*. In contrast to the results of Bosch et al. (2012) and Henriquez-Rodriguez et al. (2016), there was a reduction in apparent FUR from the growing to the fattening phase in all PT, where 3W and Duroc TT pigs exhibited higher rates than Duroc CC pigs in both GP. The same results were obtained for the real FUR, although it was only detected in the fattening phase. Considering that all 3W pigs carried the *SCD*_ T allele, and most of them were homozygous, the results were in line with those studies that demonstrated a higher rate of unsaturation by the *SCD*_T allele than *SCD*_C, without compromising overall fat content (i.e., carcass weight, backfat thickness, lean and IMF content) (Estany et al., 2014; Henriquez-Rodriguez et al., 2016; Solé et al., 2021).

The reduction in apparent FUR in fattening 3W pigs was associated with their higher SC-FIR, whereas, in Duroc pigs it was proposed to be caused by an increase in lipogenic activity, based on the stable FIR in Duroc TT pigs or the slightly reduction in Duroc CC pigs, which is also not accompanied by a decrease in their allometry coefficient. Therefore, during feed formulation, leaner pigs should have higher dietary FA requirements than fatty ones, while the opposite would be true for FA precursors (i.e., glucose, starch, etc.).

The authors are aware that lipid deposition is also determined by the catabolic processes inherent to the metabolism of these molecules. A part of the lipids deposited during the experimental period could have undergone oxidation, but we could not analyze it under our experimental conditions. Although FIR and FUR values might be somewhat underestimated, direct dietary fat deposition is highly efficient (Bruininx et al., 2011; Jebb et al., 1996).

It has been extensively studied that reducing the dietary CP content enhances IMF depots in pigs, increasing the availability of energy stimulating the lipogenic enzyme expression and fat deposition (Madeira et al., 2013). Although the moderate CP restriction applied in our study (2 %) had some effects on protein synthesis, it did no influence fat metabolism. Diets were formulated for each GP, and the protein restriction did not imply changes in fat content (Table 1 *Chapter III*) or composition (Table 2 *Chapter III*).

4. Microbiota

The gut microbiota has received special attention by the scientific community due to its potential economic and environmental impact, but also its influence upon several phenotypic traits (Guo et al., 2008; Wu et al., 2021), for instance, it is associated with the immune system (Niederwerder et al., 2016), metabolism of indigestible nutrients (Quan et al., 2019; Wu et al., 2021), synthesis of beneficial substances (i.e., vitamins), and regulation of host gene expression (Niederwerder et al., 2016; Richards et al., 2005).

Gut microbiota belongs to a dynamic ecosystem that is shaped along the intestinal tract, and adapted to the specific environmental characteristics of each intestinal segment (Zhao et al., 2015). Additionally, is it strongly influenced by endogenous factors of the host itself, including age, genetics, sex, (Wang et al., 2020; Wang et al., 2019; Xiao et al., 2018), and by the surrounding conditions, such as nutrition and environment (Pajarillo et al., 2014; Qiu et al., 2017). Therefore, it is of great interest to comprehensively understand how different factors and their interactions affect the gut microbiota.

In this regard, the effect of GP, PT, dietary CP level and their interaction were evaluated in *Chapter IV* and *Annex* of this thesis. For this purpose, the contents of ileum, cecum and distal colon in *Chapter IV*, and fecal samples in *Annex* were collected, and subsequently subjected to microbial genomic DNA extraction, PCR of the V3 and V4 regions of the microbial 16S rRNA, paired-end sequencing, taxonomy assignment of the OTU (operational taxonomic unit), and taxonomic assignment of phylotypes, with the aim of assessing for each intestinal segment (i) the differences between PT, and (ii) the differences between dietary CP level in each GP.

Alpha diversity is a measure of the variability of species, which is positively correlated with greater microbial stability, resilience to dysbiosis, and pathogen threats (Ju et al., 2008; Sommer et al., 2017). In this sense, fattening pigs presented higher microbial richness than growing pigs in all intestinal segments, which corresponds to a common development of the microbial communities with animal maturity (Ke et al., 2019; X. Wang et al., 2019). Microbial richness has also been negatively correlated with survival of invading species (van Elsas et al., 2012). In

addition, pig maturity also favored the interactions between microbial genera, tested through microbial networks. The higher microbial diversity and interactions may benefit a proper functionality of the intestinal tract, and would explain in part the higher nutrient digestibility of fattening pigs (*Chapter II* and *Chapter III*), and would enriched the metabolic pathways associated with carbohydrate and energy metabolism, coinciding with their more developed fat metabolism (*Chapter III*) (Ke et al., 2019).

Regarding pig PT, several studies have pointed out the potential of the intestinal microbiota to modulate lipid metabolism (Han et al., 2022; Xie et al., 2022; Yang et al., 2018), as it contributes to host energy (i.e., short chain FA) and bile acid metabolism (Ejtahed et al., 2020). However, the mechanisms by which it is regulated, and the degree of contribution, have not yet been fully elucidated. The microbial ratio of Firmicutes/Bacteroidetes has been associated with fat and energy metabolism, adiposity or IMF content in various studies. Some authors have described higher adiposity when the ratio increases (Guo et al., 2008; Yan et al., 2016; Yang et al., 2018), while the opposite has also been exposed (Xie et al., 2022; Yang et al., 2018). According to Magne et al. (2020) and Aguirre and Venema (2015), the great inter-individual variation in gut microbiota composition, together with the different analytical methods used between studies, and the lack of consideration of other important factors could have resulted in discrepancies in the abundance of these two dominant phylogenetic types. Therefore, this index should be treated with caution.

In addition, several microbial genera have been associated to have an important role in fat metabolism and characteristics (Fang et al., 2017; Tang et al., 2020), like those included in the families *Lachnospiraceae*, *Ruminococaceae*, *Prevotella*, *Treponema* and *Bacteroides* (He et al., 2016). In addition, higher abundance of *Lactobacillus* and *Clostridium* genera have been related with higher adiposity (Ma et al., 2022; Park et al., 2014; Wu et al., 2021). However, the mechanism by which certain gut genera contribute to fat metabolism, and its extent, is not clearly understood.

A relevant feature was elucidated between both PT, 3W pigs presented a greater alpha diversity along with a greater overall complexity in their microbial network, which increased progressively between the two GP. Although some studies have shown

opposite results, most studies in human and pigs agree that leaner individuals have higher microbial diversity than obese ones (Bergamaschi et al., 2020; Turnbaugh et al., 2009). The combination of these two characteristics contributes to greater robustness and, therefore, a greater ability to adapt and cope with disturbances (Costa-Roura et al., 2022; Dunne et al., 2002). Throughout the production period, animals may deal with a great variability of disturbances that may impact or alter the microbial community, potentially leading to dysbiosis and imparing animal health and growth. This information may potentiate the prevention and anticipation of these disturbances, designing action plans accordingly to their robustness to avoid or minimize their effects, or it could be used in genetic selection programs to enhance this attribute.

On the other hand, Duroc pigs emitted a significantly higher amount of NH_3 and CH_4 than 3W pigs (Table 5, *Annex*). Since the emission of NH_3 is positively related with the excretion of nitrogen through feces and urine (Portejoie et al., 2004), it coincides with the significantly higher excretion in Duroc pigs during the fattening period (Figure S5, *Chapter II*). As for CH_4, no differences in relative abundance were found in archaea kingdom between PT. A human study proposed a positive cooperation between H_2- utilizing methanogenic archaea with H_2 producing bacteria (e.g., *Prevotellaceae* family) (Zhang et al., 2009), this latter being more abundant in Duroc pigs (*Annex*). The transfer of hydrogen gas (H_2) between both species was thought to be implicated in higher energy uptake in obese adults. In addition, other authors have postulated that CH_4 emission production is not fully correlated with methanogen abundance (de la Fuente et al., 2019; Luo et al., 2012).

