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Genome-wide association study for intramuscular fatty acid composition in divergently selected rabbit lines

Master thesis

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Abstract

Intramuscular fat (IMF) content and composition are key traits determining meat quality. A divergent selection experiment for IMF content in *Longissimus thoracis et lumborum* muscle was carried out during nine generations in rabbits. Data from the ninth generation of selection were analysed in order to evaluate the responses to selection and examine the genetic background of IMF composition.

The high heritability and variability of IMF allowed its improvement through selection in rabbits. The direct response to selection for IMF was 0.51g/100g of muscle, representing 3.3 phenotypic standard deviations. Selection for IMF content generated also a correlated response on the intramuscular fatty acid composition. The correlated response to selection was positive for saturated (SFA) and monounsaturated (MUFA) fatty acids percentages, 1.71% and 3.24 %, respectively, with greater values in the high-IMF line. In contrast, it was negative for polyunsaturated fatty acids (PUFA) percentage (-4.96%), with greater values for low-IMF line. Most individual SFA were higher in high-IMF line except heptadecanoic (C17:0) and stearic (C18:0) acids. Whereas, most individual PUFA were higher in low-IMF line except α-linolenic acid (C18:3n3). Thus, selection for IMF content has important effects on its fatty acid composition.

Association analyses were carried out on the same divergently selected rabbit lines. The aim was to identify genomic regions associated with the IMF composition and identify putative candidate genes. Our study highlighted the polygenic nature of IMF composition. An important genomic region at 34.0-37.9 Mb on OCU1 was associated with C14:0, C16:0, SFA, and C18:2n6, explaining 3.54%, 11.20%, 11.32%, and 3.18% of the genomic variance, respectively. Besides, a genomic region at 149.0-149.9 Mb on OCU3 was associated with

almost all the traits (C14:0, C16:0, C18:0, C18:1, C18:2n6, C20:4n6, SFA, MUFA, PUFA, and PUFA/SFA). Another relevant genomic region was found to be associated at 46.0-48.9 Mb on OCU18, explaining up to 8% of the genomic variance of the ratio MUFA/SFA. Many genomic regions were found to be associated with the intramuscular fatty acid composition, harbouring several genes related to lipid metabolism such as *SCD*, *PLIN2*, and *ERLIN1*. The main genomic regions associated with the fatty acids were not previously detected for IMF content in rabbits. *MTMR2* is the only gene that was associated with both the IMF content and its composition. Our study underlined the polygenic nature of IMF in rabbits and identified several candidate genes.

Key words: Intramuscular fat, fatty acids, divergent selection, genome-wide association study, rabbits.

Resumen

El contenido y la composición de la grasa intramuscular (IMF) afectan a la calidad de la carne. Un experimento de selección divergente para el contenido de IMF en el músculo *Longissimus thoracis et lumborum* se llevó a cabo durante nueve generaciones en conejos. Se analizaron los datos de la novena generación de selección para evaluar las respuestas a la selección por el IMF y examinar la estructura genética de su composición.

La elevada heredabilidad de la IMF y su relativamente elevada variabilidad han permitido su mejora mediante la selección en conejos. La respuesta directa a la selección por el IMF fue de 0.51 g/100 g de músculo, lo que representa 3.3 desviación estándar fenotípica. La selección por el contenido de IMF dio lugar también a una respuesta correlacionada en la composición de ácidos grasos. La respuesta correlacionada a la selección fue positiva para los porcentajes de ácidos grasos saturados (SFA) y monoinsaturados (MUFA), 1.71% y 3.24%, respectivamente, con valores mayores en la línea de alta IMF. Por el contrario, la respuesta correlacionada fue negativa para el porcentaje de ácidos grasos poliinsaturados (PUFA) (-4.96%), con mayores valores en la línea de baja IMF. La mayoría de los SFA individuales fueron más altos en la línea de alta IMF, excepto los ácidos heptadecanoico (C17:0) y esteárico (C18:0). Mientras que la mayoría de los PUFA individuales fueron más altos en la línea de baja IMF excepto el ácido α-linolénico (C18:3n3). Por lo tanto, la selección por contenido del IMF tiene efectos importantes en su composición de ácidos grasos.

Los análisis de asociación se llevaron a cabo en las mismas líneas de conejo seleccionadas de forma divergente. El objetivo era identificar las regiones genómicas asociadas a la composición del IMF e identificar los posibles genes candidatos. Nuestro estudio ha mostrado la naturaleza poligénica de la composición del IMF. Una región genómica importante en 34.0-

37.9 Mb en OCU1 se asoció con C14:0, C16:0, SFA, y C18:2n6, explicando 3.54%, 11.20%, 11.32%, y 3.18% de la varianza genómica, respectivamente. Además, la región genómica en 149.0-149.9 Mb en OCU3 se asoció con casi todos los caracteres (C14:0, C16:0, C18:0, C18:1, C18:2n6, C20:4n6, SFA, MUFA, PUFA, y PUFA/SFA). Se encontró que otra región genómica relevante en 46.0-48.9 Mb estaba asociada en el OCU18, explicando hasta el 8% de la varianza genómica del ratio MUFA/SFA. Las regiones asociadas revelaron varios genes relacionados con el metabolismo de los lípidos, como *SCD*, *PLIN2*, *ERLIN1*… Las principales regiones genómicas asociadas a los ácidos grasos no se habían detectado anteriormente para el contenido de IMF en conejos. *MTMR2* es el único gen asociado con el contenido de IMF y su composición. Nuestro estudio destacó la naturaleza poligénica del IMF en conejos e identificó varios genes candidatos.

Palabras clave: Grasa intramuscular, ácidos grasos, selección divergente, análisis genómico, conejos.

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INTRODUCTION

Intramuscular fat (IMF) is a main attribute of meat quality. It affects the sensory properties of the meat, increasing juiciness and tenderness and improving its flavour. Conversely, an increase of fat and saturated fatty acid consumption is not desirable from a nutritional point of view (Reviewed by Da Silva Martins et al., 2018).

Several divergent selection experiments for IMF content were carried out in order to examine its genetic determinism in pigs (Schwab et al., 2009), chickens (Zhao et al., 2007 and Liu et al., 2020), cattle (Sapp et al., 2002), and rabbits (Martinez-Alvaro et al., 2018a). However, the effects of selection for IMF on its fatty acid composition were only studied in pigs and rabbits (Burkett, 2009 in pigs and Martinez-Alvaro et al., 2018a in rabbits). Besides, many genomic studies were performed in order to identify genomic regions associated with IMF (Ros-Freixedes et al., 2016 and Zhang et al., 2016 in pigs; Cesar et al., 2014 and Sasago et al., 2017 in cattle; Sosa-Madrid et al., 2019 in rabbits).

Rabbits offer lean meat with good nutritional properties (Hernandez and Dalle Zotte, 2020). They serve also as a good genetic model due to their short generation interval and their low cost. An example of this possibility of rabbits serving as a model is a divergent selection experiment for IMF in *Longissimus thoracis et lumborum* muscle (LM) that was carried out during nine generations at the Universitat Politècnica de València in rabbits (Martinez-Alvaro et al., 2018a). In a previous study, a Genome-Wide Association Study (GWAS) was performed to unravel the genetic background of IMF content (Sosa-Madrid et al., 2019). The present work is focused on the genomic analysis of IMF composition.

1. Intramuscular fat and meat quality

IMF represents all the lipids located between and within muscle fibres. The major components of IMF are phospholipids, triglycerides and cholesterol. Phospholipids have an important structural role, while triglycerides provide energy. IMF content affects the sensory proprieties of the meat (flavour, juiciness and tenderness), influences its technological quality, and has a direct impact on the consumer health (da Silva Martins et al., 2018 and Dalle Zotte and Szendro, 2011).

There is a general agreement on that IMF content improves tenderness, juiciness, flavour, and the overall eating quality of the meat (Reviewed by da Silva Martins et al., 2018 and Wood et al., 2008). Tenderness is one of the most important quality attributes (Wood et al., 1999). The melting of fat during cooking lubricates the meat and increases its tenderness. The IMF content was positively correlated with tenderness in pigs (0.32) and beef (0.23) (DeVol et al., 1988 in pigs and Mateescu et al., 2015 in beef). Besides, a negative correlation (-0.16) was reported between the IMF content and toughness in rabbits (Hernandez et al., 2000).

Juiciness describes the release of liquid during cooking and mastication. It increases through both the melting of fat during cooking and salivation during chewing (Thompson, 2004). Therefore, meat with high IMF content will be moister and juicier than lean meat. Positive correlations between IMF content and juiciness were reported in beef (0.27), pigs (0.21), and rabbits (0.24) (Mateescu et al., 2015 in beef, DeVol et al., 1988 in pigs, and Hernandez et al., 2000 in rabbits). Besides, juiciness and tenderness are closely related. Thompson (2004) reported a positive correlation (0.86) between tenderness and juiciness in cattle. In addition, Hernandez et al. (2000) reported a negative correlation (-0.60) between juiciness and toughness in rabbits.

The third compound of the sensory quality of the meat is flavour. IMF provides flavour through the oxidation of unsaturated fatty acids during cooking, and consequently the generation of volatile products (Elmore and Mottram, 2006). Flavour was positively associated with the IMF content in beef (0.41) and pigs (0.23) (Thompson, 2004 in beef and De Vol et al., 1988 in pigs).

Tenderness, juiciness, and flavour were also positively correlated between them. These meat quality attributes showed strong correlations with the overall liking of the meat and its acceptance by consumers. Correlations between tenderness, juiciness, and flavour with the overall liking of the meat were 0.93, 0.89, and 0.95, respectively (Thompson, 2004).

2. Intramuscular fat and the fatty acid composition

Fat is the most important source of energy, providing up to 9 Kcal per each gram. Dietary fat provides essential fatty acids that cannot be biosynthesised by the human body such as linoleic (C18:2n6) and α-linolenic (C18:3n3) acids (Das, 2006). It carries also liposoluble vitamins (A, D, E, and K) and ensures their absorption (FAO, 2014). However, consumption of high amounts of fat is associated with cardiovascular diseases (Nettleton et al., 2017). The recommended dietary intake for total fat is between 15% and 35% of the daily energy intake (FAO, 2014).

IMF is composed of phospholipids, triglycerides, and cholesterol. Triglycerides are rich in saturated (SFA) and monounsaturated (MUFA) fatty acids, while phospholipids are rich in polyunsaturated fatty acids (PUFA) (Alasnier et al., 1996). These fatty acids have different effects on consumer health. It is well known that high intakes of SFA increase low-density

lipoprotein (LDL) cholesterol levels and lead to obesity and cardiovascular diseases (Nettleton et al., 2017). However, individual SFA have different effects on cholesterol levels. Lauric (C12:0), myristic (C14:0), and palmitic (C16:0) acids increase LDL cholesterol levels in the blood, while stearic acid (C18:0) has no effects (Brown et al., 2009). The total dietary intake of SFA should not exceed 10% of the daily energy intake (FAO, 2014).

SFA are converted to MUFA through desaturation (Stearoyl–CoA desaturase *SCD).* Allman-Farinelli et al. (2005) reported that oleic acid (C18:1n9) decreases LDL cholesterol levels. In addition, replacing SFA (C12:0-C16:0) with MUFA leads to the reduction of LDL cholesterol levels (FAO, 2014). However, there is no specific recommendation about the daily intake of MUFA.

Concerning PUFA, they decrease LDL cholesterol levels and consequently decrease the risk of coronary heart diseases (Nettleton et al., 2017). PUFA ensure many biological functions such as neuronal development, antibiotic-like action, and anti-inflammatory action (Das., 2006). However, high amounts of PUFA reduce the oxidative stability of the meat and consequently reduce its shelf-life (Tao., 2015). The total daily intake of PUFA should be between 6% and 11% of the total energy intake (FAO, 2014). Regarding individual PUFA, linoleic (precursor of n-6 fatty acids) and α-linolenic (precursor of n-3 fatty acids) acids cannot be biosynthesised, they are required in the diet (Das, 2006). The recommended daily intakes for linoleic (C18:2n6) and α-linolenic (C18:3n3) acids are around 2.5% and 0,5% of the daily energy intake, respectively (FAO, 2014). The human body converts these two essential fatty acids to different long-chain fatty acids through elongation and desaturation. However, the synthesis of docosahexaenoic acid (C22:6n3; known as DHA) and eicosapentaenoic acid (20:5n3, known as EPA) from α-linolenic (C18:3n3) acid is not

efficient (Jones and Kubow, 2006). There is also a competitive inhibition between DHA and EPA. High dietary intakes of linoleic (C18:2n6) acid inhibits the conversion of α-linolenic (C18:3n3) acid to DHA (Neuringer and Connor, 1986). This latter is present in large amounts in the nervous system (40% of PUFAs) and retina (60% of PUFAs). It is also involved in brain development and visual acuity (Singh, 2005). DHA and EPA are considered as relatively essential fatty acids. The daily intake of EPA and DHA should be around 500 mg per day (Kris-Etherton et al., 2009). In general, the deprivation of essential fatty acids leads to reduced growth and several pathologies (Roqueta-Rivera et al., 2011).

Ratios PUFA/SFA, MUFA/SFA, and n-6/n-3 are health meat indicators. The ratio PUFA/SFA should be higher than 0.45, while the ratio n-6/n-3 should be lower than 4 in order to reduce the risk of cardiovascular diseases (reviewed by Hernandez and Dalle Zotte, 2020). Concerning the ratio MUFA/SFA, there is no specific nutritional recommendation, however, it is recommended to decrease SFA intake (Nettleton et al., 2017).

3. Intramuscular fat in rabbit meat

The intramuscular fat content depends on the carcass portion and the metabolic type of the muscle (oxidative or glycolytic), glycolytic muscles are leaner than oxidative ones (Alasnier et al., 1996; Rasinska et al., 2018). The intramuscular fat content for New Zealand breed was lower in *Psoas major* (1.24 g/100g of muscle), *Longissimus thoracis et lumborum (LM) (1.24 g/100g)* and *Gastrocnemius laterale (1.74 g/100g)* muscles compared to *Soleus (4.76 g/100g),* and *semimembranosus proprius (4.42 g/100g)* muscles (Alasnier et al., 1996). Martinez-Alvaro et al. (2018b) reported for two rabbit lines divergently selected for IMF that the leanest muscle is LM, with a fat content of 1.05 g/ 100 of LM, while the IMF contents of *biceps femoris* (BF), *supraspinatus* (SS), and *semimembranosus proprius* (SP) were 2.08

g/100g of BF, 2.13 g/100g of SS, and 2.64 g/100 of SP, respectively. Besides, Wang et al., (2016) found similar results for Hyla, Champagne, and Tianfu Black rabbit breeds, the IMF content was higher in BF muscle than in LM. Regarding the intramuscular fatty acid composition, SFA, MUFA and PUFA percentages were 36.8%, 20.8%, and 42.2% for LM, respectively, 35.8%, 29.9%, and 34.2% for BF, and 31.2%, 28.7%, and 40.1% for SS (Martinez-Alvaro et al., 2018b). Palmitic (C16:0), oleic (C18:1n9), and linoleic (C18:2n6) acids were the most abundant fatty acids in LM (Martinez-Alvaro et al., 2018b; Rasinska et al., 2018).

