

Study of seasonal weight loss tolerance in small ruminants – an NMR-metabolomics approach

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ABSTRACT

Small ruminants are very important in the tropics and the Mediterranean. In these regions, pasture scarcity during the dry season leads to Seasonal Weight Loss (SWL), one of the major problems in ruminant production. However, some breeds present higher tolerance to SWL. Understanding the physiological mechanisms by which breeds are able to cope with SWL is of utmost importance in animal selection. We studied two dairy goat breeds (*Majorera* and *Palmera*) and three breeds of meat-producing sheep (*Merino*, *Dorper* and *Damara*), with different levels of tolerance to SWL. The aim of this study was to characterize the metabolome of the mammary gland and milk in these two goat breeds, as well as the metabolome of muscle and liver in sheep in order to obtain a broad view of SWL tolerance physiology in animals with different production aptitudes. We used Nuclear Magnetic Resonance (NMR) to assess metabolome profiles and Gas Chromatography to determine the fatty acid composition. Goats and sheep were studied in two different work tasks. In both species, animals were divided in control and restricted-fed groups for the different breeds involved.

The NMR-metabolomics approach was shown to be adequate for the reproducible assessment of the NMR-visible aqueous metabolome of milk, mammary gland, muscle and liver of small ruminants leading to the identification of respectively 50, 46, 51 and 46 metabolites. Integration of the NMR results with data from other methodological approaches was essential for the creation of a more comprehensive analysis.

Concerning the dairy goat results, significant differences were observed in the metabolites of mammary gland and milk between control and

restricted groups, albeit with no differences between breeds. Observed differences in metabolic pathways are related to adaptation to the low-energy diet. Fatty acid analysis revealed interaction between breed and feed-restriction in mammary gland. *Majorera* breed showed more variations in metabolites, however no variations were observed in fatty acid profile. This breed seems to have higher tolerance to the low nutritional conditions while maintaining the body fatty acid depots. In the context of SWL tolerance, the *Majorera* breed was confirmed to be more tolerant to feed-restriction and should thus be preferable for breed selection in SWL prone regions.

Regarding the sheep results, *Dorper* showed fewer changes on both tissues, whereas *Merino* presented more differences between control and restricted-fed groups, mainly related with fat and protein mobilization. *Damara* presented an intermediate response, with differences in amino acids composition in muscle and in energy-related pathways in liver. Metabolic variations in *Damara* breed are a reflection of its, unique body characteristic, the fat-tail. *Dorper* and *Damara* breeds are more efficiently adapted to feed-restriction, changing the nutritional energy source without compromising the overall muscle structure. These breeds are more tolerant to SWL conditions, and thus more suitable for selection in harsh environmental conditions.

Results confirm the influence of feed-restriction, as a major promoter of SWL, in small ruminant's milk and meat production. In general, tolerant breeds performed differently under feed-restriction and proved to be more suitable for breed selection, especially in harsh environmental conditions. Under feed-restriction conditions, tolerant dairy goat breed (*Majorera* breed) kept the mammary gland structure and function,

whereas meat producer sheep breeds (*Dorper* and *Damara* breeds) maintained muscle structure.

RESUMO

Os pequenos ruminantes são um importante recurso alimentar e económico nas regiões tropicais e mediterrânicas. Durante a estação seca as pastagens tornam-se escassas e pouco nutritivas, levando os animais à Perda de Peso Sazonal (PPS). Nestas regiões a PPS é a maior limitação na produção de pequenos ruminantes. No entanto algumas raças apresentam maior tolerância à PPS, tornando-as particularmente interessantes para a compreensão dos mecanismos fisiológicos de adaptação a esta condição. Os resultados contribuirão para a definição de estratégias de seleção de raças mais adequadas às regiões áridas.

Neste trabalho foram estudadas duas raças de cabras leiteiras (raça *Majorera* e raça *Palmera*) e três raças de ovinos produtores de carne (*Merino*, *Dorper* e *Damara*), com diferentes níveis de adaptação à PPS. Com o objetivo de estudar os mecanismos fisiológicos de tolerância à PPS, foram caracterizados os metabolomas da glândula mamária e do leite das raças caprinas, e do fígado e músculo-esquelético das raças ovinas produtoras de carne. Foram utilizadas técnicas de Ressonância Magnética Nuclear (RMN) para a determinação dos perfis metabólicos, e de Cromatografia Gasosa para caracterização dos perfis de ácidos gordos. As raças caprinas e ovinas foram estudadas separadamente, e os animais de cada raça divididos em grupos controlo e de restrição alimentar.

A abordagem metabolómica por RMN confirmou ser adequada para a caracterização do metaboloma aquoso do leite, glândula mamária,

músculo e fígado em pequenos ruminantes, permitindo a identificação de 50, 46, 51 e 46 metabolitos respetivamente.

Relativamente às raças caprinas, foram identificadas diferenças nos perfis metabólicos entre grupos controlo e em restrição alimentar, em ambas as amostras. As principais diferenças observadas estão relacionadas com adaptações metabólicas à restrição nutricional. No entanto, não se observaram diferenças entre raças. Foi observada interação entre a raça e a restrição alimentar nos perfis de ácidos gordos da glândula mamária, sendo a raça *Palmera* mais suscetível ao tratamento.

A raça *Majorera* não apresentou variações nos perfis de ácidos gordos, no entanto foi a raça com mais variações nos perfis metabólicos. Estes resultados poderão indicar uma maior tolerância à restrição alimentar, uma vez que não foram solicitadas as principais reservas energéticas corporais. Em geral, a raça *Majorera* é mais tolerante à restrição alimentar, sendo por isso mais adequada à otimização de raças.

Relativamente às raças ovinas, a raça *Dorper* apresentou menos variações em ambos os tecidos. Por outro lado, a raça *Merina* foi a que apresentou mais variações entre o grupo control e o grupo de restrição alimentar, estando na sua maioria relacionadas com a mobilização de proteínas e de tecido adiposo. A raça *Damara* apresentou uma resposta intermédia entre as anteriormente mencionadas, com diferenças na composição muscular e nas vias metabólicas relacionadas com a produção de energia. As diferenças observadas nesta raça refletem a sua principal característica distintiva, a reserva adiposa na cauda (cauda-gorda). As raças *Dorper* e *Damara* demonstraram maior capacidade adaptativa à restrição alimentar através da produção de

energia por vias metabólicas alternativas, sem comprometer a estrutura muscular. Estas raças são as mais tolerantes às condições relacionadas com a PPS e, por isso, mais adequadas à seleção animal.

Os resultados deste trabalho confirmam a forte influência da restrição alimentar na produção de leite e carne em pequenos ruminantes. Em geral, as raças tolerantes responderam de forma diferente à restrição alimentar. Nestas condições, a raça caprina leiteira tolerante à PPS (raça *Majorera*) manteve a estrutura e função da glândula mamária, enquanto que as raças ovinas produtoras de carne tolerantes à PPS (raças *Dorper* e *Damara*) conservaram a estrutura muscular.

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1

INTRODUCTION

1.1 THE IMPORTANCE OF RUMINANTS IN WORLD AGRICULTURE AT A GLANCE

Animal-origin foods played a key role in human evolution and development, mainly after livestock domestication. Presently, livestock is the main source of food from animal origin and an important resource to overcome the worldwide increasing need for food production^{1,2}. In developed countries demand is mainly driven by population and urbanization growth and increasing purchasing power³. For poor populations, livestock has critical relevance, being a valuable resource of food, income, hides and fibre, manure and even draught power. In these communities livestock can also serve as financial instruments and enhance social status^{1,2}. Therefore, livestock production can then be seen in two contradictory ways. In developed countries, livestock can increase significantly the greenhouse gases emission, water consumption, use of arable land³, and effluent production⁴. On the other hand, in developing countries, livestock can be essential for community survival^{1,2}. However, this gap could be diminished with production improvement. Breeding strategies could be defined to select breeds more adapted to specific environmental conditions and with higher adaptation to harsh conditions. The improvement of these breeds could result in the need of fewer animals for the same production yields, with improved animal welfare and reduction of gas emission⁵.