The negligible effect of dietary CP level on nitrogen balance and on microbial community in *Chapter IV* and *Annex* could explain the lack of difference in GHG or NH_3 emissions between diets, reported in *Annex*. What was observed in *Chapter IV* was that pigs fed the LP-diet had greater microbial network complexity than those fed the standard CP-diet. These new microbial connections may be the resulting adaptation to counteract the nutritional effect and would lead to new metabolic pathway, as was reported in pre-gastric compartment under nitrogen restriction (Costa-Roura et al., 2020). In addition, the longer experimental period in *Annex* motivated the appearance of influential taxa in LP-fed pigs (i.e., *Blautia sp* and *Selenomonas bovis*).

CONCLUSIONS

First: Fractional protein synthesis rate was considered a valid index to measure protein synthesized per day (%/day).

a) Among pig producing types, fatty pigs (i.e., Duroc) showed higher amino acid availability, however, young lean pigs (i.e., 3W crossbreds) exhibited higher FSR in liver and *longissimus dorsi*, which may be associated with their higher protein retention rate.

b) In both pig producing types, the FSR generally decreased with animal maturity in visceral tissues, however in the case of skeletal muscles (*biceps femoris* and *longissimus dorsi* of Duroc) high synthetic activity was maintained in the fattening phase, coinciding with a higher availability of amino acids.

c) In relation to dietary crude protein content, the synthetic machinery of duodenum in Duroc pigs showed greater adaptability to non-essential amino acid restriction when crude protein was reduced by two percentage units; in contrast, 3W pigs suffered a reduction in their duodenal FSR.

Second: Fractional incorporation rate and fractional unsaturation rate were considered valid indices of direct fatty acid deposition from the diet and *de novo* synthesis of fatty acids, respectively.

a) Among pig producing types, fat deposition of 3W lean pigs appeared to be more dependent on direct incorporation of dietary fatty acids, whereas Duroc fatty pigs seemed to have a higher rate of endogenous *de novo* synthesis.

b) Real *de novo* synthesis of oleic acid (d_{35}–C18:1 *c*9) from stearic acid (d_{35}–C18:0) desaturation was higher in pigs carrying the *SCD*_T allele of the *SCD* polymorphism (rs80912566).

c) The fractional incorporation rate of D-labeled stearic acid (d_{35}–C18:0) over time differed among tissues. While fatty acid incorporation in liver increased

with age, intramuscular fat showed the opposite effect, decreasing its direct deposition.

d) Oleic acid *de novo* synthesis (d_{35}–C18:1 c9) through Stearoyl CoA Desaturase (SCD) activity increased with pig maturity.

Third: Microbiota composition throughout the intestinal tract differed according to both growth phases and producing types of pig.

a) More favorable conditions in the distal intestinal segments resulted in a more abundant, diverse, stable, and interconnected microbial community.

b) The 3W crossbred pigs presented a more stable and robust microbiota throughout the intestinal tract. This producing type harbored higher diverse and less variable among individuals in comparison with Duroc pigs, as evidenced by higher alpha diversity indices (including Shannon and Simpson indices, microbial richness and evenness), as well as a more complex microbial network architecture. On the other hand, Duroc pigs seemed to be more inefficient, contributing to a higher nitrogen excretion through urine, and the emission of higher amount of methane and ammonia.

c) As the pigs matured, their microbiota acquired higher stability, due to a generalized increase in the richness of microbial communities, and the increased microbial network complexity.

d) A moderate reduction in the crude protein content of the diet allowed an adaptation of intestinal microbiota to a more complex network to maintain its normal physiology.

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ANNEX

animals

MDPI

Article

The Impact of Producing Type and Dietary Crude Protein on Animal Performances and Microbiota Together with Greenhouse Gases Emissions in Growing Pigs

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Simple Summary: To study the effect of dietary crude protein (CP) restriction in two different pig producing types and the role of gut microbiota, 32 pure castrated male Duroc and 32 entire male hybrid (F2) piglets were raised in a three-phase feeding regime with a restriction in CP content of the diets. The average body weight of hybrid animals were higher compared to Duroc pigs. No changes were found in average daily feed intake (ADFI) of hybrid animals in comparison to Duroc pigs. Hybrid animals apparently digested more CP than Duroc and Duroc pigs emitted more CH_4 and ammonia with respect to the hybrids. Dietary protein restriction did not alter emissions of contaminant gases nor microbial community structure in terms of diversity, although some genera were affected by the dietary challenge.

Abstract: In order to reduce dietary nitrogen and achieve an efficient protein deposition as well as decrease N wastage, we challenged the nutrient utilization efficiency of two different producing types in front of a dietary crude protein (CP) restriction and studied the role of the microbiota in such an adaptation process. Therefore, 32 pure castrated male Duroc (DU) and 32 entire male hybrid (F2) piglets were raised in a three-phase feeding regime. At each phase, two iso caloric diets differing in CP content, also known as normal protein (NP) and low protein (LP), were fed to the animals. LP diets had a fixed restriction (2%) in CP content in regards to NP ones throughout the phases of the experiment. At the end of third phase, fecal samples were collected for microbiota analysis purposes and greenhouse gases emissions, together with ammonia, were tested. No changes were found in average daily feed intake (ADFI) of animals of two producing types (Duroc vs. F2) or those consumed different experimental diets (NP vs. LP) throughout the course of study. However, at the end of each experimental phase the average body weight (BW) of hybrid animals were higher compared to Duroc pigs, whereas a reverse trend was observed for average daily gain (ADG), where Duroc pigs showed greater values with respect to hybrid ones. Despite, greater CH_4 and ammonia emissions in Duroc pigs with respect to F2, no significant differences were found in contaminant gases emissions between diets. Moreover, LP diets did not alter the microbial community structure, in terms of diversity, although some genera were affected by the dietary challenge. Results suggest that the impact of reducing 2% of CP content was limited for reduction in contaminant gases emissions and highlight the hypothesis that moderate change in the dietary protein levels can be overcome by long-term adaptation of the gut microbiota. Overall, the influence of the producing type on performance and digestive microbiota composition was more pronounced than the dietary effect. However, both producing types responded differently to CP restriction. The use of fecal microbiota

as biomarker for predicting feed efficiency has a great potential that should be completed with robust predictive models to achieve consistent and valid results.

Keywords: dietary crude protein; microbiota; greenhouse gases; growing pigs; total tract digestibility

1. Introduction

In the last decade, the global concern about environmental pollution has turned into a hot issue among the pundits. Like in other sectors, the livestock production sector has drawn much attention to greenhouse gas emissions as well as the wastage of unused nutrients through the manure into the soil and water. Nitrogen waste through manure, nitrous oxide and ammonia emissions during storage, and spreading of manure are positively related to nitrogen excretion in both feces and urine [1]. Increased public concern on the livestock environmental footprint led to EU legislation to regulate the potential quota of atmospheric pollution (Integrated Pollution Prevention and Control; IPPC Directive; Directive EU 2016/2284 [2] on the reduction of national emissions of certain atmospheric pollutants), where animal nutrition is considered as a key strategy. Therefore, optimizing nutrient efficiency is essential for the sustainability of swine production systems, especially in a context where the growing demand for food must be met at an affordable cost without compromising environmental integrity [3]. Several authors have already stated the benefits of reducing dietary crude protein (CP) in essential amino acids (EAAs) in balanced diets to growing–finishing pigs in order to decrease nitrogen waste through manure without compromising animal performance and feed efficiency [4–6]; however, scarce information is available over its impact on either toxic (NH_4) or greenhouse (CH_4 and N_2O) gas emissions (ranging potentially between 5% and 7%) potential [7,8]. Moreover, due to the high individual variability of animal performances, especially in specific productions other than the lean meat market, there is still great potential to improve efficiency in livestock production systems by better adapting nutrients supply to animals' requirements, either individually or in groups (breeds or producing types).