In rabbit meat in general, SFA and PUFA are the most abundant fatty acids, with percentages around 36.9% and 34.6% of total fatty acids, respectively. MUFA group has a lower percentage (28.5%) (reviewed by Hernandez and Gondret, 2006). Rabbit meat comprises a higher percentage of PUFA compared to pork (18.5%), beef (9.5%), veal (15.2%), and chickens (25.1%) (reviewed by Dalle Zotte, 2002). Among PUFA, the ratio n-6/n-3 is not favourable in rabbit meat (6.7). However, it is more favourable than the values reported in pigs (32.5), beef (9.7), veal (36.6), and chickens (18.0) (reviewed by Dalle Zotte, 2004).

The most abundant fatty acids in rabbit meat are palmitic (C16:0), oleic (C18:1n9), and linoleic (C18:2n6) acids, representing 27.3%, 25.4%, and 20.7% of total fatty acids, respectively (reviewed by Dalle Zotte, 2004, and Hernandez and Gondret, 2006). Rabbit meat comprises high amounts of linoleic (C18:2n6) and α-linolenic (C18:3n3) acids in comparison with other species. Linoleic acid (C18:2n6) is ten times greater in rabbit meat than in beef (2.42%) and lamb (2.70%), and two times greater in rabbit meat than in pork (14.2%) (Enser et al., 1996). Besides, α-linolenic acid (18:3n3) represents around 3.3% of the total fatty acids in rabbit meat (Dalle Zotte and Szendro, 2011). It is approximately three times greater in

rabbit meat than in lamb (1.37), beef (0.70), and pork (0.95) (Enser et al., 1996). DHA (C22:6n3) and EPA (20:5n3) are present at low percentages in rabbit meat.

Taken together, rabbit meat is generally appreciated for its good nutritional proprieties (Hernandez and Dalle Zotte, 2020). It offers lean meat rich in PUFA with a favourable n-6/n-3 ratio, compared to other species. Moreover, it has the lowest cholesterol content (45 mg/ 100g of edible meat) compared to pork (61 mg/ 100g of edible meat), veal (66 mg/ 100g of edible meat), beef (70 mg/ 100g of edible meat), and chickens (81 mg/ 100g of edible meat) (reviewed by Dalle Zotte, 2004).

4. Divergent selection experiments for intramuscular fat

Divergent selection experiments are carried out in order to assess the genetic determinism of the traits of interest. As the divergent populations are subjected to the same environment and selected for the same character, the response to selection could be simply estimated as the phenotypic difference between the divergent populations. Each population is used as a control population of the other one (Blasco et al., 2018), duplicating the response per generation due to the divergence of the selection process

IMF has moderate to high heritability estimates (0.54 in rabbits and range from 0.30 to 0.57 in cattle), and a considerable variability, allowing its improvement through selection (Martinez-Álvaro et al., 2016a in rabbits and Park et al., 2018 in cattle). Several experiments of selection for IMF content have been performed, in cattle (Sapp et al., 2002), chickens (Zhao et al., 2007 and Liu et al., 2020), pigs (Schwab et al., 2009), and rabbits (Martinez-Álvaro et al., 2018a). However, the correlated response to selection on the fatty acid composition was studied only in pigs and rabbits (Burkett, 2009 in pigs and Martinez-Alvaro et al., 2018a in rabbits).

In rabbits, the divergent selection experiment for IMF was carried out at the Universitat Politècnica de València during nine generations. The experiment was successful, and the IMF content was improved over generations. The direct response to selection reached 0.09g/100g of IMF in the *Longissimus thoracis et lumborum* muscle (LM) in the third generation and 0.34 g/100 g of IMF in the LM in the eighth generation, representing 2.4 phenotypic standard deviations of the trait (Martinez-Alvaro et al., 2018a and Zomeño et al., 2013). In addition, the direct response to selection was found to be symmetrical between lines (Martinez-Álvaro et al., 2016a).

The correlated responses to selection for IMF on carcass and meat quality traits were also studied (Martínez-Álvaro et al. 2016b). Selection for IMF led to a correlated response on perinatal fat weight, with high values for the high-IMF line at the seventh generation, while differences between low-IMF and high-IMF lines were not relevant for scapular fat, reference carcass weight, meat to bone ratio, and carcass colour. Regarding the meat quality traits, the correlated response to selection was positive for the protein content with high values for the high-IMF line. Whereas, meat pH and colour (lightness, redness, and yellowness) were not affected by selection (Martínez-Álvaro et al., 2016b). Besides, Martínez-Álvaro et al. (2016b) evaluated the effects of selection for IMF on sensory traits and instrumental texture. Selection for IMF affected the instrumental firmness (9.9% greater in low-IMF line). However, differences were irrelevant for shear force, total work to cut a meat sample, cooking loss, and the sensory traits.

IMF content has high and positive genetic correlations with C18:1n9 (0.88), C18:3n3 (0.59), and MUFA (0.89). In contrast, the genetic correlations were strong and negative with C18:0 (-

0.91), C18:2n6 (-0.83), C20:4n6 (-0.89), and PUFA (-0.88) (Martinez-Álvaro et al., 2018a). As a consequence, selection for IMF generated a correlated response on its fatty acid composition. The correlated response to selection was positive for MUFA (9.2%), with greater values in high-IMF line in the eighth generation. In contrast, it was negative for PUFA (-10.3%), with greater values for the low-IMF line. Differences for SFA were not relevant between lines (Martinez-Álvaro et al., 2018a).

A Genome-Wide Association Study (GWAS) was performed in order to understand the genetic background of IMF content and to identify putative candidate genes (Sosa-Madrid et al., 2019). Four associated genomic regions with IMF were found on rabbit chromosomes (OCU) OCU1, OCU8, and OCU13, showing the polygenic nature of the trait. Two consecutive genomic windows on OCU8 explained 7.34% of the genomic variance of IMF. Main identified candidate genes were *MTMR2* on OCU1, *APOLD1, PLBD1, PDE6H, GPRC5A, KRAS* on OCU8, and *EWSR1* on OCU13. Besides, a genomic scan was performed using individuals from the ninth generation of selection in order to detect selection signatures. Sosa-Madrid et al., (2020) identified eight selection signatures (OCU1, OCU3, OCU6, OCU7, OCU16, and OCU17), harbouring several genes related to energy, carbohydrates, and lipid metabolisms (*ACER2, PLIN2, ST8SIA6, VIM, RORA, GANC, PLA2G4B*...). Sosa-Madrid et al., (2019) carried out a GWAS for IMF content on individuals from the ninth generation. In this study, we used the same individuals to perform a GWAS for intramuscular fatty acid composition.

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OBJECTIVES

A divergent selection experiment for intramuscular fat (IMF) in *Longissimus thoracis et lumborum* muscle (LM) was carried out during nine generations in rabbits. A previous Genome-Wide Association Study (GWAS) was performed in rabbits from the ninth generation of selection in order to identify genomic regions related to IMF content. The main objective of the current study is to unravel the genetic background of the IMF composition.

The specific objectives of this study are:

1. To estimate the direct response to selection for IMF content in LM muscle and the correlated responses on its composition.
2. To examine the relationship between the different fatty acids.
3. To identify the genomic regions associated with the intramuscular fatty acid composition.
4. To generate a list of candidate genes related to the IMF composition.

Chapter 1. Correlated response to selection for intramuscular fat in fatty acid composition

ABSTRACT

This study aimed to assess the relationship between intramuscular fat (IMF) content and its fatty acid composition in rabbits. Two rabbit lines divergently selected for IMF in the *Longissimus thoracis et lumborum* muscle were studied. Data from the ninth generation of selection were analysed using Bayesian methodology. Responses to selection were evaluated as the phenotypic differences between high-IMF and low-IMF lines. The direct response to selection for IMF was 0.51g/100g of muscle, representing 3.3 phenotypic standard deviations. Selection for IMF content led to changes in the fatty acid composition. The correlated response to selection was positive for saturated (SFA) and monounsaturated (MUFA) fatty acids percentages, 1.71% and 3.24 %, respectively, with greater values in the high-IMF line. In contrast, it was negative for polyunsaturated fatty acids (PUFA) percentage (-4.96%), with greater values for low-IMF line. Most individual SFA were higher in high-IMF line except heptadecanoic (C17:0) and stearic (C18:0) acids. Besides, most individual PUFA were higher in low-IMF line with the exception of α-linolenic acid (C18:3n3). The divergent lines were clearly separated by principal component analysis. High-IMF line was mainly associated with linolenic acid (C18:3n3) and SFA percentages, whereas low-IMF line was associated with stearic acid (C18:0) and PUFA percentages. In conclusion, selection for IMF changes the intramuscular fatty acid composition and may affect meat quality.

Key words: Intramuscular fat, fatty acids, divergent selection, rabbits.

INTRODUCTION

Intramuscular fat (IMF) influences several meat quality attributes (Hernández and Dalle Zotte, 2020). It affects the sensory proprieties of the meat (flavour, juiciness and tenderness), influences its technological quality, and has a direct impact on the consumer health (See reviews by Webb and O'Neill, 2008; and Dalle Zotte and Szendro, 2011 for rabbits).

Only few experiments of selection for IMF have been performed, in cattle (Sapp et al., 2002), chickens (Zhao et al., 2007; Liu et al., 2020), and pigs (Schwab et al., 2009). In rabbits, a divergent selection experiment for IMF was carried out at the Universitat Politècnica de València, during nine generations (Martinez-Alvaro et al., 2018a).

Selection for IMF led to a correlated response on the fatty acid composition in pigs (Burkett et al., 2009). Individuals with high IMF content had higher proportions of saturated (SFA) and monounsaturated fatty acids (MUFA). In contrast, pigs with low IMF content had greater proportions of polyunsaturated fatty acids (PUFA). Several reviews reported that the increase of fatness is associated with an increase of SFA and MUFA, and consequently a decrease of PUFA in several farm species (reviewed by De Smet et al., 2004 and Wood et al., 2008).

To our knowledge, there is scarce information about the correlated response to selection for IMF on its fatty acid composition in rabbits. The aim of this study was to estimate the correlated response to selection for IMF on fatty acid composition in rabbits, and to examine the relationships between IMF content and its fatty acids profile.

MATERIAL AND METHODS

Animals

Rabbits came from two lines divergently selected for IMF, 239 from the high-IMF line and 236 from the low-IMF line. The divergent selection experiment was performed during nine generations at the Universitat Politècnica de València. The base population consisted of 13 sires and 83 does. High-IMF and low-IMF lines had approximately 8 males and 40 females per generation. Rabbits were reared collectively from weaning (4 weeks) to slaughter (9

weeks). Two full sibs from the first parity of each doe were slaughtered. Carcasses were chilled for 24h at 4°C and dissected. *Longissimus thoracis et lumborum* muscle (LM) was excised from the carcass. After that, LM was ground and freeze-dried. Details of the experiment were reported by Zomeño et al. (2013) and Martinez-Alvaro et al. (2018a).

Intramuscular fat

IMF content was measured in LM by near-infrared reflectance spectroscopy (NIRS), using the calibration equation developed by Zomeño et al. (2012). Twenty percent of the samples were chemically analysed by an ether extraction with a previous acid hydrolysis in order to test NIRS results. IMF content was expressed in g/100g of muscle.

Fatty acid composition

Fatty acid profile was determined in freeze-dried LM samples. Fatty acid methyl esters (Fame) were prepared as described by O'Fallon et al. (2007) and were analysed in a Focus Gas Chromatograph (Thermo, Milan, Italy) equipped with a split/splitless injector and a flame ionization detector. The separation of methyl esters was performed in a fused silica capillary column SPTM 2560 (Supelco, PA, USA) (100 m x 0.25 mm x 0.2 μm film thickness). The carrier gas was Helium at a linear velocity of 20 cm/sec. The samples were injected with a split ratio of 1/100. The initial oven temperature was set at 140ºC held for 5 min and increased to 240 at 4ºC/min and finally maintained at that temperature for 30 min. Both detector and injector temperatures were set at 260ºC. The individual fatty acids were identified by comparing their retention times with standards of Fame supplied by Supelco (PA, USA) and quantified by using C13:0 as internal standard. Results were expressed as percentage of total fatty acids.

Twenty-three fatty acids were analysed, namely, C12:0, C14:0, C15:0, C16:0, C17:0, C18:0, C20:0, C16:1n7, C18:1n9, C18:1n7, C20:1n11, C22:1n9, C18:2n6, C18:3n6, C18:3n3, C20:2n6, C20:3n6, C20:3n3, C20:4n6, C20:5n3, C22:4n6, C22:5n3, and C22:6n3. The SFA, MUFA, n-3, n-6, PUFA groups, and their related ratios were calculated.

Statistical analysis

Descriptive statistics and response to selection

Direct and correlated response to selection, estimated as the phenotypic differences between high-IMF and low-IMF lines, were estimated with the model:

$$\mathbf{y} = \mathbf{Xb} + \mathbf{Wc} + \mathbf{e}$$

where **y** is the phenotypes vector, **X** is the incidence matrix for the fixed effects, **b** is the vector of the fixed effects of line (two levels), sex (two levels), parity order (three levels), and month (five levels), **W** is the incidence matrix for the random effect, **c** is the vector of the random effect (common litter), and **e** is the residual term.

Common litter random effect (**c**) and residuals (**e**) were assumed to be distributed as:

$$\mathbf{c} \sim \mathbf{N}(0, \mathbf{I}\sigma_c^2)$$

$$\mathbf{e} \sim \mathbf{N}(0, \mathbf{I}\sigma_e^2)$$

Where **I** is the identity matrix, σ_c^2 is the common litter variance, and σ_e^2 is the residual variance. All effects were assumed to be independent between them.

Responses to selection were estimated as the differences between high-IMF and low-IMF lines. Bayesian inference was made from the marginal posterior distributions of the differences. Features of these distributions used for the inferences were the median of the difference (D), the highest posterior density region at 95% ($HPD_{0.95}$), the probability (P_0) of D being greater than zero when D is positive or lower than zero when D is negative, and the

probability of relevance (Pr), defined as the probability of D being greater than a relevant value (r). The relevant value was assumed to be one-third of the phenotypic standard deviation of each trait. Marginal posterior distributions were estimated by Gibbs sampling (Blasco, 2017). Monte Carlo Markov chains of 60,000 samples with burn-in period of 10,000, and a lag of 10 samples were used. Gibbs sampling was performed using the Rabbit program (Institute for Animal Science and Technology, Valencia, Spain).

Multivariate analyses

Fatty acid percentages are compositional data. For each individual, all data are non-negative and sum up to a constant (100%); thus, our data are suitable for centred logratio transformation (CLR), expressed as

$$\mathrm{CLR}(x_j) = \log(x_j) - \frac{1}{J}\sum_j \log(x_j) \quad j = 1, \ldots, J$$

(Greenacre, 2018), where J is the total number of fatty acids and x_j is the proportion of the fatty acid j. Furthermore, we performed a principal component analysis on the CLR transformed data using SIMCA software (Umetrics, Umea, Sweden) in order to assess the relationships between all the fatty acids.