Ruminants are one of the major livestock groups, and encompass big ruminants: cattle (*Bos taurus* and *Bos indicus*) and buffaloes (*Bubalus bubalis*); and small ruminants: sheep (*Ovis aries*) and goats (*Capra hircus*). These animals are important sources of meat, milk^{6,7}, wool and fibre⁷ and skins production. In Mediterranean, Tropical and Sub-Tropical climate regions, goat and sheep have had an important role in economy, culture and religion⁸⁻¹⁰. In some countries small ruminants can be particularly appropriate for harvesting due to their profitable characteristics and adaptability to climate conditions (dry lands), geography (mountains) or the taste of regional products (traditional and gourmet)⁸.

Ruminants are mainly characterized by their digestive physiology that is very different from monogastric animals. Although the system has been broadly discussed and described, because it is of importance to the interpretation of some of the results obtained, we will briefly mention it in the next section.

1.2 RUMINANT DIGESTIVE AND MAMMARY PHYSIOLOGY: A BRIEF OVERVIEW

Ruminants have four stomach compartments: the reticulum, the rumen, the omasum, and the abomasum (Figure 1.1). The reticulum and the rumen are adjoining stomach compartments and the largest part the stomach. Feed enters to rumen through the oesophagus and is fermented by microbial activity. Rumen microbiota includes bacteria, protozoa, and fungi. Then, a cud is later regurgitated and remasticated. The digesta (feed being digested) is then swallowed to the omasum,

where the particle size is reduced and the excess of water is removed. The fourth compartment of the stomach is the abomasum, and is usually called “true stomach” due to the occurrence of acidic and enzymatic digestion. After that, digesta passes to the small intestine, where the digestive process is concluded¹¹.

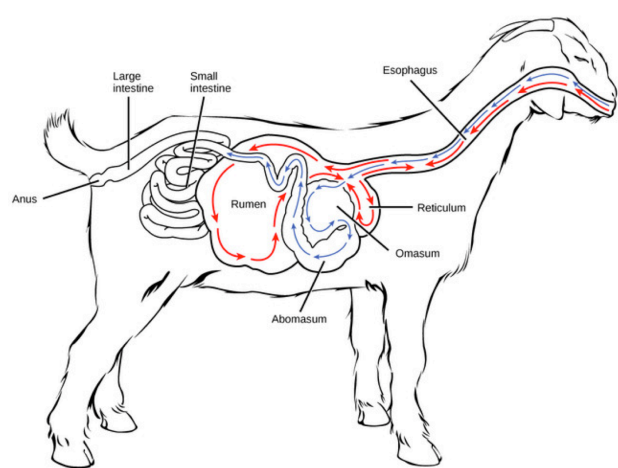


Figure 1.1. General scheme of the ruminant (goat) digestive system. ((Boundless (2016). Boundless Biology. Retrieved from <https://www.boundless.com/biology/textbooks/boundless-biology-textbook/animal-nutrition-and-the-digestive-system-34/digestive-systems-195/vertebrate-digestive-systems-748-11981/>) and (<https://creativecommons.org/compatiblelicenses/>)).

In adult ruminants, due to this particular digestive process, the diet composition can strongly affect the final digestion products. Diet composition affects the microbiota profile of the rumen and consequently carbohydrate, protein and lipid composition of final digestion products. The diet fibre content is also determinant and could affect the ratio of the three major ruminant digestion products: acetate, butyrate and propionate. Due to the low metabolization of these compounds by the rumen wall, they can reach blood stream and through it, other parts of the organism¹¹.

Milk composition can also be affected by rumen digestion products through blood stream and mammary gland. Mammary gland has a very complex anatomy and physiology (Figure 1.2). Briefly, milk is secreted by alveolar epithelial cells into the lumen and then released through the ducts. Milk constituents could arise from five pathways: I) the exocytotic pathway, responsible for the secretion of water, lactose, oligosaccharides, phosphate, calcium and citrate; II) the lipidic secretion pathway responsible for the synthesis of triacylglycerides and phospholipids; III) the transcytotic pathway, whereby proteins and macromolecules are transported; IV) the membrane transport pathway whereby ions and small molecules are transferred from blood to milk; and V) the paracellular transport pathway, responsible for direct movement of molecules between milk and interstitial space, but could be inactive during lactation in most of the species¹². For more details on mammary gland morphology and physiology, particularly in small ruminants, kindly refer to the review by Lérias et al. (2013)¹³.

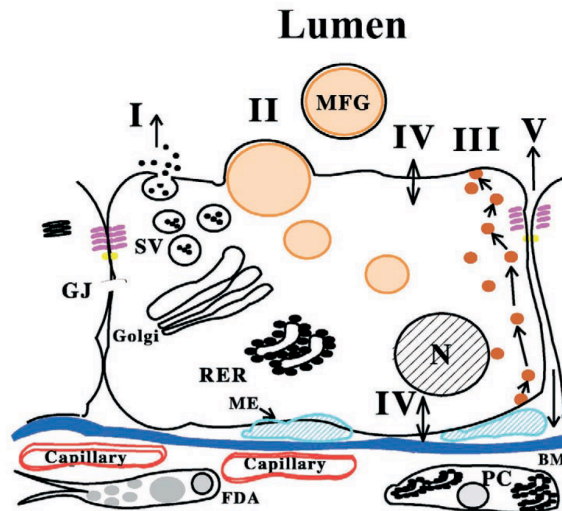


Figure 1.2. Diagram of the alveolar epithelial cell and the pathways for milk secretion. Pathways: I) exocytotic secretion, II) fat secretion, III) vesicular transcytosis, IV) membrane transport, V) paracellular transport. Key: (SV) secretory vesicle; (RER) rough endoplasmic reticulum; (BM) basement membrane; (N) nucleus; (PC) plasma cell; (FDA) fat depleted adipocyte; (JC) junctional complex including the tight and adherens junctions; (GJ) gap junction; (ME) myoepithelial cell; (MFG) milk fat globule. Adapted from McManaman and Neville, 2003.

1.3 SEASONAL WEIGHT LOSS AND ANIMAL PRODUCTION

In the tropics and the Mediterranean areas, animal production varies considerably throughout the year. In those regions, the existence of two different seasons (rainy and dry), regulate pasture availability, affecting animal production. In fact, during the dry season, due to the scarcity of pastures, animals may lose up to 40% of their body weight, a condition usually known as Seasonal Weight Loss (SWL)^{14,15}. SWL is one of the major drawbacks in animal production^{16–21}, as had been demonstrated in South Africa^{16,17}, Western Africa^{18,19}, Western Australia²⁰ and the Canary Islands^{22–24}. Meat and milk production is strongly affected by SWL. In general, quantity and quality decrease and reduce income. To counter

the effects of SWL, farmers use supplementation to balance the nutritional need of the animals. However, supplementation is expensive and difficult to implement in extensive production systems in developing countries or remote locations. An alternative method for addressing the effects of SWL is the use of breeds naturally adapted to this constraint or selected by domestication, that are able to thrive and more effectively produce in such difficult environments.

SWL topic was extensively reviewed recently and the effects of SWL in ruminants, pig and rabbits was summarized. As major sources of protein products, these species were mainly studied by proteomics approaches. Feed-restriction was used as major promoter of the SWL conditions. Identified biomarkers candidates of SWL tolerance in these animals are related with protein (muscle breakdown) and fatty acid metabolisms. During lactation and meat production animals can experience negative energy balances that are exacerbated by the pasture scarcity, increasing SWL affects²⁵. However, depending on the species and breed, animals had developed different adaptations to cope with SWL.

1.3.1 Goats and Seasonal Weight Loss

The goat versatility to adapt and produce in harsh environmental conditions are due to specific attributes of the species that can distinguish it from the other ruminants. Goats have a relatively small body size, low metabolic requirements, capacity to reduce metabolism, ability to change feeding strategies, an efficient use of high-fibre forages, the capacity to reduce nitrogen needs, and they can make an efficient use of water²⁶. Besides, goat feeding behaviour seems also to vary between

breeds and genotypes, enhancing their adaptation capacity²⁷. Therefore, goats seems to be the domestic ruminant specie more tolerant to harsh environments^{27,28} and can increase the resilience of populations more exposed to the effect of severe changes in climate, economy and health, and provide a way to maintain assets²⁹, particularly in Western and Southern Africa³⁰.