The gastrointestinal tract (GIT) of pigs harbors a very complex and dense microbial community that can be altered by diet—ileal digesta reaching the hindgut can modulate both microbial composition and activity, but on the other hand, symbiotic intestinal microbes play a key role in the host adaptation capability to dietary challenges [9]. Nowadays, interest in the existing links between the animal host and its intestinal microbiota has increased exponentially, especially with the objective of optimizing the digestion processes to find predictive biomarkers that lead to the improvement of precision feeding [10] and reduce the environmental impact.

Thus, the main objective in the present study was to explore the potential to reduce production of pollutant gases by improving CP quality, which was done by analyzing the microbial structure and function of the gut microbiota in two producing types of pigs.

2. Materials and Methods

All experimental procedures were approved by the Ethics Committee for Animal Experimentation of the University of Lleida (agreement CEEA 02-04/14) and were performed in accordance with authorization 7704 issued by the Catalan Ministry of Agriculture, Livestock, Fisheries, and Food, Spain. The care and use of animals were in accordance with the Spanish Policy for Animal Protection RD 53/2013, which meets the European Union Directive 2010/63 on the protection of animals used for experimental purposes.

2.1. Animals, Diets, Experimental Design, and Sampling Procedure

The study was conducted at the Swine Research Center of Catalonia, located in Torrelameu (CEP; Lleida, Spain). Thirty-two pure castrated male Duroc piglets (≈9 week old) with initial body

weight (BW) of 25.16 (SD 3.49) kg and 32 entire male hybrids (F2: progeny of [F1: Duroc × Landrace] dams × Pietrain sires) of the same age with initial BW of 22.63 (SD 1.72) kg were purchased from Selección Batallé® (Girona, Spain). Piglets of each genotype (32 each) were divided in 2 groups of 16, each group accommodated in one of four modules based on minimum BW variation, with each receiving differing CP content in their diets, as described below.

Animals of same genotype inside each module were accommodated in four pens (four pigs/pen) based on minimum BW variation to avoid any competition between the animals procuring the feed. Animals followed a three-phase feeding program (phase 1: 9 to 15, phase 2: 16 to 20, and phase 3: 21 to 25 week of age). Diets (described in Table 1) were formulated to be isoenergetic. They contained normal or restricted (a reduction in 2%) level of CP with regards to the recommendations of Fundación Española para el Desarrollo de la Nutrición Animal (FEDNA) [11] for pigs at each feeding phase. Moreover, in order to follow commercial conditions, essential amino acids (EAAs) supply was similar in both diets to avoid any bias that would compromise animal performance in any of both groups. Half amount of each experimental diet was thoroughly mixed with chromic oxide (Cr_2O_3) as an indigestibility marker at the rate of 900 mg/kg and given to half of the pens (8 pens in total, 4 per diet).

Table 1. Ingredients and additives (g/kg) of the three-phase experimental diets, differing in crude protein content (normal protein, NP vs. low protein, LP) for pigs of 9 to 25 weeks of age.

Ingredients	Feeding Phase [b]					
	I		II		III	
	LP [c]	NP [c]	LP	NP	LP	NP
Barley	181.6	144.4	380.0	120.0	216.7	150.0
Wheat	400.0	400.0	-	300.0	200.0	200.0
Triticale	54.8	32.1	-	100.0	-	121.1
Maize	100.0	100.0	273.9	98.4	250.0	117.1
Bakery byproducts	60.0	60.0	120.0	100.0	110.0	110.0
Rapeseed meal 00	-	-	-	100.0	36.7	100.0
Soybean meal 47% CP	71.0	133.1	118.3	79.6	11.3	23.6
Sunflower meal	-	-	-	-	60.0	56.7
Enersoy 3600	50.0	50.0	-	-	-	-
Rice b	-	-	36.4	50.0	60.0	60.0
Sugar beet pulp	30.0	30.0	8.0	-	-	-
Soybean oil	10.9	14.0	-	-	-	-
Blended animal fat 3/5 acidity	-	-	14.6	19.0	15.1	28.5
Calcium carbonate	12.1	12.0	13.0	10.1	10.7	9.9
Monocalcium phosphate	6.8	6.3	6.5	5.5	3.4	2.3
Sepiolite	-	-	6.1	-	5.0	5.0
Vitamin-mineral premix [a]	4.0	4.0	5.1	5.1	5.1	5.1
Sodium bicarbonate	-	-	3.2	2.2	2.6	0.5
Sodium chloride	3.8	3.8	4.1	2.1	2.0	2.9
L-lysine, CP 50%	10.0	7.1	6.7	5.9	8.4	6.1
DL-Methionine, 88%	2.3	1.6	1.7	0.7	0.7	0.1
L-Valine	-	-	-	-	0.3	-
L-Threonine	2.4	1.6	1.9	1.4	1.7	0.9
L-Tryptophan	0.3	0.2	0.4	0.1	0.3	0.1

[a] The vitamin and mineral premix composition is available in Morazán, et al. [12]. [b] Phases I, II, and III are 9 to 15, 16 to 21, and 22 to 25 weeks of age, respectively. [c] LP: diet with low crude protein content and NP: diet with normal crude protein content based on recommendation of FEDNA [11] for pigs of that age.

Individual variations in body weight of the animals along with pen feed consumption were recorded weekly and individual average daily gain (ADG) was calculated as the slope from linear regression of the body weight on feeding days; the individual average daily feed intake (ADFI) was estimated from the weekly pen consumption, considering the number of animals in each pen. In addition, the efficiency of animals to convert feed into body mass was expressed as gain:feed ratio.

At the last week of each feeding phase, those animals fed labeled (Cr_2O_3) diets underwent fecal spot sampling (≈50 g) by rectal stimulation at intervals of 8 h during 24 h. Representative fecal samples were stored at −20 °C until further digestibility analyses.

For microbiota analysis, an extra fecal sample (≈20 g) at the end of the third feeding phase (week 25 of age) was collected in a separate falcon tube (15 mL). These fecal samples were frozen instantly in liquid nitrogen, transferred to the laboratory, and stored at −80 °C.

Greenhouse gases (GHG; CO_2, N_2O, and CH_4), together with NH_3 emissions, were analyzed at the end of third experimental phase (week 25 of age) after the digestibility trial. A representative air sampling was obtained from the outside of the fattening installation and the midpoint of the exhaust air outlet in each module using the procedure suggested by Air Movement and Control Association (AMCA) [13] and following Seradj et al. [14]. Once we obtained the air samples inside the modules, simultaneous measurements of CO_2, NO_2, NH_3, and CH_4 concentration were analyzed using the photoacoustic technique (Innova 1312 Photoacoustic Multigas Monitor, Innova AirTech Instruments, Nærum, Denmark). Emissions were calculated after the correction of the outlet air volume to the standard temperature and pressure following Cao, et al. [15].

2.2. Laboratorial Analyses and Calculations

2.2.1. Chemical Composition

Feed samples were analyzed for their chemical composition following the procedures of Association of Official Analytical Chemists (AOAC) [16]. The DM content was determined using an oven at 60 °C for 48 h. The ash content was determined by incineration on muffle furnace at 550 °C for 4 h (ref. 942.05) to determine the organic matter (OM) content and crude protein (CP) was analyzed by the Kjeldahl method (ref. 976.05) and ether extract (EE) using Soxhlet extraction method with diethyl ether (ref. 920.39). The proportion of neutral detergent fiber (aNDFom) was determined according to Van Soest et al. [17] procedures, using α-amylase but not sulphites, and subtracting ashes from the residue, while the acid detergent fiber (ADF) and lignin (ADL) were determined by the method of Goering et al. [18].