RESULTS AND DISCUSSION

Descriptive statistics

Descriptive statistics of IMF, the fatty acid groups, and the ratios are showed in Table 1. The mean and standard deviation of IMF were 1.06 and 0.15 g/100g of LM, respectively. Percentages of SFA and PUFA groups were 36.4% and 39.2%, respectively, while MUFA group had a lower percentage (24.4%). Among PUFA, percentages of n-6 and n-3 were 37.1% and 2.1%, respectively. These results are in line with previous findings in rabbits

(Martinez-Alvaro et al., 2018a). Rabbit meat comprised a higher percentage of PUFA compared to pigs, beef, and chickens (reviewed by Dalle Zotte, 2002). Ratios PUFA/SFA, MUFA/SFA, and n-6/n-3 were 1.08, 0.67, and 17.7, respectively. The ratio PUFA/SFA (1.08) was favourable, whereas the ratio n-6/n-3 (17.7) was higher than the nutritional recommendations (0.45) (Hernandez and Gondret, 2006). There is no specific nutritional recommendation for the ratio MUFA/SFA; however, it is recommended to decrease the SFA intake (Nettleton et al., 2017).

Table 1. Descriptive statistics for IMF (g/100g of muscle), fatty acid groups (% of total fatty acids), and ratios of *Longissimus thoracis et lumborum* muscle.

Trait	Mean	SD[1]	CV[2] (%)
IMF	1.06	0.15	14
Groups			
SFA	36.4	0.94	2
MUFA	24.4	1.27	5
PUFA	39.2	1.83	4
n-3 PUFA	2.10	0.13	6
n-6 PUFA	37.1	1.76	4
Ratios			
PUFA/SFA	1.08	0.07	6
MUFA/SFA	0.67	0.03	4
n-6/n-3	17.7	0.97	5

[1]Standard deviation. [2]Coefficient of variation. IMF: Intramuscular fat. SFA: Saturated fatty acids= C12:0 + C14:0 + C15:0 + C16:0 + C17:0 + C18:0 + C20:0. MUFA: monounsaturated fatty acids = C16:1n7 + C18:1n9c + C18:1n7 + C20:1n11 + C22:1n9. PUFA: Polyunsaturated fatty acids = C18:3n3 + C20:3n3 + C20:5n3 + C22:5n3 + C22:6n3+ C18:2n6 + C18:3n6 + C20:2n6 + C20:3n6 + C20:4n6 + C22:4n6. n-3 PUFA= C18:3n3 + C20:3n3 + C20:5n3 + C22:5n3 + C22:6n3. n-6 PUFA= C18:2n6 + C18:3n6 + C20:2n6 + C20:3n6 + C20:4n6 + C22:4n6.

Table 2 shows descriptive statistics of the individual fatty acids. Overall, the most abundant fatty acids were linoleic (C18:2n6), palmitic (C16:0) and oleic (C18:1n9) acids, representing 27.7%, 25.7% and 20.3% of total fatty acids, respectively. They were followed by stearic (C18:0; 8.3%) and arachidonic (C20:4n6; 7.4%) acids. Some long chain PUFA (i.e. C20:5n3, C22:4n6, C22:5n3 and C22:6n3) were also present at a lower content. Similar results were reported in rabbits (Reviewed by Dalle Zotte, 2002 and Hernández and Gondret, 2006;

Martinez-Alvaro et al., 2018a). Compared to other species, linoleic (C18:2n6) and α-linolenic (C18:3n3) acids were greater in rabbit meat than in cattle, pigs, and chickens (reviewed by Dalle Zotte, 2004). As expected, minor fatty acids showed a higher range of variability compared to the major fatty acids.

Table 2. Descriptive statistics for individual fatty acids of *Longissimus thoracis et lumborum* muscle (% of total fatty acids) in rabbits.

Trait	Mean	SD[1]	CV[2] (%)
Saturated fatty acids			
C12:0	0.09	0.03	33
C14:0	1.26	0.26	20
C15:0	0.44	0.04	8
C16:0	25.7	1.17	4
C17:0	0.53	0.06	11
C18:0	8.34	0.66	8
C20:0	0.06	0.01	10
Monounsaturated fatty acids			
C16:1n7	1.25	0.50	39
C18:1n9	20.3	0.88	4
C18:1n7	2.56	0.20	7
C20:1n11	0.21	0.03	14
C22:1n9	0.09	0.02	19
Polyunsaturated fatty acids			
C18:2n6	27.7	1.18	4
C18:3n6	0.11	0.02	13
C18:3n3	1.16	0.14	12
C20:2n6	0.34	0.04	12
C20:3n6	0.61	0.07	11
C20:3n3	0.05	0.01	19
C20:4n6	7.45	1.05	14
C20:5n3	0.10	0.03	25
C22:4n6	0.92	0.14	15
C22:5n3	0.64	0.11	17
C22:6n3	0.14	0.05	32

[1]Standard deviation. [2]Coefficient of variation.

Response to selection

Correlated responses to selection were relevant (Pr=1) for almost all the traits (Tables 3 and 4). The direct response to selection for the ninth generation of selection was 0.51g/100g of

LM, representing 3.3 phenotypic standard deviations (Table 3). The selection experiment was successful; IMF content was clearly improved over generations. This great response to selection was expected, because of the high heritability of the trait (0.54) and its substantial variability (Martínez-Álvaro et al., 2016a). Several studies reported the improvement of IMF content through selection in pigs (Schwab et al., 2009), cattle (Sapp et al., 2002) and chickens (Zhao et al., 2007; Liu et al., 2020).

Table 3. Correlated response to selection, estimated as differences between high-IMF and low-IMF lines for IMF (g/100g of LM), fatty acid groups (% of total fatty acids), and ratios of *Longissimus thoracis et lumborum* muscle.

Trait	D[1]	HPD$_{0.95}$[2]		P_0[3]	r[4]	Pr[5]
IMF	0.51	0.48	0.55	1	0.05	1
Groups						
SFA	1.71	1.50	1.92	1	0.31	1
MUFA	3.24	2.95	3.54	1	0.42	1
PUFA	-4.96	-5.38	-4.53	1	0.61	1
n-3 PUFA	0.05	0.02	0.08	1	0.04	0.79
n-6 PUFA	-5.00	-5.40	-4.60	1	0.59	1
Ratios						
PUFA/SFA	-0.19	-0.20	-0.17	1	0.02	1
MUFA/SFA	0.06	0.05	0.07	1	0.01	1
n-6/n-3	-2.87	-3.07	-2.65	1	0.32	1

IMF: Intramuscular fat. SFA: Saturated fatty acids = C12:0 + C14:0 + C15:0 + C16:0 + C17:0 + C18:0 + C20:0. MUFA: monounsaturated fatty acids = C16:1n7 + C18:1n9 + C18:1n7 + C20:1n11 + C22:1n9. PUFA: Polyunsaturated fatty acids = C18:3n3 + C20:3n3 + C20:5n3 + C22:5n3 + C22:6n3+ C18:2n6 + C18:3n6 + C20:2n6 + C20:3n6 + C20:4n6 + C22:4n6. n-3 PUFA= C18:3n3 + C20:3n3 + C20:5n3 + C22:5n3 + C22:6n3. n-6 PUFA= C18:2n6 + C18:3n6 + C20:2n6 + C20:3n6 + C20:4n6 + C22:4n6. [1]Median of the marginal posterior distribution of the difference between high-IMF and low-IMF lines. [2]Highest posterior density region at 95%. [3]Probability of the difference being greater than zero when D>0 or lower than zero when D<0. [4]Relevant value, proposed as 1/3 of the standard deviation of the trait. [5]Probability of relevance, probability of the difference being greater than r when D>0 or being lower than r when D<0.

Regarding the fatty acid composition, high-IMF line showed greater percentages of SFA and MUFA than low-IMF line, whereas PUFA percentage was greater in the low-IMF line. Similar results were reported in pigs (Burkett et al., 2009). Among PUFA, low-IMF line

contained high proportion of n-6 PUFA, whereas differences of n-3 PUFA were not relevant (Table 3). The former differences were expected, since the high-IMF line contains greater proportion of triglycerides respect to phospholipids than the low-IMF line. Triglycerides are rich in both SFA and MUFA, while phospholipids are rich in PUFA (Alasnier et al., 1996). De Smet et al. (2004) also reported in a review the increase of SFA and MUFA compared to PUFA with fatness.

The ratios between fatty acid groups showed relevant correlated responses to selection. The ratio MUFA/SFA was greater in high-IMF line than in low-IMF line. In contrast, PUFA/SFA and n-6/n-3 were greater in low-IMF line than in high-IMF line. Therefore, selection for IMF affected also the nutritional quality of the meat.

Table 4 shows correlated response to selection for the individual fatty acids. Most individual SFA were higher in high-IMF line with the exception of heptadecanoic (C17:0) and stearic (C18:0) acids that were higher in low-IMF line. Besides, most individual PUFA were higher in low-IMF line with the exception of, α-linolenic (C18:3n3), linolenic (C18:2n6) and γ-linolenic (C18:3n6) acids percentages that were higher in high-IMF line. However, differences between lines for linolenic (C18:2n6) and γ-linolenic (C18:3n6) acids were not relevant. These exceptions could be due to the abundance of C17:0 and C18:0 in phospholipids than in triglycerides, unlike the other individual SFA, and the abundance of C18:3n3 in triglycerides than in phospholipids (Alasnier et al., 1996). Our results are in line with those obtained by Martinez-Alvaro et al. (2018a) in the eighth generation of selection.

Table 4. Correlated response to selection, estimated as differences between high-IMF and low-IMF lines for individual fatty acids of *Longissimus thoracis et lumborum* muscle (% of total fatty acids).

Trait	D[1]	HPD$_{0.95}$[2]		P_0[3]	r[4]	Pr[5]
Saturated fatty acids						
C12:0	0.06	0.06	0.07	1	0.01	1
C14:0	1.04	0.98	1.10	1	0.09	1
C15:0	0.03	0.02	0.03	1	0.01	1
C16:0	2.90	2.65	3.18	1	0.39	1
C17:0	-0.05	-0.06	-0.04	1	0.02	1
C18:0	-2.29	-2.43	-2.14	1	0.22	1
C20:0	0.02	0.01	0.02	1	0.00	1
Monounsaturated fatty acids						
C16:1n7	1.17	1.06	1.28	1	0.17	1
C18:1n9	2.15	1.95	2.35	1	0.29	1
C18:1n7	-0.09	-0.13	-0.05	1	0.07	0.93
C20:1n11	0.05	0.05	0.06	1	0.01	1
C22:1n9	-0.03	-0.03	-0.03	1	0.01	1
Polyunsaturated fatty acids						
C18:2n6	0.32	0.04	0.57	0.99	0.39	0.30
C18:3n6	0.01	0.00	0.01	1	0.01	0.69
C18:3n3	0.60	0.57	0.63	1	0.05	1
C20:2n6	-0.08	-0.09	-0.07	1	0.01	1
C20:3n6	-0.27	-0.29	-0.26	1	0.02	1
C20:3n3	-0.01	-0.01	-0.01	1	0.00	1
C20:4n6	-4.50	-4.74	-4.27	1	0.35	1
C20:5n3	-0.06	-0.06	-0.05	1	0.01	1
C22:4n6	-0.48	-0.51	-0.45	1	0.05	1
C22:5n3	-0.40	-0.42	-0.37	1	0.04	1
C22:6n3	-0.08	-0.09	-0.07	1	0.02	1

[1]Median of the marginal posterior distribution of the difference between high-IMF and low-IMF lines. [2]Highest posterior density region at 95%. [3]Probability of the difference being greater than zero when D>0 or lower than zero when D<0. [4]Relevant value, proposed as 1/3 of the standard deviation of the trait. [5]Probability of relevance, probability of the difference being greater than r when D>0 or being lower than r when D<0.

Martínez-Álvaro al. (2016a) reported in the same divergent experiment of selection that the direct response to selection was symmetrical for both high-IMF and low-IMF lines. Taken together, fatty acids showed different correlated responses to selection for IMF; however, we cannot assess from our data whether these correlated responses are symmetrical or not.

Multivariate analyses

Principal component analyses (PCA) were performed in order to visualise the correlations between the fatty acids. Close variables are positively correlated between them, whereas opposing variables (separated by 180°) are negatively correlated. Variables at 90º have low correlations. Figure 1 shows the projection of individual fatty acids on the two first principal components, explaining 60% and 9% of the total variance, respectively. The first component (P1) separated the fatty acids. Main individual SFA, except stearic acid (C18:0), were projected near each other on the positive side of P1. In contrast, most individual PUFA were projected near each other on the negative side of P1, except linolenic acid (C18:3n3) that was close individual SFA. Thus, most individual PUFA and stearic acid (C18:0) were negatively correlated with main individual SFA and linolenic acid (C18:3n3), which is in line with our previous results. As said previously, stearic and linolenic acids have an opposite tendency compared to the rest of the fatty acids of their groups. Main individual MUFA (C16:1 and C18:1n9) were positively correlated with SFA, and consequently negatively correlated with most PUFA. Besides, Figure 2 shows the projection of data on the plane defined by the two first principal components. High-IMF and low-IMF lines were clearly differentiated by selection. Individuals from high-IMF line lay on the positive side of P1, while individuals from the low-IMF line lay on its negative side.

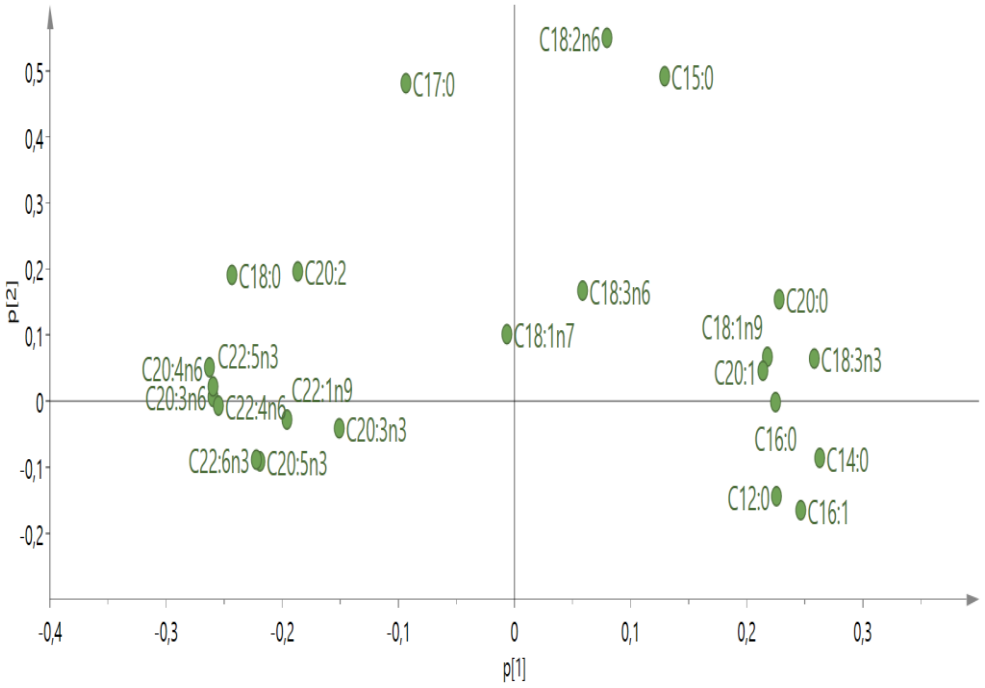

Figure 1. Loading plot of PCA. Projection of the fatty acids on the two first principal components.