The interest for goat dairy products has increased significantly worldwide³¹, especially in developed countries as a healthier substitute for bovine milk, with low allergenic risks and important nutritional properties^{32,33}, and as gourmet ingredients⁸.

1.3.2 Sheep and Seasonal Weight Loss

The capacity of sheep to tolerate harsh environments is mainly ensured by efficient fat deposition and adaptability to different feed sources. Animals can store energy supplies in adipose tissues, in periods during which food is abundant and of good quality^{20,34,35}. During scarcity periods, these supplies can be mobilized to meet energy demands, allowing animals to cope and survive dry seasons, pregnancy and lactation³⁴. Fat-tailed and fat-rump sheep breeds are the most representatives of this adaptation. Since tail and rump fat depots are the most affected by nutritional variations, they can be used as indicators of the tolerance level of the animal³⁴. Some works followed this approach to study the effect of nutritional restriction in sheep^{20,21,35–37}.

Sheep production is one of the major commercial activities in some countries of the Southern hemisphere, such as Australia, New Zealand, Argentina or South Africa^{10,38}.

Understanding the biochemical and physiological mechanisms by which, such breeds are able to cope with SWL, could result in an efficient production process that could include aspects such as increasing of production yields, earlier detection of pathologies and a more sensitive quality control. A good management of these breeds could also contribute to better policies of water and land use and climate change control. Farming animals more adapted to the environmental conditions will also contribute to enhanced animal welfare. In this context, metabolomics-based studies allow the analysis of the metabolome of a given sample and the correlation with the other Omics areas, contributing to a more comprehensive overview of the animal physiology and production potential³⁹.

1.4 THE USE OF METABOLOMICS AS AN ANALYTICAL TOOL

Metabolomics studies are generally concerned with the identification and quantification of small metabolites (compounds of low-molecular weight, MW<1.5 kDa) that result from several metabolic pathways. The metabolome is directly related to gene expression, protein activity and/or the metabolic activities^{40–42}, and is highly complementary to other Omics-based studies such as Proteomics and Transcriptomics, contributing to a more comprehensive overview of the animal physiology and production potential³⁹.

Most of the metabolomics studies are developed using Mass Spectrometry (MS) or Nuclear Magnetic Resonance (NMR) techniques. Each methodology has specific features that could represent advantages or disadvantages. The selection of the technique that better fits each

work is then depended on the objectives to be achieved⁴³. Usually, due to their complementary character, both techniques are used. In this study we selected the NMR technique to assess aqueous NMR-visible metabolome of our samples, and a comparison with MS will not be pursued.

NMR technique is based on the physical properties of atomic nuclei, specifically spin and nuclear magnetism. The spin of a nucleus is a quantum property that can exist in two states that can be thought of as a bar magnet pointing up or down. Spin can be naively visualized, like a planet rotating along its axis. Nuclear magnetism is the capacity of the nuclear particles to act as magnets and interact with other magnetic fields. These external magnetic fields can come from the molecular environment (other nuclei or electrons of the same molecule), or external sources like the magnetic field produced by an NMR spectrometer. The magnetic states generated by the two spin orientations are affected by the magnetic field in opposite ways and this results in an energy difference between them. These different spin states can be inter-converted by absorbing and re-emitting electromagnetic radiation of a specific frequency. These physical properties can be translated to chemical information in the forms of resonance frequency (chemical shift) and spin coupling (J-coupling). Chemical shift represents the slight differences in resonance frequency due to the magnetic microenvironment generated by the neighbouring nuclei and electrons while J-couplings are due to the direct magnetic interactions of chemically bonded nuclei. Therefore, chemical shift carries information about the chemical surroundings of each nucleus, and the J-coupling contains information of the directly bonded nuclei. In addition, the signal intensity is directly proportional to the number of atoms that give rise to it

and the signal can therefore be used for quantitation⁴⁴. Finally it should be noted that the biologically relevant isotopes that are ideal for NMR are ^1H , ^{13}C , ^{15}N and ^{31}P of which we will only be dealing to the first two, with the vast majority of the data being of ^1H .

1.5 NMR-BASED METABOLOMICS

Numerous metabolomics studies have been published since the debut of the concept. Since then, the number of Nuclear Magnetic Resonance (NMR)-based studies increasing steadily⁴³. NMR spectroscopy has important advantages that highlight its usefulness in metabolic studies. They include for instance being quantitative, qualitative, non-destructive, non-equilibrium disturbing⁴⁵, highly reproducible and having the ability to detect compounds within a wide range of physiochemical properties in the micromolar range^{42,45}. NMR-based metabolomics studies have been used in farm animal sciences for: in quality analysis⁴⁶, milk metabolomics^{47,48}, sample provenance discrimination⁴⁹, and diagnosis^{50,51}. The use of the NMR-metabolomics approach to small ruminants emerged recently and has been specially applied to diagnosis^{52–55}, biomarkers identification^{24,56–58} and animal welfare⁵⁹. These principal advantages of NMR make it very interesting to metabolomics studies, either applied by it self or combined with other approaches. Its simultaneous quantitative and qualitative capacity, together with the non-invasive and non-destructive nature turns NMR into a promising technique to be used in farm animal science.

1.6 OBJECTIVES OF THE STUDY

The relevance of different small ruminant breeds with tolerance to SWL effects is recognized in animal selection programs. These breeds could be of crucial importance in developed countries, to optimize production and control the negative aspects of seasonal weight loss, and in more vulnerable regions, to increase production and income. Additional information about the physiology of these breeds will help understand the mechanisms of adaptation in a more integrated view and define the selection strategies. Therefore, with this work we aim to:

- Optimize methods for: sample preparation of different tissues and fluids of small ruminant origin (goat milk and mammary gland; sheep muscle and liver), and to establish methods able of adequately profiling their metabolomes using NMR.
- Profile the metabolome and the fatty acid content of mammary gland and milk of two dairy goat breeds, with different levels of tolerance to SWL as a way to help understand which are the biochemical pathways that are determinant to such trait in dairy goats.
- Profile the metabolome of muscle and liver of three sheep breeds, with different levels of tolerance to SWL, understanding which are the biochemical pathways that are determinant to seasonal weight loss tolerance in meat producing sheep.
- Render available the information generated above to the scientific community, specifically in the fields of animal science and metabolomics.
- Render the information generated above to the general public.

1.7 THESIS OUTLINE

To accomplish the objectives, three major tasks were designed: “NMR Metabolomics: Methods Optimization for small ruminant samples”, “Study of the feed-restriction effects in dairy goats” and “NMR Metabolomics and feed-restriction effects in sheep”. Figure 1.3 represents a schematic summary of the main objective, methods and outcomes from this work.

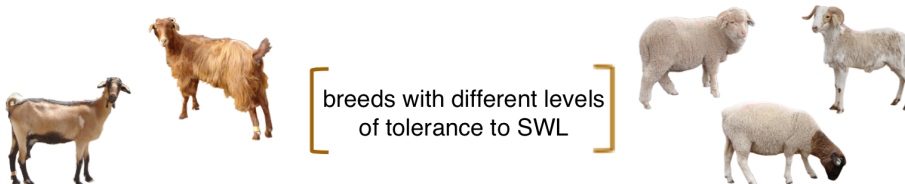
The first task, described in chapter 2, includes the optimization of methods and procedures needed to fulfil the following tasks. Principal steps of the procedures were tested and selected to be simple, quick and effective.

In chapter 3, the effects of feed-restriction in dairy goats, with special focus in mammary gland and milk aqueous metabolome and fatty acid profile are studied. In this part, major influence of feed-restriction is discussed.

Chapter 4 describes the effects of feed-restriction in meat-producer sheep. Muscle and liver metabolome was discussed in the context of SWL.

In chapter 5 major conclusions of each chapter will be highlighted. Results from chapter 3 and 4 will be integrated in the context of SWL. Future perspectives of this work are also discussed.