2.2.2. Chromium Detection

Fecal spot samples were thawed at 4 °C overnight and gently homogenized inside their collection tube; then, samples (20 g) from the same animal collected at different intervals of a day at each experimental phase were pooled together to make one grab sample per animal per day. Chromium (introduced as chromic oxide to the diets) as an external marker was detected in collected feed and fecal samples after digestion with nitro-perchloric acid (5:1) using de Vega and Poppi [19] methodology, coupled with inductively coupled plasma optical emission spectroscopy (HORIBA Jobin Yvon, Activa family, Kyoto, Japan).

The coefficient of total track apparent digestibility CTTAD of nutrients was calculated using nutrient to marker ratio in the feed and fecal samples as follows:

$$\mathrm{CTTAD} = 1 - \left(\frac{[\mathrm{Cr}]_{\mathrm{intake}}}{[\mathrm{Cr}]_{\mathrm{excreted}}} \times \frac{\mathrm{X}_{\mathrm{excreted}}}{\mathrm{X}_{\mathrm{intake}}} \right) \quad (1)$$

where X_{excreted} and X_{intake} are the nutrient concentration (g/kg) in feces and in the feed, respectively, $[Cr]_{\text{excreted}}$ and $[Cr]_{\text{intake}}$ are the concentration (ppm) of chromium in the feces and the feed, respectively.

2.2.3. DNA Extraction and NGS

Fecal samples kept at −80 °C were freeze-dried and the DNA was extracted using a QIAamp DNA Stool Mini Kit (Qiagen Ltd., West Sussex, UK) following the manufacturer's instructions. The yield and purity of extracted DNA was assessed using a Nanodrop™ (Thermo Scientific, NanoDrop 2000, Waltham, MA, USA), by measuring the absorbance intensity at 260 nm and the absorbance ratio 260/280,

respectively. Libraries were prepared using V3-V4 amplicons from the 16s rRNA gene. Sequencing was performed with Illumina Miseq (Illumina, Hayward, CA, USA), generating 902131 paired-end reads. Sequence data were analyzed following the UPARSE pipeline [20]. Taxonomic assignment of the OTUs (operational taxonomic unit) was done using the MG7 program developed by Era7 Bioinformatics [21], which uses cloud computing for the parallel massive basic local alignment search tool (BLAST) similarity analysis to infer both function and taxonomic assignment. MG7 taxonomic assignment was done based on best blast hit (BBH) obtained after searching the nt database (NCBI).

2.3. Statistical Analysis

The statistical analysis of the data was commensurate to the design of the study (completely randomized design; CRD) considering pen as the experimental unit. Data were analyzed with SAS (v 9.4; SAS Institute, Cary, NC, USA) using MIXED model procedure, assuming normal distribution of the data.

Producing parameters (BW, FI, ADG, and Gain:Feed) and coefficient of total tract of apparent digestibility of nutrients (DM, CP, and NDF) data were analyzed as follows:

$$Y_{ijklmno} = \mu + Ph_i + PT_j + Di_k + (Ph \times PT)_l + (Ph \times Di)_m + (PT \times Di)_n + \varepsilon_{ijklmn} \quad (2)$$

where Y is the dependent variable, μ is the mean value, Ph_i is the experimental phase (I, II, and III), PT_j is the producing type (Duroc and F2), Di_k is the diet (LP and NP) along with their possible interactions, and ε_{ijklmn} is the error.

GHG data together with the NH_3 emissions at the end of the third phase of study were analyzed as follows:

$$Y_{ijkl} = \mu + PT_i + Di_j + (PT \times Di)_k + \varepsilon_{ijk} \quad (3)$$

where Y is the dependent variable, μ is the mean value, PT_i is the producing type (Duroc and F2), Di_j is the diet (LP and NP) along with their possible interaction, and ε_{ijk} is the error.

Tukey multiple comparison test was applied and significant differences and tendencies were declared at $p \leq 0.05$ and $0.05 < p \leq 0.10$, respectively.

Biodiversity indices (Shannon Wiener [22], Simpson [23], and Richness [24]), Venn diagrams, Spearman correlations between biodiversity and standardized residual values from performance traits, as well as multivariate analysis (CCA, SPLS-DA) were conducted using packages "vegan", "ade4", and "mixOmics" from R (v.3.2; R Core Team, Auckland, New Zealand).

3. Results

3.1. Diet Composition

During the study, a phase feeding strategy was applied dividing the growing–finishing phase into three phases of five weeks (on average) and diets (LP and NP) were formulated based on recommendations published in FEDNA (2013) for animals of different ages. Normal and low CP diets were identical in covering the nutritional requirements of animals, except for CP where low CP diet was intentionally designed with less (≈2%) CP content compared to the normal one.

Table 1 shows the ingredients used in diets and Table 2 reveals the calculated and analyzed composition of each experimental diet. Diets were mainly composed of cereals, where soybean meal was the sole source of protein for the piglets at the initial phase (weeks 9 to 15 of age) and was partially replaced by rapeseed meal during the second and third phase of study. Sugar beet pulp was added (30 g/ kg) to cover the fiber needs mainly in initial diets. Table 2 confirms that the diets of each phase totally covered the need of standardized ileal digestible (SID) of each essential amino acid without any egregious difference in between.

Table 2. Energy and nutrients composition of the experimental diets, differing in CP content (normal, NP vs. low, LP) for pigs of 9 to 25 weeks of age.

SNutrients	Feeding Phase [d]					
	I		II		III	
	LP [c]	NP [c]	LP	NP	LP	NP
Calculated Values [a]						
ME (MJ/kg)	13.3	13.5	13.0	13.2	13.0	13.2
SID Lysine	10.2	10.1	8.4	8.4	7.6	7.6
SID Lysine/ME (g/MJ)	0.8	0.8	0.6	0.6	0.6	0.6
SID Methionine	4.0	3.7	3.4	2.8	2.5	2.3
SID Methionine + cysteine	6.4	6.3	5.5	5.5	4.6	4.8
SID Threonine	6.5	6.5	5.8	5.8	4.9	4.9
SID Tryptophan	1.8	1.9	1.7	1.7	1.5	1.5
SID Isoleucine	4.9	5.9	4.5	5.0	3.8	4.5
SID Valine	5.7	6.7	5.4	6.0	5.0	5.5
Analyzed Values (g/kg) [b]						
DM	889.5	892.5	875.8	877.4	879.3	881.5
CP	153.0	173.0	140.0	155.0	126.0	147.0
CF	35.0	36.0	36.0	39.0	46.0	54.0
aNDFom	130.0	120.0	135.0	138.0	141.0	154.0
ADFom	48.0	49.0	45.0	61.0	60.0	70.0
AEE	49.0	57.0	52.0	59.0	46.0	66.0
Starch	432.0	392.0	424.0	406.0	443.0	407.0
OM	929.0	901.0	928.3	930.6	939.3	940.4
p	4.6	4.0	4.6	5.1	4.6	5.1
k	6.0	7.0	7.0	7.0	6.0	7.0

[a] ME, metabolizable energy; SID, standardized ileal digestible amino acid calculated according to FEDNA [11]. [b] DM, dry matter; CP, crude protein; aNDFom, neutral detergent fiber expressed exclusive of residual ash; ADFom, acid detergent fiber expressed exclusive of residual ash; AEE, acid hydrolyzed ether extract; OM, organic matter; p, phosphorous; k, potassium. [c] LP, diet with low crude protein content, and NP, diet with normal crude protein content, based on recommendation of FEDNA [11] for pigs of that age. [d] Phases I, II, and III are 9 to 15, 16 to 21, and 22 to 25 weeks of age, respectively.