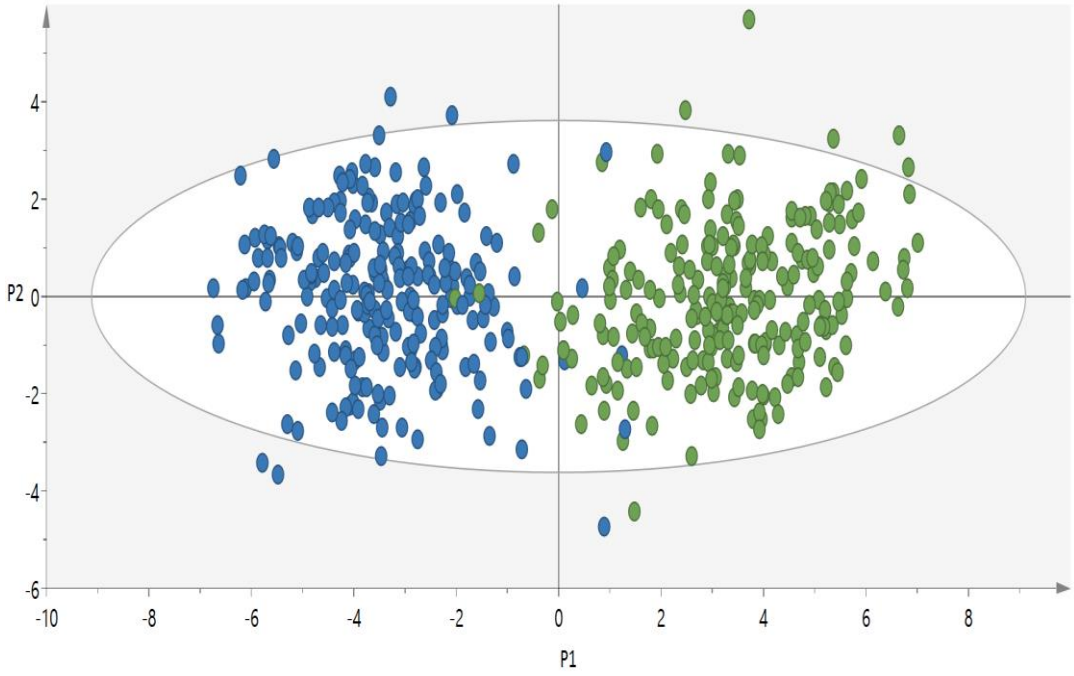

Figure 2. Score plot of PCA. Projection of data on the two first principal components. Each circle represents an observation. Colours: green, high-IMF line; blue, low-IMF line.

PCAs within each line were also performed. Figure 3 shows for low-IMF line the projection of the variable on the two first principal components, explaining 33% and 14% of the total variance, respectively. Besides, Figure 4 displays for high-IMF line the projection of fatty acids on the two first principal components, explaining 38% and 13% of the total variance, respectively. These loading plots show how the fatty acids relate to each other within each line. A similar pattern was found for both lines. Main individual SFA and linolenic acid (C18:3n3) were positively correlated between them and negatively correlated with most individual PUFA and stearic (C18:0) acid. Selection for IMF affected similarly the relationships between fatty acids in both divergent lines.

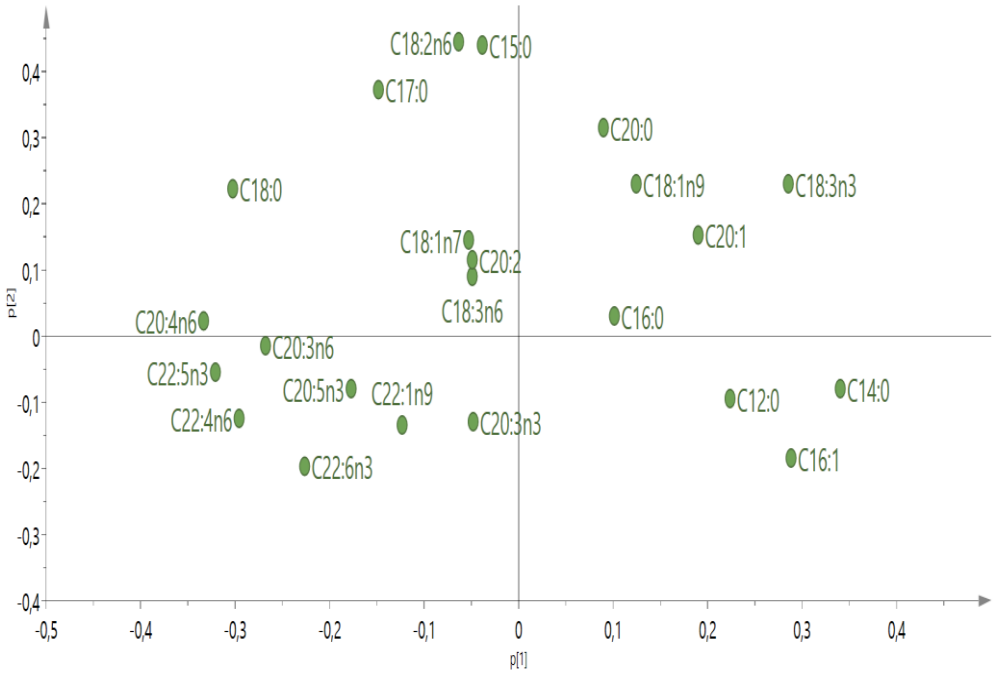

Figure 3. Loading plot of PCA for low-IMF line. Projection of the fatty acids on the two first principal components.

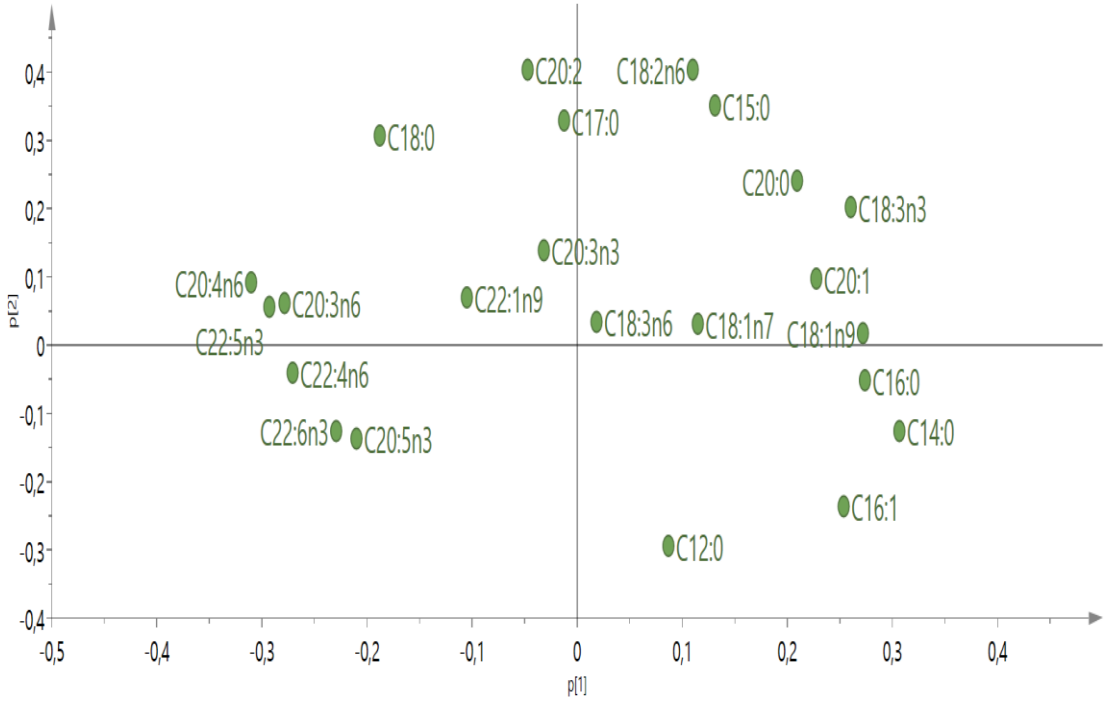

Figure 4. Loading plot of PCA for high-IMF line. Projection of the fatty acids on the two first principal components.

CONCLUSIONS

Selection for IMF content have substantial effects on the fatty acid composition. Response to selection on IMF content led to a positive correlated response on SFA and MUFA, and consequently a negative one on PUFA. Most individual SFA were higher in high-IMF line except heptadecanoic (C17:0) and stearic (C18:0) acids. Besides, most individual PUFA were higher in low-IMF line with the exception of α-linolenic acid (C18:3n3). Selection for IMF led also to changes in the meat health indicators (PUFA/SFA, MUFA/SFA, and n-6/n-3).

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Chapter 2. Novel genomic regions associated with intramuscular fatty acid composition in rabbits

ABSTRACT

Intramuscular fat (IMF) content and its composition affect the nutritional and sensory quality of meat. The high heritability and substantial variability of IMF allowed its improvement through selection in different species. Besides, selection for IMF generated a correlated response on the fatty acid composition. The increase of IMF content is associated with an increase of saturated (SFA) and monounsaturated (MUFA) fatty acids, and consequently the decrease of polyunsaturated fatty acids (PUFA). In the current study, we used two rabbit lines divergently selected for IMF to carry out a genome wide association study (GWAS), using Bayes B, for the intramuscular fatty acid composition. The studied traits were the major fatty acids: C14:0, C16:0, C18:0, C16:1n7, C18:1n9, C18:3n3, C18:2n6, C20:4n6, the SFA, MUFA, PUFA groups and the ratios PUFA/SFA, MUFA/SFA, n-6/n-3. The main objectives were to identify genomic regions associated with the intramuscular fatty acid composition and to generate a list of candidate genes. Genomic regions associated with the fatty acid composition were spread across different rabbit chromosomes (OCU). An important region at 34.0-37.9 Mb on OCU1 was associated with C14:0, C16:0, SFA, and C18:2n6, explaining 3.54%, 11.20%, 11.32%, and 3.18% of the genomic variance, respectively. Besides, the genomic region at 149.0-149.9 Mb on OCU3 was associated with almost all the traits. Another relevant genomic region was found to be associated at 46.0-48.9 Mb on OCU18, explaining up to 8% of the genomic variance of the ratio MUFA/SFA. The associated regions harbour several genes related to lipid metabolism such as *SCD*, *PLIN2*, and *ERLIN1*… The main genomic regions associated with the fatty acids were not previously detected for IMF content in rabbits. Nonetheless, *MTMR2* is the only gene that was associated with both the IMF content and composition in rabbits. We identified several genomic regions associated with the fatty acid composition. Our study highlighted the polygenic nature of the fatty acid

composition in rabbits and elucidated its genetic background. Further analyses throughout the associated regions would be needed to validate these associations.

Key words: Intramuscular fat, fatty acids, divergent selection, genome-wide association study, rabbits.

INTRODUCTION

Intramuscular fat (IMF) content and its fatty composition are key traits influencing meat quality (Hernández and Dalle Zotte, 2020). The increase of IMF improves the juiciness, tenderness, and flavour of the meat (Wood et al., 2008). IMF content and its fatty acid composition determine also the nutritional value of the meat. Individual saturated (SFA) and monounsaturated (MUFA) fatty acids could be biosynthesised. However, linoleic (precursor of n-6 fatty acids) and alpha linoleic (precursor of n-3 fatty acids) are both polyunsaturated fatty acids (PUFA) that cannot be biosynthesised, they must be obtained from the diet (Das, 2006). High intakes of SFA increase low-density lipoprotein (LDL) cholesterol which is associated with coronary heart disease (Brown et al., 2009). In contrast, PUFA decrease LDL cholesterol (Sacks and Katan, 2002). Meat consumers are interested in the fatty acid composition of the meat, demanding healthy products rich in PUFA respect to SFA.

The high heritability of IMF and its substantial variability allowed its improvement through selection in cattle (Sapp et al., 2002), chickens (Liu et al., 2020; Zhao et al., 2007), and pigs (Schwab et al., 2009). In rabbits, a divergent selection experiment was carried out at the Universitat Politècnica de València during nine generations revealing a correlated response on the fatty acid composition (Martinez-Alvaro et al., 2018a; Sosa-Madrid et al., 2019). We have shown in the first chapter that selection for IMF changed the intramuscular fatty acid composition. The high-IMF line had greater percentages of SFA and MUFA than the low-

IMF line. In contrast, the low-IMF line had greater percentages of PUFA than the high-IMF line.

Several association analyses were performed in order to unravel the genetic background of the IMF composition. Previous Genome-Wide Association Studies (GWAS) reported genetic markers and genes associated with the fatty acid composition in pigs (Ros-Freixedes et al., 2016; Zhang et al., 2016) and cattle (Cesar et al., 2014; Sasago et al., 2017; Wang et al., 2019). The main candidate genes were Stearoyl-CoA desaturase-1 (*SCD*), Leptin receptor (*LEPR*), Fatty Acid Synthase (*FASN*), and Fatty Acid Elongase (*ELOVL*). These studies highlighted the complexity and the polygenic nature of the fatty acids in livestock species (Cesar et al., 2014; Ros-Freixedes et al., 2016; Sasago et al., 2017; Wang et al., 2019; Zhang et al., 2016). To our knowledge, there are no previous association analyses for the IMF composition in rabbits. A previous study identified four genomic regions related to IMF content using two rabbit lines divergently selected for IMF (Sosa-Madrid et al., 2019). IMF content and its fatty acid composition can share genomic regions with pleotropic effects, since they have moderate to high genetic correlations (Martinez-Alvaro et al., 2018a). In this study, we carried out a GWAS for intramuscular fatty acid composition using the same previous rabbit lines divergently selected for IMF.

We performed GWAS to identify genomic regions associated with the intramuscular fatty acid composition and to detect putative candidate genes.

MATERIAL AND METHODS

Animals

The study was carried out using two rabbit lines divergently selected for IMF during the 9th generation of selection. The base population of the IMF selection experiment consisted of 13 sires and 83 does (Zomeño et al., 2013). High (high-IMF) and low (low-IMF) lines had approximately 8 males and 40 females per generation. Rabbits were weaned at 4 weeks of age and housed collectively until slaughter. Two full sibs of the first parity of each doe were slaughtered at 9 weeks of age. Carcasses were chilled for 24h at 4°C. From each animal, *Longissimus thoracis et lumborum* muscle (LM) was excised, minced and freeze-dried. IMF content was measured in LM by near-infrared reflectance spectroscopy (NIRS) and expressed in g/100 g of LM (Zomeño et al., 2012). Twenty percent of the samples were chemically analysed by an ether extraction with a previous acid hydrolysis to confirm NIRS results.

More details of the experiment can be found in previous works (Martinez-Alvaro et al., 2018a; Zomeño et al., 2013). The present study was carried out on 478 individuals from the ninth generation of selection (239 rabbits of each line).