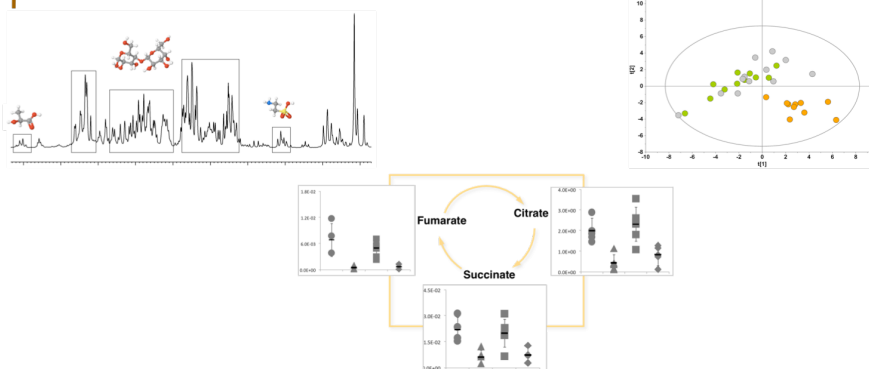
Seasonal Weight Loss (SWL) major constraint in small ruminants production



goat breeds:
Majorera and *Palmera*

sheep breeds:
Merino, *Dorper* and *Damara*

NMR - Metabolomics



Metabolome characterization

Study of physiological adaptation to SWL

Figure 1.3. Graphical abstract. Summary of the key objectives, methods and outcomes from the thesis.

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2

NMR METABOLOMICS: METHODOLOGY OPTIMIZATION FOR SMALL RUMINANT SAMPLES

The work presented in this chapter was included in the publication:

- Palma M, Almeida AM, Matzapetakis M. NMR-Metabolomics profiling in small ruminant tissues and fluids: establishment of novel methodologies for animal and food sciences [in preparation]

Mariana Palma performed all the experiments, except field work and sample collection.

2.1 SUMMARY

NMR-based metabolomics studies involve the analysis of biological tissues and fluids through a specific set of proceedings. Here, we describe the methods we have established for sample preparation, NMR data acquisition, data collection and data analysis that allowed us to successfully profile the metabolomes of various samples of major interest for research in animal sciences, specifically in ruminants. We have established valid methodologies to study the metabolomes of working samples: aqueous extracts of mammary gland and the milk serum of dairy goats; and aqueous extracts of muscle and liver of sheep. We also describe important information on the validation procedures we have used and how these procedures may be of use by other researchers in this novel field.

2.2 INTRODUCTION

Livestock is an essential resource to overcome the worldwide increasing need for food production. Understanding the physiology of different species is determinant for production optimization. Metabolomics-based studies provide information about the metabolome, that combined with other Omics-based studies allow an enhanced overview of the animal physiology that can help improve its production potential¹. Intrinsic attributes of Nuclear Magnetic Resonance (NMR) spectroscopy such as its quantitative, qualitative, non-destructive and non-equilibrium disturbing nature² make it ideal for metabolic studies. Moreover, it is highly reproducible and has the ability to detect compounds within a wide range of physicochemical properties in the micromolar to millimolar range^{2,3}.

The wide range of applications of NMR have been demonstrated by the increasing number of NMR-based studies⁴. In animal and human sciences^{5–16}, NMR-metabolomics studies have been applied to several types of biological samples such as urine¹⁷, tears¹⁸, saliva¹⁹, serum²⁰, amniotic fluid²¹, exhaled breath condensate²², and several tissues^{6,7,23}. In farm animals, NMR techniques have been applied to assess food quality and safety parameters^{23–27}, and determine its origin^{28–30}, proving its broad range of applications in this area. However, its application in small ruminants is quite recent and has been specially applied to diagnosis^{31–34}, biomarkers identification^{35–38} and animal welfare³⁹.

Most of the NMR-metabolomics studies are based on 1D spectra (¹H NOESY) of liquid samples that allow the identification and quantification of metabolites, require low optimization of acquisition parameters and are

usually fast to acquire. Additional techniques can be used to assist metabolome profiling, depending on the type of sample and the kind of analysis required. Alternatively, the high-resolution magic angle spinning (HR-MAS) approach can also be applied to wet solid samples, and requires only a small intact piece of tissue, without any previous preparation such as extractions^{4,40}.

For liquid state NMR, tissue extraction and sample preparation is usually straightforward, done in a few steps and in a way that allows keeping the integrity of the sample as much as possible. The Methanol/chloroform (M/C) method^{2,7,23,41} proved to be more suitable than perchloric acid extraction, especially due to the low variability of the technique, the use of small solvent volumes and the higher metabolite yields obtained⁴². M/C extraction method also avoids oxidation of sample metabolites and proteins⁴³. The method has been used in several NMR-based metabolomics studies^{6,7,15,44}.

After acquisition, data interpretation can be achieved via a complete spectral assignment and quantification - targeted approach (profiling) or via an untargeted approach (chemometric method). Profiling requires the identification and quantification of all detectable metabolites in a mixture⁴⁵. Although more time consuming, this approach is considered to be more reliable than the chemometric. Signal assignment is achieved using spectral decomposition, assisted by databases of compound spectral data to allow peak identification. Quantification is achieved by comparison of peak volumes with those of standards⁴⁶. Targeted analysis yields information of the composition of each sample, however it is limited by the number of compounds that can be reliably identified. NMR metabolomics employing profiling has been used in small ruminants,

especially for diagnosis and biomarker identification^{37,39,47}. This approach has the added advantage of providing information about the physiological metabolome profile of the studied animals, which can be useful for future works. In contrast, chemometric analysis is based on the quantification of signals in discrete regions of the spectra without any signal interpretation prior to the analysis. This is only possible when results are analysed with statistical tools. While chemometrics is fast and convenient it has the possible drawbacks of missing low concentrated spectral signals that are located nearby high concentrated ones⁴⁸ and is also affected by small variations in chemical shifts⁴³. The chemometric approach does not allow sample profiling, however it is a useful and faster option when the profile has already been established, or when only the variation of few compounds is needed. Its use was already described in production animals for diagnosis and quality control^{35,49}.

Sample preparation for NMR-metabolomics is usually an easy and swift process³ although, for each new sample type, several optimization steps have to be conducted to ensure sample preparation reproducibility and maximize results. Hence, development and adaptation of methodologies that can facilitate the implementation of these studies are required. Although, NMR-based metabolomics studies are an already established area, its use in farm animals is still limited. As such, protocol optimizations could help further develop this topic. In this work we describe the sample preparation methods that better suited for our studies on milk, mammary gland, muscle and liver samples from small ruminants. We further highlight major optimizations needed to complete and thoroughly profile the metabolome. Outcomes are of importance not only to metabolomics research in the context of biomarkers identification and physiological/pathological conditions, but also to food composition

determination and quality control. Results could also be useful to other production species such as pig, poultry or cattle and ultimately for a more general use of NMR-based metabolomics in animal and agricultural sciences.

2.3 MATERIAL AND METHODS

2.3.1 Biological material

Goat mammary gland samples were obtained from two dairy breeds (*Majorera* and *Palmera*), from Canary Islands, and were separated in a control (N=9) and an experimental group (N=10). Animals and nutritional trials were described by Lérias *et al.*⁵⁰. Sheep muscle and liver samples were obtained from ram lambs of *Merino* breed and were divided into a control (N=11) and an experimental group (N=10). Animals and sample collection were described by Scanlon *et al.*⁵¹ and Alves *et al.*⁵². After collection, samples were immediately frozen in liquid nitrogen and kept at -80°C until preparation.

Milk samples were obtained from the dairy goats described above⁵⁰. After collection, samples were immediately frozen in liquid nitrogen and kept at -80°C until preparation.

2.3.2 Tissue sample preparation

Mammary gland, muscle and liver were individually ground to a fine powder, keeping the samples frozen during the whole process with liquid nitrogen, with a porcelain mortar and pestle. Tissue fraction extraction

procedures were performed in glassware, and samples and solvents (methanol, chloroform and water) were kept on ice throughout the process. Tissue fraction extractions were performed following the methanol/chloroform (M/C) method⁴¹, with some adjustments.

Mammary gland and muscle samples

Mammary gland samples weighted 200 mg and muscle 500 mg. A total of 1.2 ml of a chloroform/methanol mixture (1:2, v/v) was added to each sample and vortexed for 1 minute. Then 0.4 ml of chloroform were added and vortexed for 1 minute, and subsequently 0.4 ml of water was added and vortexed again for 1 minute. The homogenate was centrifuged at 1935 g for 20 minutes at 4°C in a Beckman Coulter Avanti J25I (Pasadena, CA, USA) centrifuge. The methanol/water fraction was separated by pipetting and dried in a vacuum concentrator Labconco CentriVap Concentrator (Kansas City, MO, USA). Evaporation lasted between 2 and 3 hours. Dried residue was frozen at -80°C until NMR experiments.