3.2. Performance Parameters

A summary of performance parameters is provided in Table 3. As it was conceived, the effect of experimental phase was significant ($p < 0.01$) on all the studied parameters related with performance. No changes were found in ADFI of animals of two producing types (Duroc vs F2) or those that consumed different experimental diets (NP vs. LP) throughout the course of study ($p > 0.05$). In each phase, animals of two producing types were, on average, fed same amount of feed on the daily basis ($p > 0.05$); however, during the last phase, LP diet was consumed more than NP (3.2 vs 2.9 kg/day; $p = 0.03$).

At the entry of animals to the fattening facilities (9 weeks old) the piglets were distributed based on minimum BW variation to yield iso-weighed pens considering producing type (Duroc and F2) and diet (LP or NP). The data provided for the initial BW (Table 3) show the least variation between genotypes (23.3 vs. 24.5 SEM 1.17; Duroc and F2, respectively) and diets (23.9 vs 23.9 SEM 1.14; LP and NP, respectively), which did not differ statistically ($p = 1.0$ for genotype and diet at the initial BW). However, at the end of each experimental phase, the average BW of F2 animals (52.9, 80.5, and 103.8; at the end of P1, P2 and P3, respectively) were higher ($p < 0.05$) compared to Duroc pigs (49.9, 73.6, and 96.5; at the end of P1, P2, and P3, respectively).

Composition of diet (LP or NP) did not influence the BW throughout the feeding phases ($p > 0.05$). Thus, no variations in BW were found between animals fed different diets.

F2 animals showed numerically higher ADG with respect to Duroc ones in all the feeding phases, although differences did only reach statistical significance during the second (weeks 15 to 18 of age) phase (0.99 vs. 0.84 $p < 0.01$; F2 vs. Duroc, respectively). Variations in performance led to a higher

overall ADG in animals of F2 with respect to Duroc (0.85 vs. 0.76 for F2 and Duroc pigs, respectively, $p < 0.01$). Protein level in the diet did not influence the ADG of the animals throughout the experiment ($p > 0.05$). Neither producing type of the animals (Duroc and F2) nor CP content of the diet (LP and NP) influenced the efficiency of animals to convert feed into body mass expressed as gain:feed ratio ($p > 0.05$). No interactions were found between producing type of the animal and diet composition (PT × Di) in performance parameters measured during the experiment ($p > 0.05$).

Table 3. Growth performance (BW, ADG, ADFI, gain:feed) in growing–finishing pigs as affected by dietary CP (normal, NP vs. low, LP) for pigs of 9 to 25 weeks of age.

Parameters [a]	Genotype		SEM	Diet [b]		SEM	*p*-Value [c]			
	Duroc	F2		LP	NP		PT	Di	Ph × PT	Ph × Di
ADFI (Phase I), kg/day	1.3	1.4	0.66	1.4	1.4	0.07	0.92	0.23	0.22	0.01
ADFI (Phase II), kg/day	2.3	2.6	0.66	2.4	2.5	0.07				
ADFI (Phase III), kg/day	3.0	3.0	0.66	3.2 [e]	2.9 [f]	0.07				
Overall ADFI, kg/day	2.2	2.4	0.66	2.3	2.2	0.04				
Initial BW (at 9 weeks of age), kg	23.3	24.5	1.17	23.9	23.9	1.14	<0.01	0.70	<0.01	0.08
BW (end of Phase I, 15 weeks of age), kg	49.9 [b]	52.9 [a]	0.87	51.6	51.1	0.83				
BW (end of Phase II, 21 weeks of age), kg	73.6 [b]	80.5 [a]	1.06	76.0	78.1	1.02				
Final BW (end of Phase III, 25 weeks of age), kg	96.5 [b]	103.8 [a]	1.55	100.0	100.4	1.53				
ADG (Phase I), kg/day	0.65	0.70	0.023	0.69	0.67	0.023	0.01	0.74	0.03	0.16
ADG (Phase II), kg/day	0.84 [b]	0.99 [a]	0.025	0.90	0.93	0.024				
ADG (Phase III), kg/day	0.78	0.86	0.042	0.84	0.80	0.042				
Overall ADG, kg/d	0.76 [b]	0.85 [a]	0.023	0.81	0.80	0.022				
Gain:feed (Phase I), g/g	0.39	0.58	0.120	0.49	0.48	0.018	0.57	0.88	0.06	0.37
Gain:feed (Phase II), g/g	0.22	0.34	0.119	0.28	0.28	0.009				
Gain:feed (Phase III), g/g	0.16	0.26	0.120	0.20	0.22	0.010				
Overall gain:feed, g/g	0.26	0.39	0.119	0.32	0.33	0.008				

[a] ADFI, average dairy feed intake; BW, body weight; ADG, average dairy gain; Phases I, II, and III are 9 to 15, 16 to 21, and 22 to 25 weeks of age, respectively.[b] LP, diet with low crude protein content and NP, diet with normal crude protein content based on recommendation of FEDNA [11] for pigs of that age. [c] Ph, phase of study; PT, producing type; Di, diet. Different upper case superscripts (a, b) and (e, f) within rows denote differences among producing types and diets, respectively ($p < 0.05$). No interaction ($p > 0.05$) was found between the producing type and the diet (PT × Di).

3.3. Coefficient of Total Tract Apparent Digestibility (CTTAD) of the Nutrients

Results from CTTAD determination are showed in Table 4. Dry matter digestibility during the third phase of the study (week 22 to 25 of age) was lower, compared with the other two phases (0.934 vs 0.945 and 0.948 $p < 0.01$; P3 vs P2 and P1, respectively). No differences were found in total tract digestibility of DM between Duroc and F2 or between animals fed NP or LP diets ($p > 0.05$). As the CP content recommended for each feeding phase reduced gradually in diets from first to third phase, the coefficient of total tract digestibility of CP decreased accordingly (0.736, 0.701, and 0.683 for P1, P2, and P3, respectively, $p < 0.01$). Besides, in overall, animals of F2 genotype apparently digested more CP than Duroc (0.724 vs 0.689 for F2 vs Duroc, respectively, $p < 0.05$), where no effect of diet was observed for the total tract digestibility of CP. The apparent digestibility of NDF was similar to that of CP and decreased ($p < 0.01$) along the phases of the experiment up to 21.6% at the third phase (0.304 and 0.286 for P1 and P2, respectively). No effects of producing type or CP content of the diet were observed in digestibility of NDF content ($p > 0.05$). Moreover, no interactions between main factors were found in CTTADs (DM, CP, and NDF) measured during the experiment ($p > 0.05$).

Table 4. Coefficient of total tract apparent digestibility (CTTAD) of growing–finishing pigs as affected by CP (normal, NP vs. low, LP) from 9 to 22 weeks of age.

CTTAD [a]	Phase of Study [b]			SEM	PT		Diet [c]		SEM	*p*-Value [d]		
	I	II	III		Duroc	F2	LP	NP		Ph	PT	Di
DM	0.948 [a]	0.945 [a]	0.934 [b]	0.0020	0.944	0.940	0.944	0.941	0.0018	0.01	0.10	0.10
CP	0.736 [a]	0.701 [b]	0.683 [c]	0.0117	0.689 [b]	0.724 [a]	0.703	0.710	0.0095	0.01	0.01	0.60
NDF	0.304 [a]	0.286 [b]	0.216 [c]	0.0164	0.273	0.264	0.281	0.257	0.0134	0.01	0.70	0.20

[a] CTTAD, coefficient of total tract apparent digestibility of DM, dry matter; CP, crude protein; NDF, neutral detergent fiber.[b] Phases I, II, and III are 9 to 15, 16 to 21, and 22 to 25 weeks of age, respectively.[c] LP, diet with low crude protein content and NP, diet with normal crude protein content based on recommendation of FEDNA [11] for pigs of that age. [d] Ph, phase of study; PT, producing type; Di, diet. Different upper case superscripts (a, b, c) within rows denote differences among phases of the study and genotype of animals, respectively ($p < 0.05$). No interactions ($p > 0.05$) were found between the producing type and the diet (PT × Di), the phase of study and the producing type (Ph × PT), or the phase of study and the diet (Ph × Di).