Phenotypes

Fatty acids were quantified by gas chromatography. Fatty acid methyl esters (FAME) were prepared as described by O'Fallon et al. (2007). Further, they were analysed by gas chromatography (FOCUS, Thermo, Milan, Italy). The gas chromatograph was equipped with a split/splitless injector and a flame ionization detector. The separation of methyl esters was performed in a fused silica capillary column SPTM 2560 (Supelco, PA, USA) (100 m x 0.25 mm x 0.2 μm film thickness). The carrier gas was Helium at a linear velocity of 20 cm/sec.

The samples were injected with a split ratio of 1/100. The oven temperature was set at 140ºC during 5 min, then increased to 240ºC at 4ºC/min and maintained at 240ºC temperature for 30 min. Both detector and injector were at 260ºC. The individual fatty acids were determined by comparing their retention times with standards of FAME supplied by Supelco (PA, USA) and quantified by using C13:0 as internal standard. Results were expressed as percentage of total fatty acids.

The studied traits were the main individual fatty acids C14:0, C16:0, C18:0, C16:1n7, C18:1n9, C18:2n6, C18:3n3, C20:4n6, the groups SFA, MUFA, PUFA, and the ratios PUFA/SFA, MUFA/SFA, n-6/n-3. Other minor individual fatty acids such as C12:0, C15:0, C17:0, C20:0, C18:1n7, C20:1n11, C22:1n9, C18:3n6, C20:2n6, C20:3n6, C20:3n3, C20:5n3, C22:5n3, and C22:6n3 were not studied individually, however, they were considered to estimate SFA, MUFA, and PUFA groups. The studied fatty acids accounted for more than 93% of total fatty acids.

Genotypes and quality control

Rabbits were genotyped using the Affymetrix Axiom OrcunSNP Array (Affymetrix Inc., Santa Clara, CA, USA). The Single Nucleotide Polymorphisms (SNP) array contained 199,692 molecular markers. Quality control was performed using Axiom Analysis Suite v.4.0.3 of Thermo Fisher Scientific. SNPs with a call rate greater than 0.95, a minor allele frequency (MAF) greater than 0.05, and a known chromosome position were retained for the association analyses. In addition, individuals with a missing genotype frequency greater than 0.05 were excluded from the dataset. The missing genotypes were imputed by Beagle software v.4.1 (Browning and Browning, 2016). After quality control, 475 animals (low-IMF: 236; high-IMF: 239) and 90,235 SNPs remained in the dataset.

Genome-Wide Association Study (GWAS)

The association analyses were performed using a Bayesian Multiple-Marker Regression (BMMR) under the following Bayes B model:

$$\boldsymbol{y} = \boldsymbol{Xb} + \sum_{j=1}^{k} \boldsymbol{z}_j \alpha_j \delta_j + \boldsymbol{e}$$

where $\boldsymbol{y}$ is the phenotypes vector. $\boldsymbol{X}$ is the incidence matrix for fixed effects and $\boldsymbol{b}$ is the vector of the fixed effects of month (five levels), sex (two levels) and parity order (three levels). $\boldsymbol{z_j}$ is the genotypes vector for a SNP at locus j (j = 1, …, k, where k is the number of SNPs after quality control). α_j is the random substitution effect for SNP j, with a normal distribution $\mathbf{N}$ (0, $\mathbf{I}\sigma_\alpha^2$). δ_j is a random 0/1 variable (δ_j = 1 represents the presence of SNP j with probability 1- π and δ_j = 0 represents the absence of SNP j with probability π). Besides, $\boldsymbol{e}$ is the residual term, assumed to be distributed as e ~ $\mathbf{N}$ (0, $\mathbf{I}\sigma_e^2$). Residuals were considered to be independent and normally distributed.

The prior proportion of SNPs with zero effects (π) was fixed to 0.9988 (Sosa-Madrid et al., 2019). The prior variances (σ_α^2 and σ_e^2) for all the traits were calculated using the heritability estimates reported by Martinez-Alvaro et al. (2018a). Marginal posterior distributions of the model unknowns were estimated using Monte Carlo Markov chains. A total of 500,000 iterations were performed with a burn-in of 100,000 and a lag of 40. In this study, 1982 non-overlapping genomic windows of 1 Mb were a priori allocated to the 21 autosomes, with an average of 45 SNPs per 1 Mb window. The contributions of the windows to the genomic variance were computed as the posterior distribution of the genomic variance explained by

SNPs within each 1 Mb genomic window. All GWAS were performed using the GenSel software (Garrick and Fernando, 2013).

The Bayes factor (BF) was estimated to evaluate the statistical relevance of the association between SNPs and traits. The BF was calculated as follows (Bouwman et al., 2011):

$$BF = \frac{\frac{\hat{p}_j}{1-\hat{p}_j}}{\frac{1-\pi}{\pi}}$$

where $\hat{p}_j$ is the posterior probability of SNP *j*.

Genomic regions associated with the fatty acid composition

The genomic windows exceeding 1.0% of the genomic variance of the trait were considered to be associated with the trait. In addition, the genomic windows exceeding 0.5% of the genomic variance of the trait and having SNPs with a BF greater than 10 were also considered as associated with the trait. These thresholds of 1% and 0.5% represent 20 and 10 times the expected percentage of the genomic variance explained by each genomic window, respectively. SNPs with a BF greater than 10 were considered having enough evidence to be considered as associated with the trait (Kass and Raftery, 1995). The associated genomic windows were extended to ± 500 Kb from the first and last associated SNP in order to consider nearby associated SNPs, taking into account the linkage disequilibrium. These new genomic windows are termed as "extended regions".

Identification of candidate genes

Genes were retrieved from the extended regions. Candidate genes were obtained from the Ensembl Genes 98 database using the *Oryctolagus cuniculus* as the reference genome

(Cunningham et al., 2019). The biological functions were retrieved from the Database for Annotation, Visualization and Integrated Discovery (DAVID) v.6.8 (Jiao et al., 2012). The genes related to lipid metabolism have been also investigated using the gene ontology (GO) (Binns et al., 2009).

Linkage disequilibrium analysis

We examined the linkage disequilibrium (LD) in the most relevant extended regions. LD analyses were performed using plink software (Chang et al., 2015) and LDheatmap function from R (Shin et al., 2006).

Internal validation

Permutation test

Results should be validated in order to overcome the problem of multiple testing. The phenotypes were resampled 100,000 times to remove any possible relationship between the phenotypes and genotypes, using the following covariates: sex, month, parity order, and the three first components of the principal component analysis of the genotypes (explaining 36% of the variance) in order to avoid populations structures. Permutation testing was performed on the new data corresponding to the null hypothesis. Empirical p-values (EMP1) were provided for each SNP. EMP1 was estimated as K/N, where N is the total number of permutations and K is the number of times the SNP comes out associated in the simulated data. SNPs with EMP1 values close to 10^{-5} were considered as true positives, as suggested by Casto-Rebollo et al. (2020). Permutation testing was performed using Plink software (Chang et al., 2015).

Within line GWAS

GWAS within line were performed to compare the SNPs effects. Confidence intervals of the SNPs effects (effect ± 2SD) were calculated in both lines. SNPs with overlapping confidence intervals were considered as true positive associations. In contrast, SNPs with non-overlapping confidence intervals were discarded since we assumed that these associations were caused by a sampling effect.

RESULTS AND DISCUSSION

Descriptive statistics of the intramuscular fatty acid composition

Table 5 shows descriptive statistics of the studied traits. The fatty acids profile of rabbit meat mainly comprises SFA and PUFA (36.4% and 39.2%, respectively), while MUFA had a lower percentage (24.4%). The most ubiquitous fatty acids were linoleic (C18:2n6), palmitic (C16:0) and oleic (C18:1n9) acids, representing 27.7%, 25.7% and 20.3% of total fatty acids, respectively. Ratios PUFA/SFA, MUFA/SFA, and n-6/n-3 were 1.08, 0.67, and 17.7, respectively. These results are in agreement with previous results in rabbits (Martinez-Alvaro et al., 2018a).

Table 5. Descriptive statistics for fatty acid composition (% of total fatty acids) of *Longissimus thoracis et lumborum* muscle in rabbits.

Trait	Mean	SD[1]	CV[2] (%)
C14:0	1.26	0.26	20
C16:0	25.7	1.17	4
C18:0	8.34	0.66	8
SFA	36.4	0.94	2
C16:1n7	1.25	0.50	39
C18:1n9	20.3	0.88	4
MUFA	24.4	1.27	5
C18:2n6	27.7	1.18	4
C18:3n3	1.16	0.14	12
C20:4n6	7.45	1.05	14
PUFA	39.2	1.83	4
PUFA/SFA	1.08	0.07	6
MUFA/SFA	0.67	0.03	4
n-6/n-3	17.7	0.97	5

[1]Standard deviation, [2]coefficient of variation, SFA: saturated fatty acids = C12:0 + C14:0 + C15:0 + C16:0 + C17:0 + C18:0 + C20:0, MUFA: monounsaturated fatty acids = C16:1n7 + C18:1n9 + C18:1n7 + C20:1n11 + C22:1n9, n-3 = C18:3n3 + C20:3n3 + C20:5n3 + C22:5n3 + C22:6n3, n-6 = C18:2n6 + C18:3n6 + C20:2n6 + C20:3n6 + C20:4n6 + C22:4n6, PUFA: polyunsaturated fatty acids = C18:3n3 + C20:3n3 + C20:5n3 + C22:5n3 + C22:6n3 + C18:2n6 + C18:3n6 + C20:2n6 + C20:3n6 + C20:4n6 + C22:4n6.

Genomic regions associated with the intramuscular fatty acid composition

The association analyses were performed using the 475 individuals and 90,235 SNPs that remained in the dataset after quality control.

Although samples came from two rabbit lines, we did not consider the line as a fixed effect. Allele frequencies differ among lines due to the random genetic drift and selection. As the animals came from a divergent selection experiment for IMF, genes with large causal variants will show extreme frequencies for alternate alleles between lines due to selection. We expect that high-IMF line will show genes with high frequencies given a reference allele, while the same genes will have lower frequencies in low-IMF line. These genes will show intermediate frequencies when both lines are used. The line effect was not considered, to take advantage from these extreme differences caused by selection and detect the genes with large causal variants. We performed also GWAS with the line effect. The same associated regions were obtained with lower percentages of the genomic variances of the traits (Results not shown).

The genomic windows that surpassed the thresholds levels were spread across several chromosomes (all rabbit chromosomes (OCU), except OCU7, OCU11, and OCU20). The associated regions with SFA, MUFA, PUFA, and the ratios were displayed in Tables 6, 7, 8 and 9, respectively. The associated SNPs were tested using GWAS within line and permutation testing. The validated SNPs with a BF greater than 50 were presented in additional file 1.

Table 6. Genomic regions associated with saturated fatty acid percentage of *Longissimus thoracis et lumborum* muscle in rabbits.

OCU[1]	Region (Mb)[2]	Trait	VAR[3]	PPA[4]	rSNP[5]	Number of genes[6]	PCG[7]
1	29.1-29.9	C18:0	0.52	0.55	21	3	-
	34.0-37.9	C14:0	3.54	0.51	38	13	*ACER2, PLIN2*
		C16:0	11.20	0.51	36		
		C18:0	0.87	0.33	1		
		SFA	11.32	0.45	22		
	121.0-121.9	C14:0	0.62	0.69	27	13	*MTMR2*
		C18:0	0.50	0.59	27		
3	149.0-149.9	C14:0	1.11	0.96	19	8	*ENSOCUG00000000157*
		C16:0	3.65	0.98	15		
		C18:0	1.72	0.99	15		
		SFA	0.98	0.51	16		
	154.0-154.9	SFA	0.91	0.39	12	4	-
4	67.0-67.9	C18:0	0.71	0.75	14	8	-
5	7.2-7.9	C16:0	0.71	0.50	15	1	-
6	0-0.9	C16:0	1.33	0.60	12	-	-
8	15.0-15.9	SFA	0.52	0.43	25	7	-
	21.0-21.9	C18:0	0.57	0.75	69	6	*PIK3C2G, PLCZ1, PLEKHA5*
9	64.0-64.9	C16:0	0.62	0.38	2	10	*NPC1*
		SFA	0.96	0.37	2		
	69.0-69.5	SFA	0.56	0.32	2	4	-
10	43.0-43.9	SFA	0.59	0.35	8	3	-
13	126.0-126.9	C18:0	0.96	0.87	9	23	*HEYL, MFSD2A, PPT1*
14	82.0-82.9	C18:0	0.51	0.55	18	19	*ADIPOQ*
15	10.0-10.9	C16:0	0.63	0.40	17	11	*LRAT, TLR2*
		SFA	0.63	0.32	16		
18	48.0-48.9	C18:0	0.52	0.52	42	32	*ERLIN1, ENSOCUG00000014801 ENSOCUG00000001375*
		SFA	0.55	0.41	2		

[1]Rabbit chromosome, [2]associated region, [3]percentage of the genomic variance explained by the associated region, [4]posterior probability of association, [5]number of relevant SNPs with a Bayes factor higher than 10 in the extended region, [6]number of protein coding genes in the extended region, [7]putative candidate genes related to lipid metabolism retrieved from DAVID database. SFA: Saturated fatty acids = C12:0 + C14:0 + C15:0 + C16:0 + C17:0 + C18:0 + C20:0.

Table 7. Genomic regions associated with monounsaturated fatty acid percentage of *Longissimus thoracis et lumborum* muscle in rabbits.

OCU[1]	Region (Mb)[2]	Trait	VAR[3]	PPA[4]	rSNP[5]	Number of genes[6]	PCG[7]
1	29.1-29.9	C16:1	0.81	0.48	16	3	-
	36.0-36.9	C16:1	0.92	0.34	3	2	-
	121.0-121.9	C18:1	0.63	0.34	2	13	*MTMR2*
		MUFA	0.61	0.38	18		
3	149.0-149.9	C18:1	1.06	0.64	17	11	*ENSOCUG00000000157*
		MUFA	1.38	0.77	18		
4	67.0-67.9	C18:1	0.66	0.41	14	8	-
5	7.2-7.9	C16:1	0.82	0.61	22	1	-
		MUFA	0.99	0.64	22		
6	8-9.9	C16:1	0.71	0.64	22	22	*SMG1*, *GDE1*
		C18:1	1.43	0.50	12		
		MUFA	0.67	0.47	10		
8	21.0-21.9	C16:1	0.63	0.65	8	6	*PIK3C2G*, *PLCZ1*,
		C18:1	0.72	0.57	4		*PLEKHA5*
		MUFA	1.01	0.66	16		
9	12.0-12.5	C16:1	1.05	0.60	17	7	*PPARG*
10	42.2-42.9	MUFA	0.53	0.41	16	1	-
13	126.0-126.9	C18:1	1.28	0.73	9	23	*HEYL*, *MFSD2A*, *PPT1*
		MUFA	0.99	0.69	9		
14	82.0-82.9	C18:1	0.58	0.30	7	14	*ADIPOQ*
17	12.0-13.9	C16:1	1.55	0.66	35	14	*ALDH1A2*, *LIPC*, *MYO1E*
		MUFA	0.81	0.56	36		
18	47.0-48.9	C16:1	1.21	0.43	7	35	*GOT1*, *ERLIN1*,
		C18:1	0.83	0.37	2		*ENSOCUG00000014801*,
		MUFA	1.90	0.53	46		ENSOCUG00000001375
21	10.0-10.9	C18:1	0.72	0.44	6	2	-

[1]Rabbit chromosome, [2]associated region, [3]percentage of the genomic variance explained by the associated region, [4]posterior probability of association, [5]number of relevant SNPs with a Bayes factor higher than 10 in the extended region, [6]number of protein coding genes in the extended region, [7]putative candidate genes related to lipid metabolism retrieved from DAVID database. MUFA: monounsaturated fatty acids = C16:1n7 + C18:1n9 + C18:1n7 + C20:1n11 + C22:1n9.