Liver samples

Liver samples weighted approximately 175 mg. A total of 3 ml of a water/methanol mixture (1:2, v/v) were added to each sample and vortexed during 1 minute. Then 2 ml of chloroform were added and vortexed for 1 minute, and another 1 ml of water was added and vortexed again for 1 minute. Homogenate was centrifuged at 1935 g for 20 minutes at 4°C in a Beckman Coulter Avanti J25I (Pasadena, CA, USA)

centrifuge. The methanol/water fraction was separated by pipetting and dried in a vacuum concentrator Labconco CentriVap Concentrator (Kansas City, MO, USA). Evaporation lasted between 5 and 7 hours. Dried residue was frozen at -80°C until NMR experiments.

2.3.3 Milk sample preparation

Milk samples (~1 ml) were thawed on ice and weighed (925.4 to 1243.0 mg). Samples were ultracentrifuged at 110.000 g for 75 minutes at 4°C. Protein pellet (bottom layer) and the lipidic fraction (top layer) were removed and the milk serum (middle layer) was further centrifuged at 21.600 g for 20 minutes at 4°C. Both centrifugations were conducted in a Beckman TL-100 (Pasadena, CA, USA) ultracentrifuge. Milk serum was filtered using a Vivaspın2 3 kDa cut-off spin filter during 2 hours, for 3.220 g, at 4°C in an Eppendorf 5810R (Hamburg, Germany) centrifuge. Serum sample was frozen at -80°C until preparation for NMR experiments.

2.3.4 NMR experiments

Sample preparation

All samples were thawed on ice just before preparation for NMR experiments. Sample preparation was made as follows: to the dried extract of mammary gland and muscle samples were added 600 μ l of phosphate buffer (150 mM; pH 7.0 (pD 7.4); with 1 mM DSS); to the dried extract of liver were added 800 μ l of phosphate buffer (100 mM; pH 7.4 (pD 7.8); with 0.5 mM DSS); and to 300 μ l of milk serum were added 300

μ l phosphate buffer (150 mM; pH 7.0 (pD 7.4); with 26.7 μ M TSP). After resuspension, all tissue samples were centrifuge at 141 000 g for 10 minutes, and the supernatant was transferred to an NMR tube. Milk samples were centrifuged inside the NMR tube, for 1 minute in a homemade centrifuge for NMR tubes.

NMR data acquisition

Proton (^1H) NMR spectroscopy was performed on an 800 MHz Bruker AvanceII⁺ (Ettlingen, Germany) spectrometer equipped with a room temperature triple resonance HCN Z-gradient probe, at 298 K. ^1H 1D-NOESY spectra were collected as follows: pulse sequence: “noesypr1d”; spectral width: 12 ppm; mixing time: 0.1 s; relaxation delay: 1 s; acquisition time: 4 s. For all sample types, J-Resolved and 2D ^1H - ^1H COSY spectra were also collected on selected samples using the standard Bruker parameters for profiling, in order to assist in resonance assignment. All NOESY spectra were processed with TopSpin3.2 using exponential apodization with 1Hz line broadening and zero filling up to 128K points (Bruker Biospin, Billerica, MA, USA).

2.3.5 Data analysis

Targeted analysis - Metabolite Profiling

Metabolite profiling was performed using Chenomx NMR Suite 8.0 software, with the internal reference library version 9, following the general recommendations of the software manual.

The concentration of the internal standard, DSS, was used to calibrate peak volumes. No pH markers were used since the solutions were buffered. For the tissue samples, mass corrected concentrations were obtained by normalization with the mass of each tissue sample.

Assignment ambiguities were examined using the combination of J-Resolved and COSY spectra together with data from external sources such as published literature and the metabolite database of the Biological Magnetic Resonance Bank (www.bmrb.wisc.edu).

Untargeted analysis - Data Binning

Spectral binning was performed with Chenomx NMR Suite 8.0 using uniform binning with size of 0.04 ppm, from 0 to 9.5 ppm. The regions for urea (5.65-5.88 ppm), water (4.68-5.15 ppm) and DSS (0.01-0.08 ppm) were excluded.

2.3.6 Statistical analysis

Statistical analysis was performed using SIMCA-P 13.0.3.0 software (Umetrics AB, Umeå, Sweden). Multivariate analysis tests were performed to evaluate any clustering behaviour of the samples' groups. The first approach was made using the unsupervised Principal Component Analysis (PCA), that allows evaluation of sample clustering (scores) and identify metabolites responsible for group clustering (loadings). When PCA could not reveal any clustering between groups, data was analysed through a supervised Partial Least Squares Discriminant Analysis (PLS-DA) and an Orthogonal Partial Least Squares

Discriminant Analysis (OLPS-DA). These methods evaluate repeated variables between groups and so they are better geared at highlighting differences between groups.

To compare paired groups such as control versus test groups for example, we tested the statistical significance of the results using a t-test in Microsoft Excel. We used test type-3 with 2 tails, considering $p < 0.05$ to reject null-hypothesis.

A schematic summary of the methodologies used in these samples is presented in Figure 2.1.

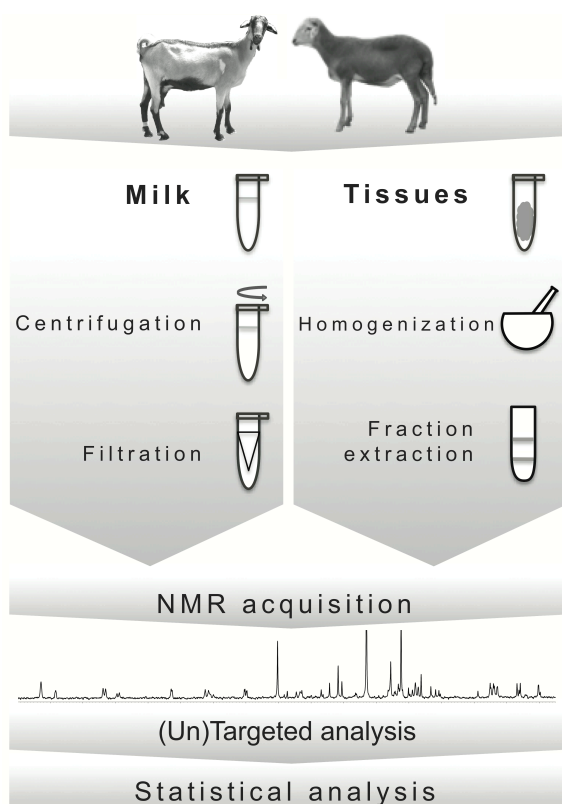


Figure 2.1. Summary of the methodology for NMR-based metabolomics of small ruminants, for tissue samples (muscle, mammary gland and liver) and milk serum.

2.4 RESULTS AND DISCUSSION

The increasing interest in NMR-metabolomic studies led to the development or adaptation of existing techniques to efficiently meet the new requirements. Although literature highlights the proficiency and reproducible character of the presented methods^{4,42} it is the first time that such samples (goat mammary gland and sheep muscle and liver) were tested and evaluated altogether. Accordingly, we tested the suitability of these methods specifically to these samples, and discuss the main optimizations needed to design a protocol easy to implement and follow, quick to fulfil and that could be easily used to the most common needs in animal and agriculture productions.

2.4.1 Tissue samples

The procedure for tissue aqueous fraction extraction was tested in triplicate. The solvent-volume/tissue proportion was tested, and results revealed that the M/C method is capable of extracting metabolites in a reproducible way as long as the ratio of solvents is kept constant, in the range of tissue mass that was used. This is especially important for biopsy sampling since the amount of tissue collected could vary slightly between animals. For NMR-profiling studies, the amount of tissue should be sufficient to produce extracts with concentrations that ensure the detection of the least concentrated compounds of interest while keeping the signal of the most concentrated ones within the dynamic range of the instrument (10^5 - 10^6). The lower limit of detection can be improved with the increase of the number of acquisition scans, however this can lead it to non-practical experiment lengths. Similarly very concentrated samples

can be diluted or collected with a lower receiver gain but this can influence the detection limit of the least concentrated components. Based on these considerations, in the studied samples, the amount of tissue that better fulfil these conditions are in the range of 200 mg for mammary gland, 500 mg for muscle, and 175 mg for liver.