3.4. Gas Emissions

During the last (week 25 of age; ≈100 kg BW) of the experiment, the emissions (expressed as g per animal per day) of greenhouse gases (CH_4, CO_2, and NO_2) and ammonia (NH_3), were monitored and the results are tabulated in Table 5. Duroc pigs significantly ($p < 0.01$) emitted (g animal/day) more CH_4 (10.7 vs 5.6) and ammonia (3.3 vs 0.5) than F2 animals ($p < 0.05$). No differences were observed in NO_2 and CO_2 emissions either between diets or between producing types ($p > 0.05$).

Table 5. The impacts of crude protein content (normal, NP vs. low, LP) on emission of greenhouse gases and ammonia (g/animal/day) at the end of growing–finishing period (ca. 100 kg BW, week 25 of age).

Emission (g/animal/day)	PT		Diet [a]		SEM	*p*-Value [b]	
	Duroc	F2	LP	NP		PT	Di
Methane	10.7 [a]	5.6 [b]	7.6	8.7	0.53	0.01	0.2
Carbon Dioxide	1496.8	1441.8	1619	1319.6	122.28	0.8	0.2
Nitrous Oxide	0.17	0.09	0.14	0.12	0.026	0.1	0.7
Ammonia	3.3 [a]	0.5 [b]	1.7	2.1	0.40	0.01	0.5

[a] LP, diet with low crude protein content and NP, diet with normal crude protein content based on recommendation of FEDNA [11] for pigs of that age.[b] PT, producing type; Di, diet. Different upper case superscripts (a, b) within rows denote differences among genotype of animals ($p < 0.05$). No interaction ($p > 0.05$) was found between the producing type and the diet (PT × Di).

3.5. Fecal Microbial Characterization

A total number of 7,155,810 reads were obtained, with an average of 119,263 reads per sample. Good's coverage index resulted in an average of 99.86%, indicating that most of the microbial population present in the samples was covered by the analysis.

All four groups (F2 and Duroc with two level of protein in the diet) shared the 64.13% of the OTUs present in the analysis (see Figure 1) and only an average of 0.54% of the OTUs was specific in each group. Duroc animals showed numerically less specific OTUs than F2 (2.79 vs 3.88%), as well as the animals fed LP diets compared to NP diets (2.59 vs 3.47%). In general, the shared proportion of OTUs was high (96.12, 97.21, 97.41, and 96.53% in Duroc, F2, NP, and LP diets, respectively), indicating a very stable core population in the feces from finishing pigs.

Richness index of diversity showed significant differences between genotypes and diets, with diversity being higher in NP diets and F2 animals (Table 6; $p < 0.05$ in both cases).

Table 6. Effect of either producing type or diet on the microbial diversity.

Indices	PT		Diet [a]		SEM	*p*-Value [b]	
	Duroc	F2	NP	LP		PT	Di
Shannon	3.134	3.178	3.139	3.174	0.0238	0.19	0.36
Simpson	0.894	0.889	0.889	0.894	0.0029	0.18	0.21
Richness	542.4	579.5	575.6	546.2	11.28	0.02	0.07

[a] LP: diet with low crude protein content and NP: diet with normal crude protein content based on recommendation of FEDNA [11] for pigs of that age. [b] PT, producing type; Di, diet.

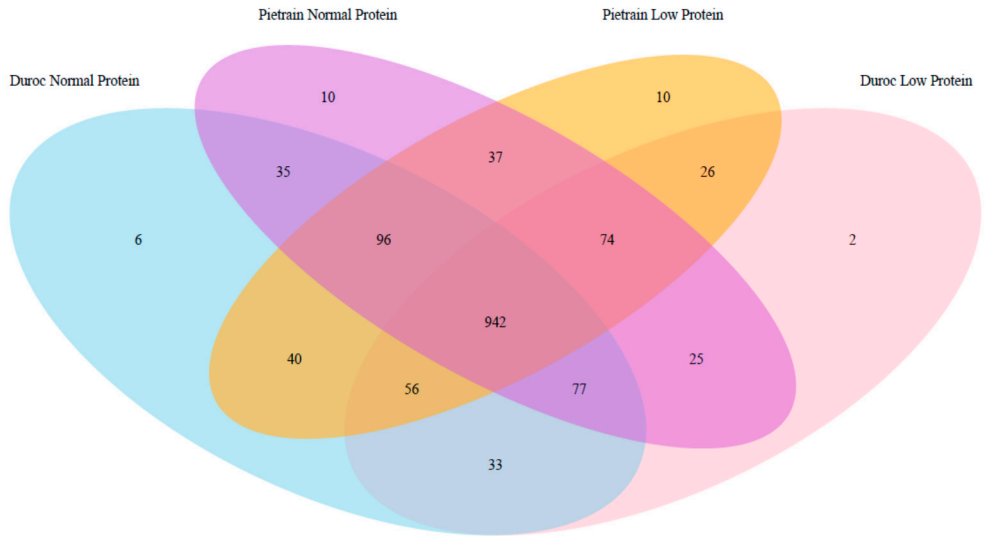

Figure 1. Venn diagram showing the number of shared OTUs (operational taxonomic unit) between animals belonging to Duroc or F2 (Pietrain) producing types and fed with either normal protein (NP) or low protein (LP) diets.

Reads belonging to the kingdom Archaea accounted for an average of 0.15% of the total number of sequenced reads and no differences were found in the relative abundance of archaea between either producing types or diets ($p > 0.05$).

Nineteen different phyla were identified within the kingdom Bacteria, although only nine phyla (Actinobacteria, Bacteroidetes, Fibrobacteres, Firmicutes, Fusobacteria, Proteobacteria, Spirochaetes, Tenericutes, and Verrucomicrobia) presented mean abundances above 0.1%. Firmicutes was the most abundant phyla in both genotypes (72.4 ± 6.13% in Duroc and 73.5 ± 4.81% in F2), followed by Bacteroidetes and Proteobacteria. Among main phyla, a genotype effect was found on Bacteroidetes, Proteobacteria, and Verrucomicrobia phyla (Figure 2), with Duroc having higher abundances in the former phylum and lower in rest.

Diet did not affect the microbial composition of the animals at phyla level ($p > 0.05$), but in NP diets, Duroc animals presented a lower proportion of Firmicutes than F2 (interaction effect, $p < 0.05$). Hence, the Bacteroidetes:Firmicutes ratio tended to be lower in Duroc animals ($p = 0.08$, Supplementary Materials Figure S1).