Table 8. Genomic regions associated with polyunsaturated fatty acid percentage of *Longissimus thoracis et lumborum* muscle in rabbits.

OCU[1]	Region (Mb)[2]	Trait	Var[3]	PPA[4]	rSNPs[5]	Number of genes[6]	PCG[7]
1	29.1-29.9	PUFA	0.56	0.34	15	3	-
	31-31.9	C18:2n6	1.34	0.27	2	7	-
		PUFA	0.6	0.31	16		
	34-37.9	C18:2n6	3.18	0.20	2	13	*ACER2, PLIN2*
		C20:4n6	1.57	0.47	33		
		PUFA	2.41	0.37	11		
2	85.0-85.9	C18:2n6	1.24	0.24	8	3	-
3	25.0-26.9	C18:2n6	6.05	0.65	41	6	*FGF1, NR3C1*
	149.0-149.9	C18:2n6	0.68	0.24	1	8	*ENSOCUG00000000157*
		C20:4n6	0.82	0.92	16		
		PUFA	2.25	0.97	15		
5	7.2-7.9	PUFA	0.91	0.66	22	1	-
6	0-0.9	PUFA	0.55	0.57	14	-	-
	5.0-5.9	C18:2n6	0.61	0.21	8	15	*LITAF, SNX29*
8	21.0-21.9	PUFA	0.78	0.67	14	5	*PIK3C2G, PLCZ1*
	50.0-50.9	C18:2n6	1.06	0.30	-	6	-
	55.3-55.9	C18:2n6	4.77	0.30	11	1	-
		C18:3n3	0.99	0.38	18		
9	63.0-63.9	C18:2n6	0.61	0.23	1	4	-
	64.0-65.9	PUFA	1.9	0.46	30	16	*NPC1, OSBPL1A*
	115.0-115.9	C18:2n6	1.21	0.38	16	3	-
10	43.0-43.9	PUFA	0.58	0.47	17	3	-
12	7.0-7.9	C20:4n6	1.48	0.93	10	8	-
13	33.0-34.9	C18:2n6	3.42	0.49	13	60	*CRP, DCAF8, FCER1A, PIGM, ATP1A2*
14	156.2-156.9	C18:3n3	0.73	0.74	17	3	-
15	10.0-10.9	C18:2n6	2.41	0.39	16	11	*LRAT, TLR2*
		PUFA	0.87	0.53	19		
	70.0-71.9	C18:2n6	3.59	0.50	4	12	*ANXA3, GK2*
16	62.0-62.9	C18:2n6	0.59	0.23	6	9	*HSD11B1*
	71.0-72.9	C18:3n3	1.67	0.43	10	7	*ENSOCUG00000006240*
17	9.0-10.9	C18:2n6	3.87	0.41	21	10	
	12.0-13.9	C18:2n6	3.55	0.46	34	14	*ALDH1A2, LIPC, MYO1E*
		PUFA	0.84	0.61			
18	23.0-23.8	C18:2n6	1.18	0.36	17	8	*NRBF2*
19	21.0-21.9	C18:2n6	0.52	0.21	2	15	*ADAP2*
21	10.0-10.9	C18:3n3	0.55	0.68	6	2	-

[1]Rabbit chromosome, [2]associated region, [3]percentage of the genomic variance explained by the associated region, [4]posterior probability of association, [5]number of relevant SNPs with a Bayes factor higher than 10 in the extended region, [6]number of the protein coding genes in the extended region. [7]putative candidate genes related to lipid metabolism, PUFA: polyunsaturated fatty acids = C18:3n3 + C20:3n3 + C20:5n3 + C22:5n3 + C22:6n3 + C18:2n6 + C18:3n6 + C20:2n6 + C20:3n6 + C20:4n6 + C22:4n6.

Table 9. Genomic regions associated with PUFA/SFA, MUFA/SFA, and n-6/n-3 ratios of *Longissimus thoracis et lumborum* in rabbits.

OCU[1]	Region (Mb)[2]	Trait	Var[3]	PPA[4]	rSNPs[5]	Number of genes[6]	PCG[7]
1	29.1-29.9	PUFA/SFA	0.53	0.32	14	3	-
	31.1-31.9	PUFA/SFA	0.59	0.30	11	7	-
	34.0-37.9	PUFA/SFA	2.98	0.38	10	13	*ACER2, PLIN2*
		n-6/n-3	1.45	0.29	17		
3	118.0-118.9	MUFA/SFA	0.58	0.36	1	11	-
	149.0-149.9	PUFA/SFA	2.45	0.98	15	8	*ENSOCUG00000000157*
5	7.3-7.9	PUFA/SFA	0.84	0.61	22	1	-
6	0.0-0.9	PUFA/SFA	1.09	0.74	14	-	-
8	51.0-51.9	n-6/n-3	0.67	0.42	3	7	-
	53.0-53.8	MUFA/SFA	0.53	0.28	11	14	*DGKH*
9	64.0-65.9	PUFA/SFA	2.44	0.50	38	16	*NPC1, OSBPL1A*
10	5.0-5.6	MUFA/SFA	0.63	0.37	19	4	*ITGB8*
	43.0-43.9	PUFA/SFA	0.52	0.45	8	3	-
14	78.2-78.9	MUFA/SFA	0.52	0.23	5	14	*ATP11B*
15	8.0-8.9	n-6/n-3	0.83	0.48	30	13	-
	10.0-10.9	PUFA/SFA	1.60	0.71	18	11	*LRAT, TLR2*
16	62.0-62.9	MUFA/SFA	0.59	0.17	3	9	*HSD11B1*
	71.0-72.9	n-6/n-3	1.04	0.26	10	7	*ENSOCUG00000006240*
18	11.0-11.9	MUFA/SFA	0.86	0.32	3	5	*SAMD8*
	21.0-21.9	MUFA/SFA	0.93	0.52	22	3	-
	46.0-48.9	MUFA/SFA	7.91	0.59	54	41	*GOT1, ERLIN1 ENSOCUG00000014801, ENSOCUG00000001375*

[1]Rabbit chromosome, [2]associated region, [3]percentage of the genomic variance explained by the associated region, [4]posterior probability of association, [5]number of relevant SNPs with a Bayes factor higher than 10 in the extended region, [6]number of the protein coding genes in the extended region, [7]putative candidate genes related to lipid metabolism retrieved from DAVID database, SFA: Saturated fatty acids = C12:0 + C14:0 + C15:0 + C16:0 + C17:0 + C18:0 + C20:0, MUFA: monounsaturated fatty acids = C16:1n7 + C18:1n9 + C18:1n7 + C20:1n11 + C22:1n9, n-3 = C18:3n3 + C20:3n3 + C20:5n3 + C22:5n3 + C22:6n3, n-6 = C18:2n6 + C18:3n6 + C20:2n6 + C20:3n6 + C20:4n6 + C22:4n6, PUFA: polyunsaturated fatty acids = C18:3n3 + C20:3n3 + C20:5n3 + C22:5n3 + C22:6n3 + C18:2n6 + C18:3n6 + C20:2n6 + C20:3n6 + C20:4n6 + C22:4n6.

On OCU1, a large genomic region at 34.0-37.9 Mb was associated with C14:0, C16:0, C18:0, and SFA, explaining 3.54%, 11.20%, 0.87%, and 11.32% of the genomic variances, respectively (Table 6). The same region was associated with C18:2n6, C20:4n6, PUFA, PUFA/SFA, and n-6/n-3, explaining 3.18%, 1.57%, 2.41%, 2.98%, and 1.45% of the genomic variances, respectively. This genomic region harbours two genes related to lipids metabolism,

alkaline ceramidase 2 (*ACER2*) and perilipin 2 *(PLIN2). ACER2* promotes the hydrolysis of ceramides, the generation of sphingosine, and consequently the biosynthesis of sphingolipids (Spiegel and Milstien, 2003; Xu et al., 2006). Besides, the protein encoded by *PLIN2* covers the intracellular lipid droplets and plays a major role in their stabilization (Xu et al., 2019). In pigs, *PLIN2* was considered as a useful marker for lean growth and its expression was positively associated with IMF content (Gandolfi et al., 2011; Gol et al., 2016). In rabbits, a selection signature for IMF was located in this genomic region, and presented high values for cross population – composite likelihood ratio (XP-CLR) and cross population – extended haplotype homozygosity (XP-EHH) (Sosa-Madrid et al., 2020). Hence, this region harbours genes of pleiotropic effect given its association with the IMF content and its composition. Moreover, this region disclosed two validated SNPs with high BF (Additional file 1). The first SNP at position 34.70 Mb was highly associated with C16:0 (BF = 177) and SFA (BF = 115), while the second SNP at 35.18 Mb was associated with SFA (BF = 59).

On the same chromosome (OCU1), the genomic window at 121.0-121.9 Mb was associated with C14:0, C18:0, C18:1, and MUFA. This region harbours the *MTMR2* gene involved in lipid metabolic processes according to DAVID database. *MTMR2* was also associated with the IMF content in rabbits, the genomic region at 120.6-121.9 Mb on OCU1 accounted for 2.03% of the genomic variance of IMF content (Sosa-Madrid et al., 2019). Besides, *MTMR2* gene was located at a signature of selection for IMF in pigs (Kim et al., 2015).

The genomic region at 25.0-26.9 Mb on OCU3 accounted for 6.05% of the genomic variance of linoleic acid (C18:2n6). Two candidate genes were retrieved, *FGF1* and *NR3C1*. *FGF1* was also considered as a candidate gene for IMF in chickens (Ye et al., 2014), while an eGWAS (expression Genome-Wide Association Study) reported the implication of *NR3C1*

gene in lipid metabolism in pigs (Ballester et al., 2017). On the same chromosome, the genomic region at 149-149.9 Mb was associated with almost all the studied traits except C16:1, C18:3n3, MUFA/SFA, and n-6/n-3. This region includes *ENSOCUG00000000157* novel gene related to lipid biosynthesis. The novel gene is also known as *ST3GAL1* in humans, pigs, and mice. The validated SNPs at this region showed high values of the BF (up to 141 for the ratio PUFA/SFA, 114 for C18:0 and 101 for C14:0) (Additional file 1). Moreover, these associations were corroborated by high values of the posterior probability of association (PPA). The PPA of C14:0, C16:0, and C18:0 were 0.96, 0.98, and 0.99, respectively (Table 6).

The associated genomic region at 7.2-7.9 Mb on OCU5 did not map to genes related to lipid metabolism, however, it encompasses the highest BF. The validated SNP at position 7.43 Mb on OCU5 was associated with C16:0 (BF = 459), C16:1 (BF = 77), PUFA (BF = 193) and the ratio PUFA/SFA (BF = 390) (Additional file 1).

Three different regions on OCU6 were associated with the fatty acid composition. The first region at the start of the chromosome (0-0.9 Mb) was associated with C16:0, PUFA, and PUFA/SFA. However, it did not map to putative candidate genes related to lipid metabolism. The second genomic window at 5-5.9 Mb was associated with C18:2n6, and included two genes related to lipid metabolism (*LITAF* and *SNX29)*. *LITAF* regulates the response to lipopolysaccharide according to gene oncology (GO: 0032496), whereas, *SNX29* affects phosphatidylinositol (family of lipids) binding (GO: 0035091). *SNX29* was also a candidate gene for IMF in pigs (Dong et al., 2014). The third region at 8-9.9 Mb was associated with C16:1, C18:1, and MUFA. This region harbours two genes related to lipid metabolism (*SMG1* and *GDE1)* according to DAVID database.

On OCU8, the genomic region at 21.0-21.9 Mb was associated with C18:0, C16:1, C18:1, MUFA, and PUFA. This region harbours *PIK3C2G*, *PLEKHA5*, and *PLCZ1* genes related to lipid metabolism. *PIK3C2G* affects phosphatidylinositol binding (GO: 0035091), while *PLEKHA5* controls lipid binding (GO: 0032266, GO: 0070273, and GO: 0010314). On the other hand, *PLCZ1* regulates lipid metabolic and catabolic processes (GO: 0006629 and GO: 0016042, respectively). On the same chromosome, the genomic region at 53-53.8 Mb was associated with MUFA/SFA. This region harbours *DGKH* gene involved in triglycerides degradation (Aung et al., 2013). The genomic region at 55.3-55.9 Mb explained 4.77% of the genomic variance of C18:2n6, however, it did not contain putative candidate genes.

The genomic region at 12.0-12.5 Mb on OCU9 accounted for 1.05% of the genomic variance of C16:1. This region harbours *PPARG* gene involved in adipocyte differentiation (reviewed by Janani and Ranjitha Kumari, 2015). *PPARG* was also associated with fat deposition in sheep (Guo et al., 2014). In pigs, two polymorphisms in the transcriptional regulatory region of *PPARG* were related to IMF content (Wang et al., 2013). Moreover, a correlation (0.64) was detected between mRNA *PPARG* and IMF content in Korean cattle (Jeong et al., 2013). On the same chromosome, the region at 64-65.9 Mb was found to be associated with C16:0, SFA, PUFA, and PUFA/SFA. This region harbours *NPC1* and *OSBPL1* genes. *NPC1* is associated with lipid transport (Sleat et al., 2004). On the other hand, *OSBPL1* is involved in lipid transport (GO: 0006869) and binding (GO: 0008289).

On OCU10, the genomic region at 5-5.6 Mb was associated with the ratio MUFA/SFA. This region harbours the *ITGB8* gene related to lipid metabolic process. This gene was also located at a signature of selection for IMF content in pigs (Kim et al., 2015).

Two genomic regions on OCU13 were found to be associated with the fatty acids. The first region at 33-34.9 Mb accounted for 3.42% of the genomic variance of linoleic acid (C18:2n6). This region includes five genes related to lipid metabolism according to DAVID database (*CRP, DCAF8, FCER1A, PIGM, and ATP1A2)*. The second region at 126.0-126.9 Mb was associated with C18:0, C18:1, and MUFA, harbouring *HEYL*, *MFSD2A*, and *PPT1* genes. *MFSD2A* regulates the docosahexaenoic acid (C22:6n3) transport (Nguyen et al., 2014). Both *MFSD2A* and *PPT1* genes were associated with n-3 and n-6 groups in cattle (Feitosa et al., 2018). Validated associated SNPs at this region showed high values of the BF (96 for C18:0, 84 for C18:1, and 72 for MUFA; Additional file 1).