It was suggested that the pH of the extraction solvents could affect the efficiency of the methanol/chloroform/water extraction⁵³. We tested this hypothesis on sheep muscle samples following the protocol described by Blackwell et al.⁵⁴. After the initial extraction, the tissue dry extracts were washed separately with an acidic solution (formic acid 1 %) and a basic solution (ammonia hydroxide 2 %). We could not observe any significant peaks in NMR spectra, suggesting that the pH 7 solution was able to solubilise all the water soluble components that would be sufficiently abundant to be visible in an NMR spectrum.

In an effort to detect the small molecule composition on intact, untreated tissues we also performed High Resolution Magic Angle Spinning (HR-MAS) experiments on mammary gland samples. Compared to the spectra of aqueous extracts the HR-MAS spectra were populated by mostly broad peaks (Figure 2.2). It was possible to detect a number of components such as amino acids (tyrosine, valine, leucine and isoleucine), lactate and various lipids. However, the quantification of these compounds was difficult due to inhomogeneous peak broadening, difficulties in shimming and the presence of the broad signals that are due to a combination of lipids and protein background. The highly similar structure of the majority of the lipids present in our tissue samples would result in severe peak overlap making their identification impractical⁴⁰. However HR-MAS has been used successfully in farm products,

specially for origin determination and authenticity tests^{9,55–57}, we finally selected the tissue extraction approach followed by ^1H 1D NOESY acquisition. This choice was related to the extended time required for HR-MAS optimization, and the small amount of sample available that in some cases could be insufficient for the entire test.

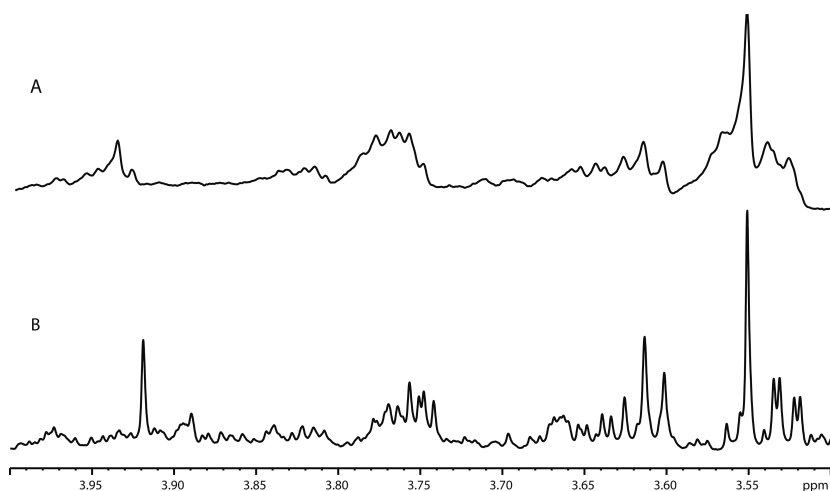


Figure 2.2. Example of spectra of mammary gland aqueous fraction acquired on an 800 MHz spectrometer. A) ^1H 1D-NOESY with the HRMAS probe at 277 K, B) ^1H 1D-NOESY with the HCN Z-gradient probe at 298 K.

2.4.2 Milk

Milk samples were also tested to determine the procedure that better fits the NMR requirements for sample profiling. Since metabolomics studies are focused on small metabolites only, protein presence and the proportion of metabolite that could be observed in the NMR sample were

the major concern during method optimization. We tested two methods, one based on the separation of milk components through centrifugation only⁵⁸ and other based on filtration with a low molecular weight protein cutoff filter¹¹. All tests were made in triplicate. However, none revealed to be ideal for our samples, probably due to the fact of being completely raw milk samples, with a high fat content without any pre-treatment. The use of the centrifugation method alone revealed the presence of proteins in the end of the process (detected in NMR spectra) that hindered the correct identification and quantification of metabolites. On the other hand, the filtration method was difficult to complete due to the clotting of the filters with big molecules and fat. So, after tested, we concluded that the sequential use of both methods was the best option to obtain NMR spectra with enough quality to be profiled, especially when studying raw milk. It should be noted that, this procedure does not ensure the complete recovery of the aqueous metabolome, as portions of it can still be associated with the lipids and the proteins that were removed by ultracentrifugation. The reproducibility of the method however, was found to be very good and thus this extraction method can be used in order to compare the metabolomes of different sample types.

We also observed the formation of some cloudiness in the sample after adding the phosphate buffer prior to NMR acquisition. This is likely due to the formation of calcium phosphate, by adding the phosphate buffer to serum. We tested its dissolution by adding EDTA (10 μ l of 0.5 M EDTA in the 600 μ l NMR sample), and its precipitation by centrifugation. Both approaches were efficient to remove the cloudiness. However, by using EDTA we were also adding extra peaks from free-EDTA, Mg-EDTA and Ca-EDTA between 2.5 and 3.7 ppm⁵⁹. It is described that EDTA peaks could interfere with the peaks from samples' metabolites in this region,

where we have other important metabolites like acetylcarnitine, citrate, creatine, and glucose for example⁵⁹. So, we followed the procedure with a 1 minute centrifugation step just before the NMR acquisition, using a centrifuge to NMR tubes.

2.4.3 Data analysis and biological interpretations

Tissue Samples

The profiling of the aqueous fraction of goat mammary gland samples led to the identification of 46 metabolites. In order to estimate the percentage of metabolites that were identified in the assigned spectra compared to the total present in the spectrum, we calculated the area of the residual spectrum after subtraction of the assigned peaks, with the area of the total spectrum. Water region was excluded in all spectra. For mammary gland, the area of the assigned peaks corresponds to 69%. Using similar analysis on the sheep samples, we conducted profiling leading to the identification of 51 metabolites in muscle and 46 in liver. The peak area of the assigned peaks corresponds to 69% and 74% of the total in muscle and liver samples respectively. It should be noted that this measure corresponds to the mass and not the number of metabolites.

Regarding data analysis, we used goat mammary gland and sheep liver samples to compare the results of the analysis with the targeted (profiling) and the untargeted approaches (chemometric analysis).

For mammary gland, profiled data were analysed using univariate as well as multivariate analysis. Univariate analysis was able to identify changes in 4 metabolites between the two study groups. Multivariate analysis

using both the binning data and the profiled data, was performed to evaluate differences between these two groups. PCA scores plot of the binning data (chemometric analysis) show no separation between groups (Figure 2.3A), although with profiling information it is possible to see some separation in the second principal component (Figure 2.3B). Given the poor separation of the groups through PCA, we performed PLS analysis to evaluate separation. PLS scores plot of the binning data (Figure 2.3C) revealed some separation along the first principal component, with good quality parameter. Permutation test do not validate this model, although the obtained values are close to the acceptable values. This is probably due to the low number of samples that were available in this study. PLS scores plot of the profiling data (Figure 2.3D) presented separation of the groups with acceptable quality parameters, albeit the permutation test do not validate the model.