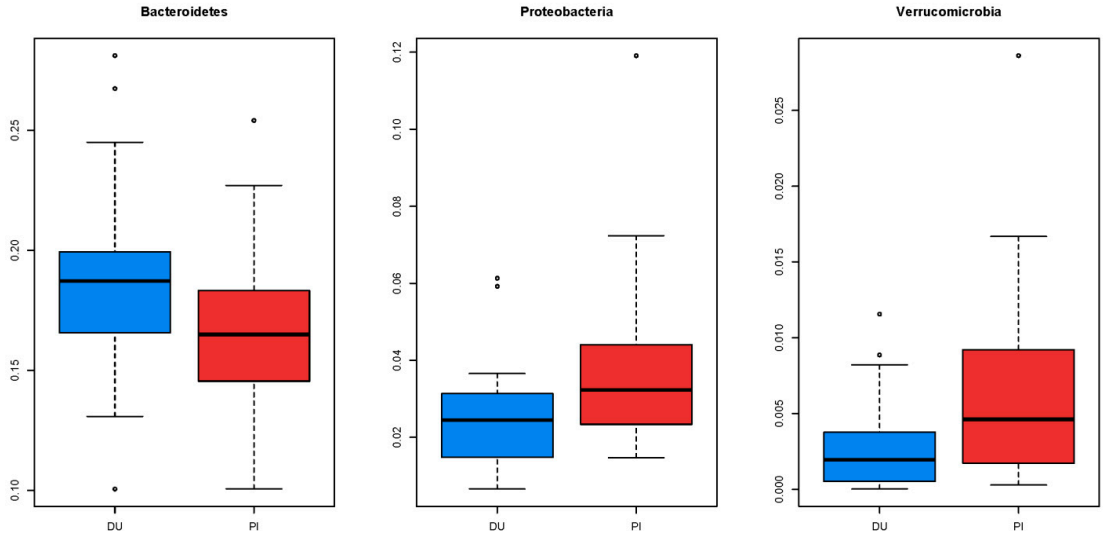

Figure 2. Boxplot of mean abundances of phyla affected by the producing type (DU = Duroc; PI = F2; $p < 0.05$).

A description of the main families identified within the four most abundant phyla is shown in Figures S2 and S3. Duroc animals showed higher abundances in families *Atopobiaceae* ($p = 0.007$) and *Coriobacteriaceae* ($p = 0.004$) from phylum Actinobacteria, family *Prevotellaceae* ($p = 0.049$) from phylum Bacteroidetes, and family *Lachnospiraceae* ($p = 0.003$) from phylum Firmicutes. On the other hand, F2 animals showed higher abundances in family *Eggerthellaceae* ($p < 0.001$) from phylum Actinobacteria; families *Paludibacteraceae* ($p = 0.006$), *Porphyromonadaceae* ($p < 0.001$), and *Tannerellaceae* ($p < 0.001$) from phylum Bacteroidetes; families *Clostridiaceae* ($p = 0.003$) and *Peptococcaceae* ($p = 0.001$) from phylum Firmicutes; and family *Succinivibrionaceae* ($p = 0.036$) from phylum Proteobacteria (Figure S2).

Diet only affected family *Geobacteraceae* ($p = 0.001$) from phylum Proteobacteria, having higher abundances in animals fed NP diets than those fed LP diets. Interaction effects were present in families *Clostridiales Family XIII, Incertae Sedis* ($p = 0.038$, NP > LP in Duroc animals), and *Eubacteriaceae* ($p = 0.036$, F2 > Duroc in LP diets) from phylum Firmicutes and family *Geobacteraceae* ($p = 0.019$, Duroc animals fed NP diets were higher than the rest of animals) from phylum Proteobacteria (Figure S3).

SPLS-DA analysis enabled the selection of the most predictive or discriminative taxons in the data that helped to classify the samples according to either diet or genotype effect (see Figure S4); from the 10 most predictive taxons, only those with relative abundances higher than 0.01% were considered. In this scenario, five taxons were found to be the most responsible of the differences between genotypes (two varieties of *Holdemanella biformis*, uncultured Coriobacteriales bacterium, uncultured Bacteroidetes bacterium, and uncultured *Prevotellaceae* bacterium) and two related with differences between diets (*Blautia sp.* canine oral taxon 143 and *Selenomonas bovis*).

We compared all the detected phyla and the most abundant bacterial genera (>0.1%, N = 34), with those producing and digestive efficiency parameters studied in the trial (Table 7) using Spearman correlation rank between performance parameters and abundant genera. Only significant correlation values ($p < 0.05$, $r > |0.58|$) were considered. In Duroc animals, up to nine different genera and two phyla either positively or negatively were correlated with performance (BW, ADG, or digestibility), meanwhile in F2 animals, this correlation was only found in two genera and one phylum. LP diets also presented more correlations between microbiota and performance (five genera and two phyla) than in NP diets (three genera and one phylum).

Table 7. Spearman rank correlations between standardized residual values and microbiota ($p < 0.05$, r > |0.58|). Positive and negative correlations are highlighted in green and red, respectively.

Phyla / Genus	ADG 110d	ADG 150d	ADG 70d	CPd std 130d	DMd std 130d	NDFd std 130d	BW std 110d	BW std 150d
Actinobacteria				Duroc				
Bacteroidetes						Duroc		
Deferribacteres	LP							LP
Fibrobacteres				F2/LP		LP		
Proteobacteria					NP			
Collinsella	LP							
Coriobacterium				LP			LP	
Faecalibacterium		NP				Duroc		
Fibrobacter				F2/LP		LP		
Geobacter				F2				
Holdemanella					Duroc			
Lactobacillus			Duroc					
Mitsuokella		NP				Duroc		
Oribacterium		NP						
Parabacteroides						Duroc		
Prevotella				LP				
Roseburia				Duroc		Duroc		
Ruminococcus					Duroc			
Selenomonas	LP							
F: B ratio						Duroc		
P: (F+B) ratio					NP			

ADG: average daily gain; CPd: crude protein digestibility; DMd: dry matter digestibility; NDFd: neutral detergent fiber digestibility; BW: body weight.

4. Discussion

4.1. Effect of Diet

Concern about the environmental pollution caused by the livestock sector has arisen exponentially and this concern involves greenhouse gases (mainly methane and carbon dioxide) emitted and nutrients wasted through the manure on the soil and water. In the present study, we tried to analyze the impact of a dietary challenge as CP reduction in EEAA-balanced diets on both fecal microbiota and environmental impact through pollutant gas emissions. Although we are aware of the use of balance studies to approach this issue, our main aim was to investigate the role of gut microbiota when adapting to CP restriction and their potential impact on the animal host.

Experimental animals were kept under commercial conditions and they were raised in a three-phase feeding regime with the 2% restriction in LP diets that were fixed through the experiment. In agreement with the existing literature [25–28], no differences were observed in performance between diets (Table 3), and the pigs were able to adjust their metabolism without compromising growth rate. In order to avoid confounding factors, we decided to keep the energy:Lys ratio constant, since an excess in energy in the diet can lead to an increase in the fatness, and thus affect both metabolism and performance [29]. No significant differences were found in contaminant gases emissions (see Table 5) between diets, suggesting that the impact of reducing 2% of CP content was limited. Osada et al. [5] found a reduction in 39% of GHG emissions when reducing a 2.5% CP content in the feed, but animals under their study were growing (38 kg of mean BW, compared with 100 kg of mean BW in our study), and hence more affected by changes in CP content. Lack of response in our study may suggest that our animals already arrived to a steady state in N deposition, although it should be confirmed by N balance studies.

LP diets did not alter the microbial community structure, in terms of diversity, although some genera were affected by the dietary challenge. These results are in agreement with a recent study [30], and suggest the hypothesis that moderate change in the dietary protein levels (2% in our case) can be overcome by at long adaptation of the gut microbiota. However, results do not allow us to get a once for all conclusion in this sense.

More than 95% of total sequences were shared across the animals used in this trial, which suggests a highly predominant and stable core population. SPLS-DA analysis showed *Blautia sp.* canine oral taxon 143 and *Selenomonas bovis* as the most influential taxa in the discrimination by diet. *Blautia* is a genus in the bacterial family Lachnospiraceae that phylogenetic analysis places within the Clostridium coccoides group, also referred to as the Clostridium Cluster XIVa [31]. The common feature of acetogenic

Blautia spp. may be to become a sink for reductive capability (H^+) and so alternative pathway to methane synthesis [32]. *Selenomonas spp.* is genus from phyla Firmicutes with a great activity as lactate producer; in both cases, the high proportion of cereals in the LP diet could have influence the proliferation of these types of bacteria.