Stearic (C18:0) and oleic (C18:1) acids were associated with the genomic window at 82-82.9 Mb on OCU14. This region harbours the *ADIPOQ* gene that encodes for the adiponectin involved in fatty acids metabolism (Karbowska and Kochan, 2006). *ADIPOQ* was also associated with IMF in cattle (Barendse, 2011). Moreover, a previous study reported a strong correlation between its expression and IMF (Wang et al., 2009). On the same chromosome, the genomic region at 78.2-78.9 Mb was associated with the ratio MUFA/SFA, presenting *ATP11B* gene involved in phospholipid transport (GO: 0015914).

The genomic region at 10-10.9 Mb on OCU15 was associated with C16:0, SFA, C18:2n6, PUFA, and the ratio PUFA/SFA. Two genes related to lipid metabolism mapped to this region (*LRAT* and *TLR2)*. On the same chromosome, the genomic region at 70-71.9 Mb accounted for 3.59% of the genomic variance of linoleic acid (C18:2n6), harbouring *ANXA3* and *GK2* genes related to lipid metabolism.

Two regions on OCU16 were associated with the fatty acids. The first region at 62.0-62.9 Mb was associated with C18:2n6 and MUFA/SFA, harbouring *HSD11B1* gene related to lipid metabolic process. The second region at 71-72.9 Mb was associated with C18:3n3 and n-6/n-3, explaining 1.67% and 1.04% of the genomic variances, respectively. This region harbours *ENSOCUG00000006240* novel gene known as *NR5A2* in humans. *NR5A2* regulates cholesterol homeostasis (GO: 0042632).

On OCU17, the region at 9-10.9 Mb explained 3.87% of the genomic variance of C18:2n6, however, it did not contain candidate genes. Besides, the region at 12-13.9 Mb was associated with C16:1, MUFA, C18:2n6, and PUFA. This genomic region harbours three genes related to lipid metabolism (*ALDH1A2, LIPC,* and *MYOE1). LIPC* regulates lipid metabolic process (GO: 0006629), whereas *MYOE1* controls lipid binding (GO: 0035091).

The genomic region at 11-11.9 Mb on OCU18 was associated with the ratio MUFA/SFA, harbouring *SAMD8* gene related to ceramides biosynthetic process (GO: 0046513). Besides, a large genomic region at 46-48.9 Mb on OCU18 was associated with the majority of the traits, explaining 7.9% of the genomic variance of the ratio MUFA/SFA. This region harbours the following genes: *GOT1, ERLIN1, ENSOCUG00000001375,* and *ENSOCUG00000014801*. *ERLIN1* promotes lipid binding; it encodes for proteins that bind to cholesterol and interacts with the sterol regulatory element binding protein (SREBP). This latter stimulates the cholesterol and fatty acid synthesis in liver (Goldstein et al., 2006; Horton et al., 2002). Moreover, a previous GWAS reported that *ERLIN1* was associated with lipid metabolism in cattle (Zhang et al., 2020). The novel genes *ENSOCUG00000001375* and *ENSOCUG00000014801* are known in pigs and humans as Stearoyl-CoA Desaturase (*SCD*). *SCD* gene is responsible for the biosynthesis of MUFA from SFA. A strong association

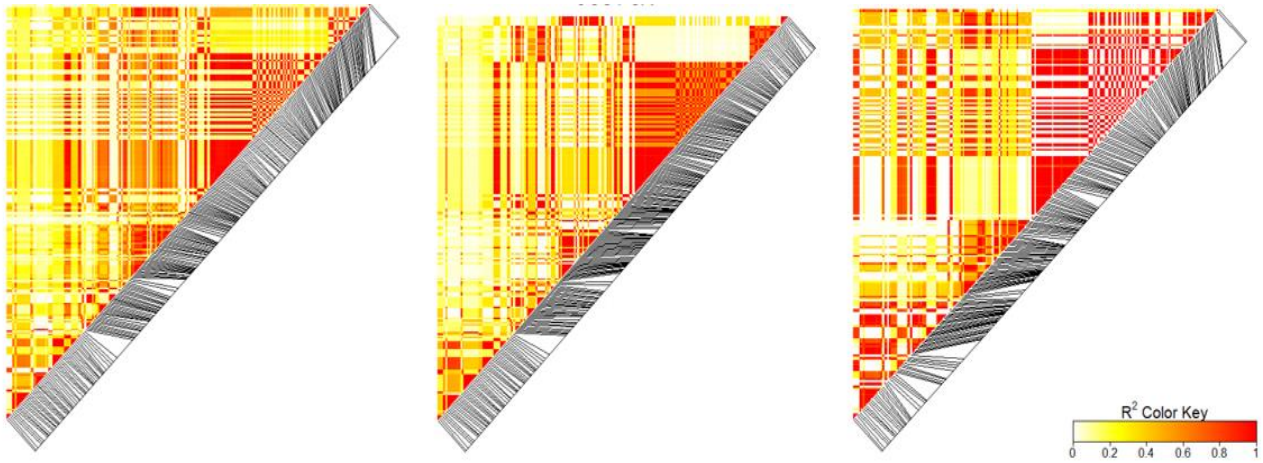
between *SCD* and the fatty acid composition was reported in pigs (Maharani et al., 2013; Ros-Freixedes et al., 2016), cattle (Wang et al., 2019), and goats (Avilés et al., 2016). In addition, an eGWAS study showed a high correlation (0.78) between *SCD* and *PPARG* expressions (Puig-Oliveras et al., 2016).

The genomic region at 21.0-21.9 Mb on OCU19 was associated with linoleic acid (C18:2n6) and disclosed the *ADAP2* gene related to phosphatidylinositol bisphosphate binding (GO: 1902936).

Many other genes were identified in the associated genomic regions; however, their functional annotations did not show a direct relationship with lipids metabolism. The current study identified several regions associated with the fatty acid composition, showing its polygenic nature in rabbits. However, we did not detect genomic regions with mayor effects like those found in pigs (Ros-Freixedes et al., 2016).

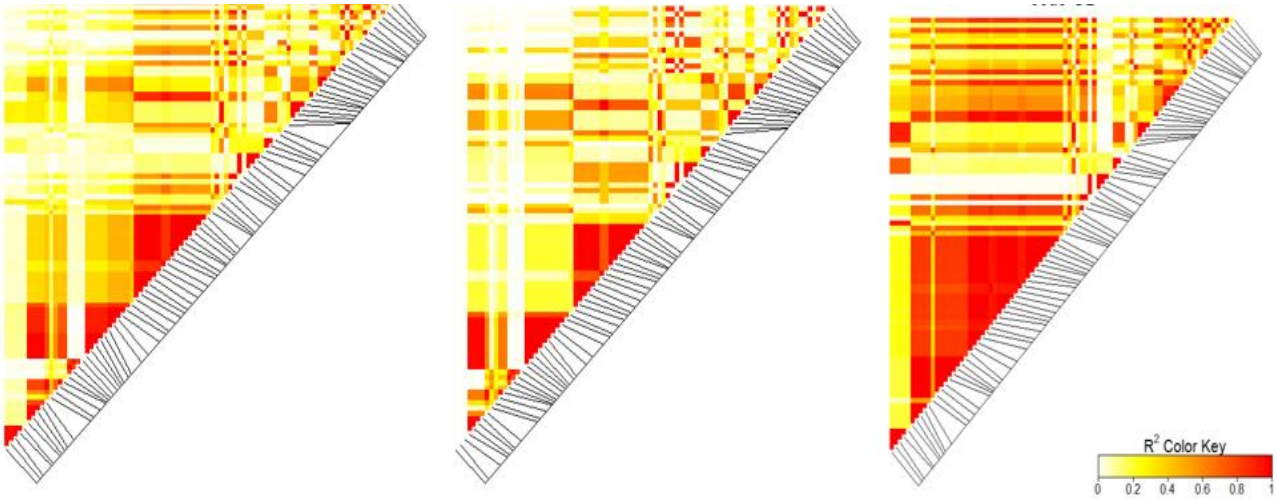
Besides, the linkage disequilibrium (LD) was assessed throughout the most relevant QTLs (at 34-37.9 Mb on OCU1, at 148.5-150.2 Mb on OCU3, and at 46.0-49.2 Mb on OCU18). Different LD patterns were detected (Figures 5, 6, and 7). The extended region at OCU1 displayed several short blocks for both high-IMF and low-IMF lines, indicating the presence of several causal variants. Correlations between SNPs were greater in high-IMF line than in low-IMF line (Figure 5). Besides, the genomic region at 148.5-150.2 Mb on OCU3 displayed different LD patterns between lines. Correlations between SNPs were greater in low-IMF line than in high-IMF line (Figure 6). On the other hand, the QTL at 46.0-49.2 Mb on OCU18 formed a strong block ($r^2 = 1$) for both lines, while correlations between SNPs were greater

for high-IMF line (Figure 7). Thus, the QTLs were exposed to different selection pressures for IMF.



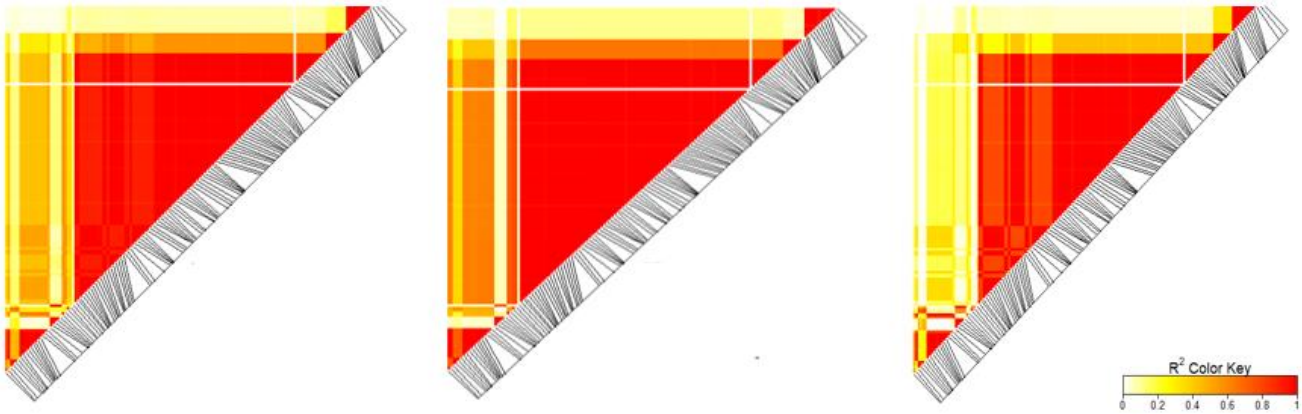
(a) **(b)** **(c)**

Figure 5. Linkage disequilibrium plot at 34-37.9 Mb on chromosome 1. Colours from white to red indicate r^2 value. (a) All lines. (b) High-IMF line. (c) Low-IMF line.



(a) (b) (c)

Figure 6. Linkage disequilibrium plot at 148.5-150.2 Mb on chromosome 3. Colours from white to red indicate r^2 value. (a) All lines. (b) High-IMF line. (c) Low-IMF line.



(a) (b) (c)

Figure 7. Linkage disequilibrium plot at 46.0-49.2 Mb on chromosome 18. Colours from white to red indicate r^2 value. (a) All lines. (b) High-IMF line. (c) Low-IMF line.

Taken together, the present study underlined different genomic regions associated with the fatty acid composition in rabbits. The results were corroborated by high values of the BF. Manhattan plots of SFA, PUFA, and MUFA groups were displayed in Figures 8, 9, and 10, respectively. The present study highlighted also the polygenic nature of the fatty acid composition and its complexity in rabbits. Similar findings were reported by previous studies for the fatty acid composition in pigs (Ros-Freixedes et al., 2016; Zhang et al., 2016) and cattle (Cesar et al., 2014; Wang et al., 2019). To our knowledge, this is the first GWAS for the fatty acid composition in rabbits. A pervious study was performed on the IMF content (Sosa-Madrid et al., 2019). Comparing the results, *MTMR2* is the only gene that was associated with both IMF content and its composition. In addition, *ACER2* and *PLIN2* genes were associated with the fatty acid composition and were also detected at a selection signature for IMF content (Sosa-Madrid et al., 2020).

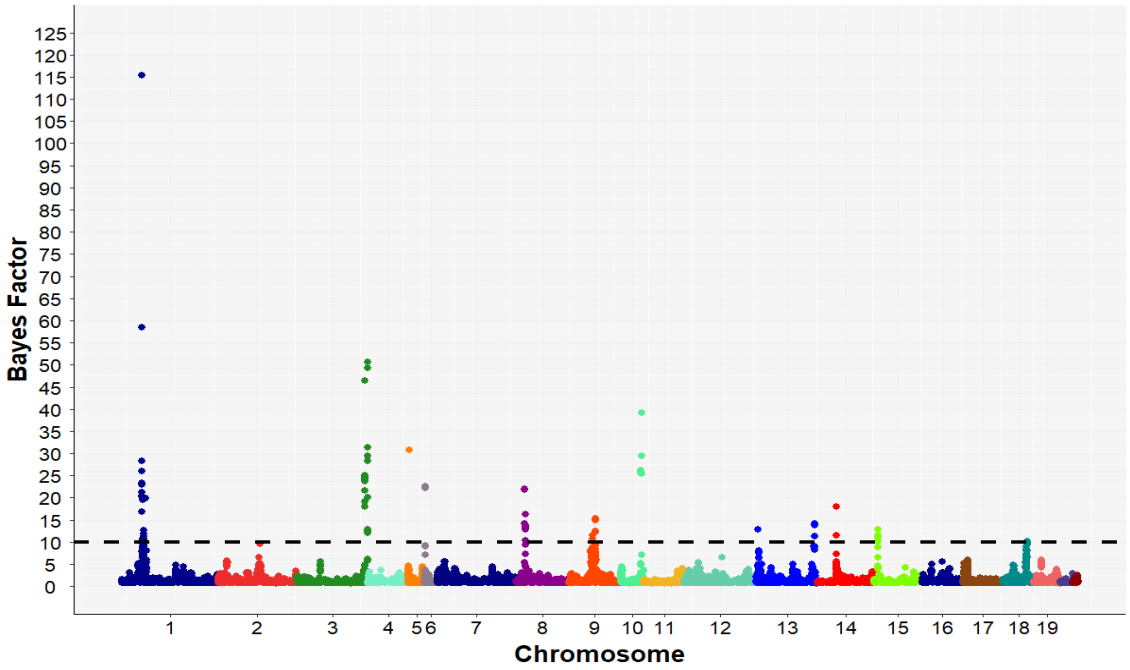


Figure 8. Manhattan plot of genome-wide association study for saturated fatty acids.

The black dashed line indicates the Bayes factor threshold of 10. Each dot represents a SNP. X-axis: chromosomes. Y-axis: Bayes factor.