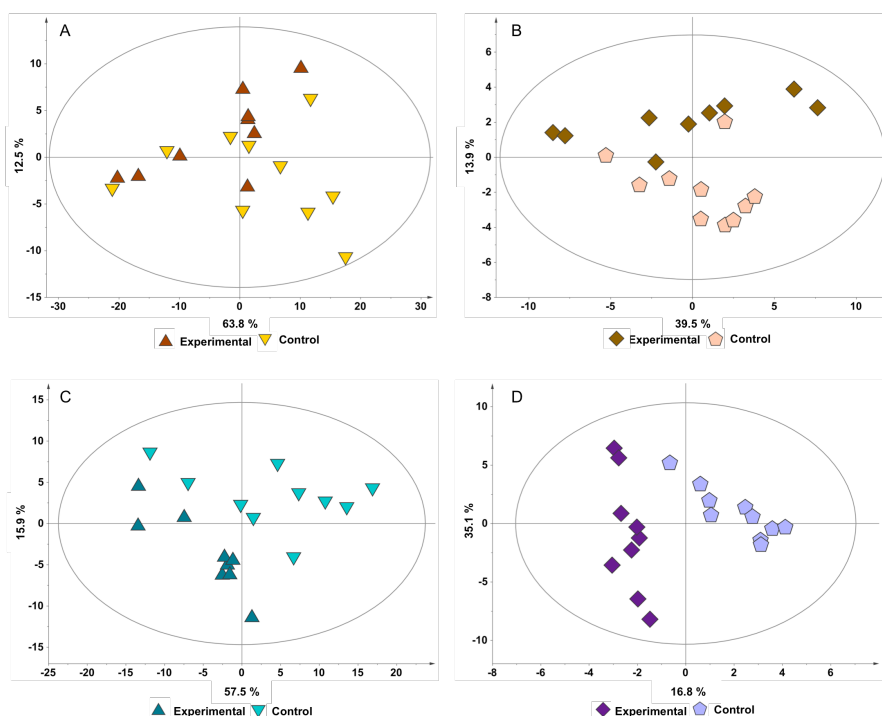


Figure 2.3. Multivariate analysis of the goat mammary gland (aqueous fraction). A) PCA scores plot of chemometrics approach data (NC = 2; PC1 = 63.8%; PC2 = 12.5%), B) PCA scores plot of profiling approach data (NC = 3; PC1 = 39.5%; PC2 = 13.9%), C) PLS scores plot of chemometrics approach data (NC = 3; PC1 = 57.5%; PC2 = 15.9%; $R^2 = 0.811$; $Q^2 = 0.701$; $p_{CV-ANOVA} = 0.016$; permutation test: 100 permutations, $R^2 = (0.0, 0.454)$, $Q^2 = (0.0, -0.296)$), D) PLS scores plot of profiling approach data (NC = 3; PC1 = 16.8%; PC2 = 35.1%; $R^2 = 0.606$; $Q^2 = 0.540$; $p_{CV-ANOVA} = 0.211$; permutation test: 100 permutations, $R^2 = (0.0, 0.736)$, $Q^2 = (0.0, -0.173)$).

The same comparison was made with liver samples, where univariate analysis of the profiled data led to the identification of 10 metabolites with differences between the two experimental groups. PCA scores plot of the binning data revealed no separation between the two groups (Figure 2.4A). PCA scores plot of the profiling data shown some separation of the two groups in the second component, albeit with two outliers in each

study group (Figure 2.4B). PLS scores plot of the binning data (Figure 2.4C) showed some separation of the study groups by the second principal component, albeit with some disperse points in both breeds. Quality parameters are not acceptable and the permutation test does not validate the model. The PLS scores plot of the profiling data (Figure 2.4D) presented separation of the two study groups by the first principal component, although with few disperse points. Quality parameters are not acceptable and the permutation test does not validate this model.

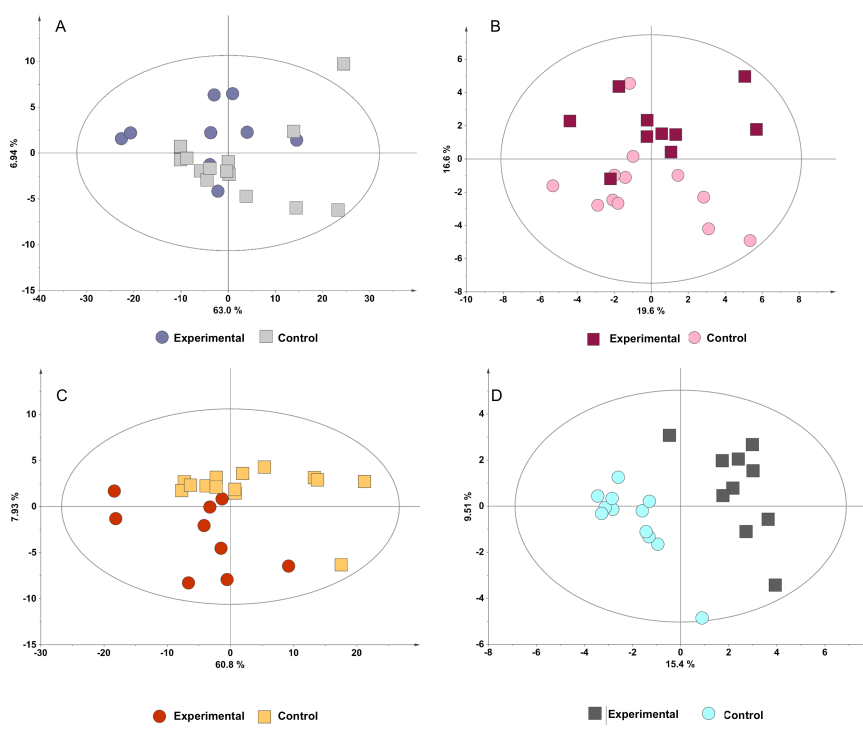


Figure 2.4. Multivariate analysis of the sheep liver (aqueous fraction). A) PCA scores plot of chemometrics approach data (NC = 2; PC1 = 63.0%; PC2 = 6.94%), B) PCA scores plot of profiling approach data (NC = 2; PC1 = 19.6%; PC2 = 16.6%), C) PLS scores plot of chemometrics approach data (NC = 2; PC1 = 60.8%; PC2 = 7.93%; $R^2 = 0.688$; $Q^2 = 0.305$; $p_{CV-ANOVA} = 0.1285$; permutation test: 100 permutations, $R^2 = (0.0, 0.502)$, $Q^2 = (0.0, 0.085)$), D) PLS scores plot of profiling approach data (NC = 2; PC1 = 15.4%; PC2 = 9.51%; $R^2 = 0.249$; $Q^2 = 0.502$; $p_{CV-ANOVA} = 0.011$; permutation test: 100 permutations, $R^2 = (0.0, 0.707)$, $Q^2 = (0.0, -0.239)$).

These results suggest that both approaches (profiling and chemometrics) are able to pick up differences between groups. However, in this case, the profiling approach seems to be more refined and robust in that analysis. This discrepancy could be due to several reasons. Firstly, the number of variables used in the chemometric approach, is much larger

and it includes many points with just noise. Also, chemometrics analysis can be sensitive to slight variations in the baseline, differences in peak positions and to the fact that intense peaks will overshadow the influence of weaker ones that are overlapped with them. All the above are easily dealt with by the pragmatic analysis performed during profiling and thus are not as much of an issue. On the other hand, the analysis using chemometrics is very fast as it does not require prior interpretation of the spectra. Factors responsible for the separation of the data groups, by PCA or PLS, can be evaluated by the loading plot of each principal component. In chemometrics approach, of both samples, it was possible to identify some regions of the spectra with important differences. Within these, we identified the regions corresponding to the metabolites that had in turn being previously identified to have significant differences in the univariate analysis of profiled data. Other variations were originated from compounds with isoelectric point near the buffer pH and some unidentified peaks. Within the latter some singlet peaks that were not possible to identify through the profiling approach were included. Another limitation of using the quantitative analysis is the number of compounds in the databases that could limit the identification of some less common metabolites, especially in uncommon samples.

In general, and in order to observe differences between sample groups, it is necessary to perform either univariate or multivariate analysis of the data. The profiled data are amenable to univariate analysis which will reveal changes on specific metabolites. Multivariate methods represent a more powerful way to discern differences between datasets. They can be applied to both targeted (profiling analysis) and untargeted data (chemometric analysis) and can reveal correlated variations within groups of data. For a more detailed explanation about the multivariate

methods and the differences among them kindly refer to the works of Worley and Powers⁴⁸ and Bro and Smilde⁶⁰.

Differences in metabolite composition between tissues could be observed in the 1D NMR representative spectra in Figure 2.5 (A – sheep liver; B – sheep muscle; C – goat mammary gland).