4.2. Effect of Genotype

The authors are aware of the effect of castration on both parameters and the close interaction of breed and sex. However, in the real situation, breeds used to produce commercial male fatty pigs, are submitted to castration, whereas meat hybrids are not. Entire crossbreed animals are focused to produce low-price lean meat, whereas castrated fatty pigs (such as pure Duroc) are used to produce cured products with some specific features such as higher levels of intramuscular fat and precocity. The two extreme producing types allow us to study the animal's resilience in a nutritional change (i.e., dietary treatments) considering the key role of gut microbiota in such adaption processes. Effort has been done to study microbes' interspecies interactions rather than titers and/or properties of singular microbes, considering that microbiota interactions may explain relevant aspects of gut functioning in swine, as has been demonstrated recently in human intestine [33,34].

In this study, F2 animals had both higher body weight at slaughter (103.8 vs 96.5, $p < 0.01$) and ADG (0.85 vs 0.76, $p = 0.01$), although gain:feed ratio did not statistically differ between producing types ($p = 0.57$). Compared to other studies with a similar design [30], we could observe a consistency in the observed performance values for F2 animals.

In terms of microbiota profile and among all the diversity indices analyzed, Richness was the only one in which significant differences were found, where F2 animals had a higher number of OTUs in relation to Duroc animals ($p < 0.05$, Table 6). In general, it has been suggested that an increase in microbial diversity is positive in terms of resistance and resilience to dysbiosis and potential pathogens [35]; however, microbial community composition alone does not necessarily provide understanding of community function, since a large degree of functional redundancy exists in the gut microbial ecosystem [36]. Moreover, results depicted in Figure 1 suggest that apart from having differences in diversity, both groups shared a large number of OTUs, indicating a very stable core of microbial population. In a recent study, Holman et al. [37] found a shared proportion among at least 90% of GI samples regardless of experimental variables, including *Clostridium, Blautia, Lactobacillus, Prevotella, Ruminococcus*, and *Roseburia*, which all were found in our study. These genera represent bacteria that are well adapted to the swine gut and may serve as potential markers of a typical swine gut microbiota. No differences were found between producing types when archaea abundance was analyzed (0.12 vs 0.18 % in Duroc and F2 respectively, $p < 0.05$); however, the enteric methane emission was almost 2-fold in Duroc animals compared with F2 (Table 5, 10.7 vs 5.6 g/an/day, $p = 0.01$). Such results are in agreement with the previous ones that indicate the methanogens abundance is not fully correlated with methane production, where certain key species may be responsible of a substantial part of the overall emission [38,39]; however, some influence of the differential size of the hindgut in both productive types cannot be discarded.

Duroc animals presented a lower proportion of *Firmicutes* than F2 in NP diets (interaction effect, $p < 0.05$). Hence, the Firmicutes: Bacteroidetes (F:B) ratio tended to be lower in Duroc animals ($p = 0.08$, Figure S1). Balance between Firmicutes and Bacteroidetes has been recently investigated as an indirect measure of obesity in monogastric animals, including pigs [40]. Distal gut microbiota of obese and lean mice, as well as obese and lean humans, have been compared and the results revealed a statistically significant reduction in the relative abundance of Bacteroidetes and a significant greater proportion of Firmicutes in obese groups than in lean controls [41,42]. Guo et al. [40], also found a significant inverse correlation between body weight and Bacteroidetes abundance. Duroc animals are supposed to have a more active fat metabolism, compared with F2 animals, which are representative of lean pigs. However, to our best knowledge, there is no information regarding F:B ratios in different genotypes. Taking into account that obesity can be considered as a metabolic disease [43], it can be

hypothesized that this ratio is only altered in unbalanced situations and not in animals with a more active fat metabolism, that in fact is developed after the nutrients' absorption [44]. Moreover, it has been described in the literature than in other monogastric animals, such as humans, the age plays an important role in the composition of the gut microbiota. In fact, old age individuals are related with an increase in the abundance of Bacteroidetes [45]. Pure bred Durocs are known to arrive at maturity earlier than commercial crossbreeds [46], and thus, it could be expected a higher contribution of Bacteroidetes in those animals.

4.3. Use of Fecal Microbiota as Biomarker for Feed Efficiency

In order to investigate whether the gut microbiota could be related with any of the studied efficiency parameters, we standardized the main performance data (ADG, BW, as well as CP, NDF, and DM digestibility) following a residuals linear regression, including factors such as producing type and initial weight of the animals (see Table 7). In this scenario, we wanted to explore the individual response of the pigs and to see that the differential effect was due to the diet or the producing type. Some recent studies have been trying to get some insight on the links between feed efficiency and gut microbiota in pigs [47–49], but the results were inconclusive and more information must be gathered from different breeds and conditions to have an overall overview on the adequacy of using microbiota as biomarker for predicting feed efficiency in pigs.

In the present study, up to 14 genera and five phyla were found to be linked to any of the performance parameters analyzed (Table 7). Fibrobacteres showed positive correlation with efficiency of CP utilization in LP diets and F2 pigs and negative correlation with NDF utilization in LP diets. Metzler-Zebeli et al. [47] found higher abundance of this phyla in highly efficient animals. Although their results were not significant, it can be speculated that a better utilization of CP (especially in diets with CP limitation, e.g., LP diet, and in breeds with higher growth potential, e.g., F2 crossbreed) can be related to an increase in this bacterial phyla. Deferribacteres was positively associated with both ADG (at 110 days) and BW (at 150 days) in LP diets, suggesting a microbial adaptation when CP is limiting. Serino et al. [50], reported changes in Deferribacteres as part of adaptation to a high-fat diet. It can be hypothesized that limiting CP content can unpair the N requirements for the gut microbiota and promote certain groups more involved in N utilization, like Deferribacteres.

Genera including *Prevotella* (negative correlation with CP digestibility in LP diets), *Lactobacillus* (negative correlation with ADG in Duroc animals), and *Ruminococcus* (positive correlation with DM digestibility in Duroc animals) correlated with the performance parameters. Generally, data from Table 7 showed a great influence of some specific bacterial genera over NDF digestibility in Duroc animals (up to four genera, one phyla, and the F:B ratio) and over CP digestibility in LP diets (three genera and one phyla). Other associations should be further investigated.

5. Conclusions

To conclude, the influence of the producing type on pollutant gas emissions and fecal microbiota composition was more pronounced than the dietary CP quality effect, which lacked major significant influence. Both producing types appeared to respond differently to CP restriction and quality in terms of microbial composition and gas emissions, but further research should be conducted to confirm these preliminary results. The use of fecal microbiota as biomarker for predicting feed efficiency has a great potential, although it should be completed with robust predictive models.

Supplementary Materials: The following are available online at http://www.mdpi.com/2076-2615/10/10/1742/s1, Figure S1: Bacteroidetes to Firmicutes ratio in animals belonged to either Duroc (DU) or F2 (PI) producing type; Figure S2: Distribution of families within the main phyla (Actinobacteria in blue, Firmicutes in green, Bacteroidetes in red, and Proteobacteria in violet) presented in the fecal microbiota of the animals under the study, according to the producing type; Figure S3: Distribution of families within the main phyla (Actinobacteria in blue, Firmicutes in green, Bacteroidetes in red, and Proteobacteria in violet) presented in the fecal microbiota of the animals under the study, according to the level of protein in the diet; Figure S4: Main discriminative taxons found in the sPLS-DA, with abundance higher than 0.1% (T_1735 = *[Eubacterium] biforme*; T_518637

= *Eubacterium biforme* DSM 3989; T_349920 = uncultured Coriobacteriales bacterium; T_152509 = uncultured Bacteroidetes bacterium; T_370804 = uncultured *Prevotellaceae* bacterium; T_1151491 = *Blautia sp.* canine oral taxon 143; T_416586 = *Selenomonas bovis*).

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