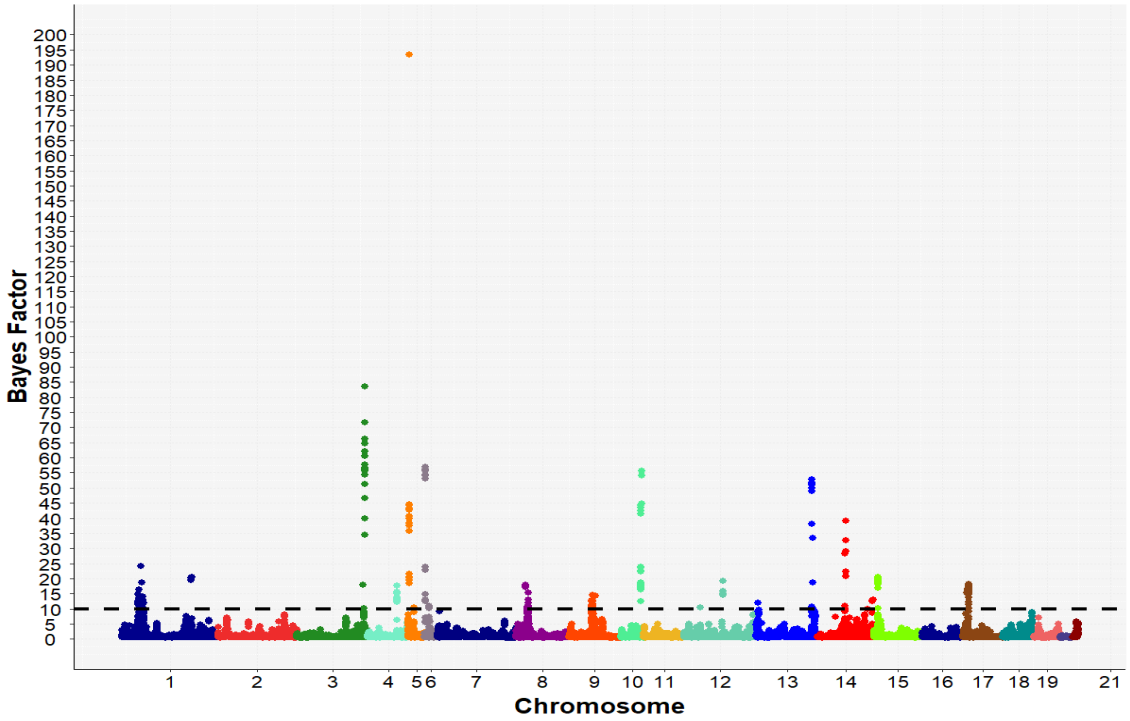


Figure 9. Manhattan plot of genome-wide association study for polyunsaturated fatty acids.

The black dashed line indicates the Bayes factor threshold of 10. Each dot represents a SNP. X-axis: chromosomes. Y-axis: Bayes factor.

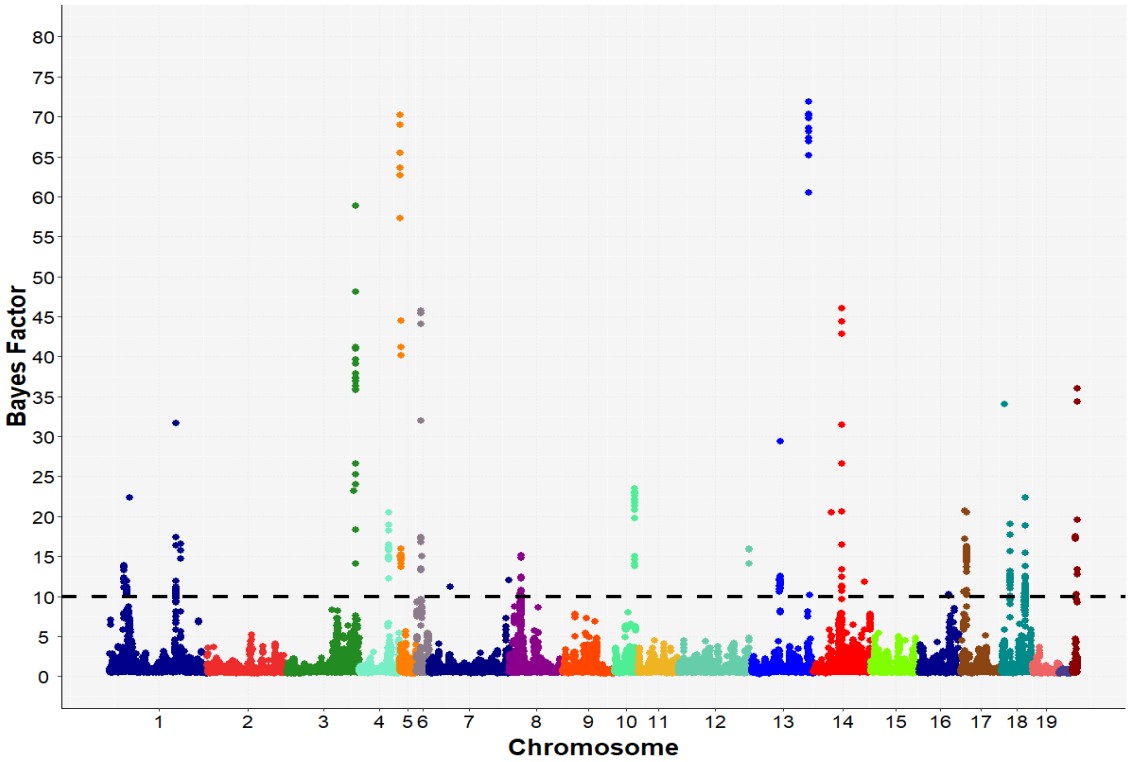


Figure 10. Manhattan plot of genome-wide association study for monounsaturated fatty acids. The black dashed line indicates the Bayes factor threshold of 10. Each dot represents a SNP. X-axis: chromosomes. Y-axis: Bayes factor.

CONCLUSIONS

Association analyses for the fatty acid composition were carried out on two rabbit lines divergently selected for IMF. Several associated regions were detected. The most relevant regions were found at 34-37.9 Mb on OCU1, at 149.0-149.9 Mb on OCU3, and at 46.0-48.9 Mb on OCU18. The associated regions disclose several genes related to lipid metabolism such as *SCD*, *PLIN2*, *ERLIN1*, and *PPARG*. The main genomic regions in which we found genes related to lipid metabolism were not detected in our previous experiment for IMF. *MTMR2* is the only gene that was associated with both the IMF content and its composition. To our knowledge, this is the first GWAS for the fatty acid composition in rabbits. Our study highlighted the polygenic nature of the fatty acid composition. Further analyses throughout the associated regions would be needed in order to validate their importance.

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CONCLUSIONS

The current study was carried out on two rabbit lines divergently selected for Intramuscular fat (IMF) in *Longissimus thoracis et lumborum* muscle (LM). First, we evaluated the direct and correlated responses to selection for IMF in the ninth generation of selection. Second, we performed a Genome-Wide Association Study (GWAS) in order to identify genomic regions associated with IMF composition in rabbits and identify candidate genes.

Our study has shown that:

1. Selection improved the IMF content of LM in rabbits. The direct response to selection for IMF was 0.51g/100g of LM, representing 3.3 phenotypic standard deviations.
2. Selection for IMF content led to changes in its fatty acid composition. The correlated response to selection was positive for saturated (SFA) and monounsaturated (MUFA) fatty acids percentages and was negative for polyunsaturated fatty acids (PUFA) percentage (-4.96%). Most individual SFA were higher in high-IMF line except heptadecanoic (C17:0) and stearic (C18:0) acids. Besides, most individual PUFA were higher in low-IMF line with the exception of α-linolenic acid (C18:3n3).
3. The genomic regions associated with the fatty acid composition were spread across different rabbit chromosomes, showing its polygenic nature. The most relevant regions were found at 34-37.9 Mb on OCU1, at 149.0-149.9 Mb on OCU3, and at 46.0-48.9 Mb on OCU18.
4. The associated regions harbour various genes related to lipid metabolism such as *SCD, PLIN2,* and *ERLIN1*… Compared to the previous GWAS for IMF content, *MTMR2* is the only gene that was associated with both the IMF content and composition.

To our knowledge, this is the first GWAS for intramuscular fatty acid composition in rabbits. Further analyses would be necessary to validate our results.

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ADDITIONAL FILES

Table. Relevant validated SNPs associated with the fatty acid composition

SNP[1]	OCU[2]	Position (Mb)	Trait (BF)
Affx-151830797	1	34705560	C16:0 (177), SFA (115)
Affx-151927561	1	35179856	SFA (59)
Affx-151792344	3	149055073	C14:0 (69), C16:0 (84), PUFA (84), PUFA/SFA (141)
Affx-151888563	3	149077577	C14:0 (57), C16:0 (56), PUFA (54)
Affx-151792814	3	149087928	C16:0 (52), C18:0 (114), C20:4 (69)
Affx-151975641	3	149101068	C16:0 (69), C20:4 (50), PUFA/SFA (51)
Affx-151949682	3	149115014	C14:0 (59), C16:0 (64), C18:0 (57), PUFA (56), PUFA/SFA (52), C20:4 (55)
Affx-151994687	3	149134444	C16:0 (57), C18:0 (59), PUFA (51), PUFA/SFA (58), C20:4 (55)
Affx-151920839	3	149197645	C14:0 (56), C16:0 (54), C18:0 (70), C20:4 (51), PUFA (56), PUFA/SFA (58),
Affx-151789023	3	149207220	C14:0 (101), C16:0 (65), C18:0 (104), C20:4 (83), MUFA (59), PUFA (72), PUFA/SFA (62)
Affx-152003840	3	149217588	C14:0 (59), C16:0 (58), C18:0 (51), PUFA (65), PUFA/SFA (60), C20:4 (54)
Affx-151786411	3	149234804	C14:0 (58), C16:0 (55), C18:0 (61), C20:4 (52), PUFA (61), PUFA/SFA (56)
Affx-151793488	3	149240549	C14:0 (56), C16:0 (62), C18:0 (51), C20:4 (52), PUFA (58), PUFA/SFA (56)
Affx-151894635	3	149251760	C16:0 (51), C18:0 (51)
Affx-151868763	3	149264636	C14:0 (58), C16:0 (53), C18:0 (62), PUFA (66), PUFA/SFA (51)
Affx-151934711	3	149273765	C14:0 (58), C16:0 (50), C18:0 (58), C20:4 (52), PUFA (62), PUFA/SFA (53)
Affx-151907463	3	149286297	C16:0 (60), C18:0 (59), PUFA (57), C20:4 (53)
Affx-151923390	3	154972330	SFA (51)
Affx-151847514	4	67788761	C18:0 (51)
Affx-152012838	4	67872134	C18:0 (51)
Affx-151814852	4	67904655	C18:0 (52)
Affx-151914628	4	67923053	C18:0 (51)
Affx-151810058	4	67929173	C18:0 (65)
Affx-151880575	5	7431430	C16:0 (459), PUFA (193), PUFA/SFA (390), C16:1 (77)
Affx-151831838	6	549906	PUFA (53), PUFA/SFA (64)
Affx-151791100	6	657737	C16:0 (67), PUFA (54), PUFA/SFA (83)
Affx-151842345	6	664526	C16:0 (65), PUFA (54), PUFA/SFA (82)
Affx-151992141	6	684896	C16:0 (61), PUFA (56), PUFA/SFA (81)
Affx-151791216	6	693029	C16:0 (71), PUFA (56), PUFA/SFA (83)
Affx-151826602	6	698127	C16:1 (71), PUFA (56), PUFA/SFA (85)
Affx-151889938	6	733376	C16:0 (69), PUFA (57), PUFA/SFA (83)
Affx-151945410	6	740441	C16:0 (69), PUFA (54), PUFA/SFA (78)

Affx-151823382	6	1146191	C14:0 (132)
Affx-151789305	6	1161738	C14:0 (132)
Affx-151932297	6	1175389	C14:0 (129)
Affx-151997763	6	1215639	C14:0 (140)
Affx-151864289	8	51740066	N6/N3 (88)
Affx-151863229	8	51756376	N6/N3 (100)
Affx-151990338	8	51761577	N6/N3 (88)
Affx-151915915	9	115376710	C18:3 (51)
Affx-151854394	9	115384314	C18:3 (50)
Affx-151818021	9	115406129	C18:3 (50)
Affx-152018237	10	43045953	C14:0 (56)
Affx-151916647	10	43067880	C14:0 (56)
Affx-151937052	10	43090348	C14:0 (56)
Affx-151897718	10	43124828	C14:0 (52)
Affx-151875091	10	43139227	C14:0 (55)
Affx-151818186	10	43664397	PUFA (56)
Affx-151962520	10	43699274	PUFA (54), PUFA/SFA (52)
Affx-151801407	12	7091872	C20:4 (105)
Affx-151800822	12	7143734	C20:4 (115)
Affx-151949474	12	7216496	C20:4 (66)
Affx-151793001	12	7472389	C20:4 (124)
Affx-152017966	12	7517424	C20:4 (89)
Affx-151921449	12	7562895	C20:4 (90)
Affx-151872137	12	7573695	C20:4 (86)
Affx-151821085	12	7639744	C20:4 (79)
Affx-151824799	12	7656657	C20:4 (82)
Affx-151827797	13	126467634	C14:0 (51), C18:0 (91), C18:1 (84), MUFA (65)
Affx-151853066	13	126472947	C14:0 (57), C18:0 (90), C18:1 (73), MUFA (72), PUFA (51)
Affx-151826588	13	126480058	C14:0 (56), C18:0 (90), C18:1 (73), MUFA (69), PUFA (51)
Affx-151855184	13	126492873	C14:0 (58), C18:0 (95), C18:1 (72), MUFA (70)
Affx-151824219	13	126507986	C14:0 (55), C18:0 (85), C18:1 (68), MUFA (70), PUFA (52)
Affx-151888968	13	126521481	C14:0 (54), C18:0 (89), C18:1 (68), MUFA (67), PUFA (53)
Affx-151886979	13	126529738	C14:0 (60), C18:0 (88), C18:1 (72), MUFA (70), PUFA (51)
Affx-151921036	13	126557366	C14:0 (59), C18:0 (84), C18:1 (75), MUFA (67), PUFA (51)
Affx-151976256	13	126562498	C14:0 (57), C18:0 (96), C18:1 (70), MUFA (68)
Affx-152015424	13	127805799	C18:0 (58), C18:1 (68), MUFA (61)

Affx-151954273	13	128419505	C16:1 (51)
Affx-152014349	13	128426279	C16:1 (50)
Affx-151882633	13	128741930	C14:1 (93), C16:1 (140)
Affx-151912621	14	156287074	C18:3 (66)
Affx-151999519	18	42619650	C16:1 (58)
Affx-152000019	21	10314592	C18:1 (127), C18:3 (344), C20:4 (118)
Affx-151797748	21	10323986	C18:1 (128), C18:3 (357), C20:4 (121)

[1]Validated SNPs with a Bayes factor greater than 50. [2]Rabbit chromosome. BF: Bayes factor. SFA: Saturated fatty acids= C12:0 + C14:0 + C15:0 + C16:0 + C17:0 + C18:0 + C20:0. MUFA: monounsaturated fatty acids = C16:1n7 + C18:1n9 + C18:1n7 + C20:1n11 + C22:1n9, n-3 = C18:3n3 + C20:3n3 + C20:5n3 + C22:5n3 + C22:6n3. n-6 = C18:2n6 + C18:3n6 + C20:2n6 + C20:3n6 + C20:4n6 + C22:4n6. PUFA: polyunsaturated fatty acids = C18:3n3 + C20:3n3 + C20:5n3 + C22:5n3 + C22:6n3+ C18:2n6 + C18:3n6 + C20:2n6 + C20:3n6 + C20:4n6 + C22:4n6.