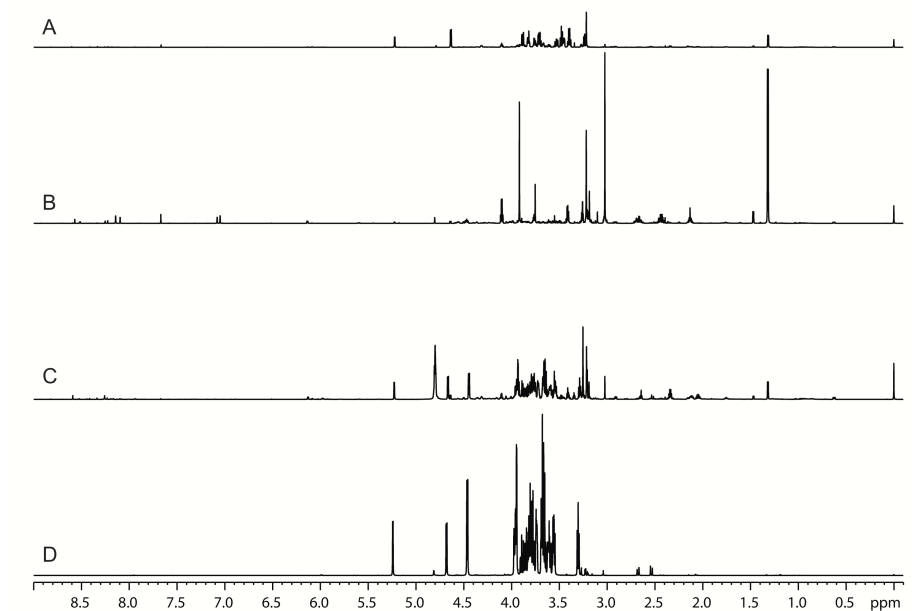


Figure 2.5. Representative spectra at 800 MHz, with a HCN Z-gradient probe at 298 K. A) aqueous fraction of sheep liver, B) aqueous fraction of sheep muscle, C) aqueous fraction of goat mammary, D) goat milk serum.

Milk

Using the profiling approach the signals of 50 metabolites were identified in milk serum. These metabolites account for 93% of the total peak area in spectra of the sera. A representative 1D NMR spectrum from goat milk

is presented in Figure 2.5D. Following this approach, and considering two study groups³⁶ we identified significant differences in 17 metabolites. Using the binning information, in the chemometrics approach, the PCA analysis was able to separate roughly the control and the experimental samples (Figure 2.6A). PCA scores of the profiling data (Figure 2.6B) showed a sharp separation of the groups, indicating that both approaches came to similar conclusions. This observation revealed that, in this sample, both methods are capable of extracting essential information for group samples, although they are based on different levels of information. Loading plots showed the bins responsible for these differences. As in mammary gland, some are from compounds sensitive to minimal variations in pH; others correspond to some unidentified peaks, especially in the aromatic region of the spectra; and others to the peaks of the compounds identified with significant differences between groups in the quantitative approach.

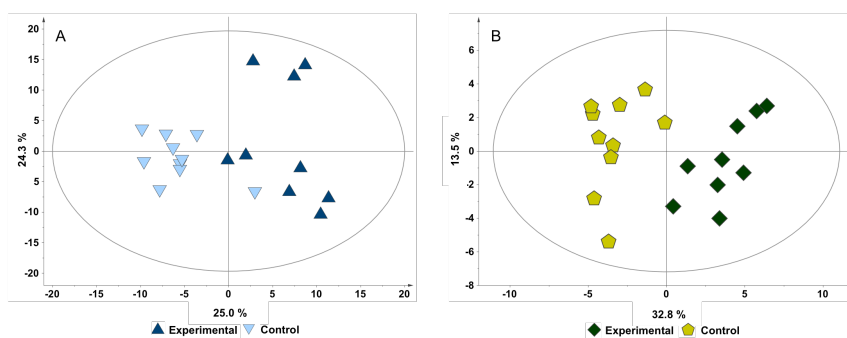


Figure 2.6. Multivariate analysis of the goat milk serum. A) PCA scores plot of chemometrics approach data (NC = 3; PC1 = 25.0%; PC2 = 24.3%), B) PCA scores plot of profiling approach data (NC = 3, PC1 = 32.8%; PC2 = 13.5%).

2.5 CONCLUSIONS

With this work, we offer guidelines and recommendations regarding the study of metabolomics of mammary gland, muscle, liver and milk samples from small ruminants.

Concerning the number of compounds identified we followed the mean (20 to 60) of the NMR detectable metabolites in a typical tissue^{4,43}, so we can conclude that the presented methods are adequate for the nature of these studies.

Regarding data analysis, we can conclude that both quantitative and chemometric analysis retrieve reliable results, even with small groups of samples. The choice of one of them should be taken considering the specific objective of each work. Generally, for a completely new approach to a specific sample, we recommend the profiling of the metabolome, considering that the metabolome itself is a brand new and useful piece of information. After a certain degree of knowledge is obtained for a given sample, or when some type of specific information is pursued, such as the presence/absence of a given metabolite for example, it is simpler to perform a chemometric analysis and check information about its specific region in the spectra. Also, when only the surveillance of general differences between groups is required, the chemometrics approach seems to be the better choice, given the reduced time needed for data analysis and the lack of need for pre-existent databases. This approach could be especially helpful in studies concerning diagnosis through a specific biomarker, quality control and authenticity tests, particularly in meat and dairy products.

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3

STUDY OF THE FEED-RESTRICTION EFFECTS IN DAIRY GOATS

The work presented in this chapter was included in the publications:

- Palma M, Hernández-Castellano L, Castro N, Arguëllo A, Capote J, Matzapetakis M, Almeida AM. NMR-Metabolomics profiling of mammary gland secretory tissue and milk serum in two goats breeds with different levels of tolerance to seasonal weight loss. *Molecular BioSystems*. (2016) 12:2094-2107.
- Palma M, Alves SP, Hernández-Castellano L, Capote J, Castro N, Arguëllo A, Matzapetakis M, Bessa RJB, Almeida AM. Fatty acid composition of mammary gland and milk of two goat breeds under seasonal weight loss condition. *Journal of Dairy Research*. [submitted]

Mariana Palma designed and performed all the experiments, with the exception of field work, sample collection and GC data acquisition.

3.1 SUMMARY

Goats are of special importance as they produce a variety of dairy products. Scarcity of pastures during the dry season leads to seasonal weight loss (SWL), which affects milk production. In this work, we studied the effect of feed-restriction in two dairy goat breeds: *Majorera* breed (tolerant to SWL) and *Palmera* breed (susceptible to SWL). We compared the metabolome of aqueous fraction and the fatty acids profile of mammary gland and milk serum in both breeds. Metabolite profiling analysis has led to the identification of 46 metabolites in aqueous extract of mammary gland and 50 metabolites in milk serum. Significant differences were observed, in mammary gland biopsies and milk serum, between control and restricted-fed groups in both breeds, albeit with no differences between breeds. Variations seem to be related to metabolism adaptation to the low-energy diet and indicative of a breed-specific microbiota. Regarding the fatty acids profiles, we observed an interaction between breed and feed-restriction in mammary gland, highlighting the *Majorera* breed tolerant to feed-restriction. Milk showed more metabolites and fatty acids variations due to feed-restriction and no significant interaction between breed and feed-restriction was observed.

3.2 INTRODUCTION

The Canary Islands is an archipelago located in the Atlantic Ocean, off the coast of the Sahara desert in Africa. It is a sub-tropical climate region, formed by seven islands. The geography of the archipelago determines the existence of different climate conditions within islands. Precipitation decreases from west to east, making the western island (La Palma) rainy and forested whereas the eastern islands (Fuerteventura and Lanzarote) dryer and arid¹. This climate pattern allowed the development of different ecosystems in the western and eastern islands with very different fauna and flora, conditions that also determined the agriculture and animal production systems, as well as human occupation.

Goat breeds in the Canary Islands evolved from the crossing of an initial goat population of African descent and several Iberian (Spanish and Portuguese) breeds, introduced into the archipelago during its colonization^{2,3}. Presently, it is considered that three major breeds of goats exist in the Canary Islands: The *Palmera*, the *Majorera* and the *Tinerfeña*. The *Palmera* breed (Fig. 3.1A), has its origins in the La Palma Island, and is adapted to a rainy climate^{4,5}. On the other hand, the *Majorera* breed (Fig. 3.1B), originally from Fuerteventura and Gran Canaria islands is adapted to arid environments, having acquired resistance to SWL^{2,6}. The origins of these goats and their phenotypes clearly reflect such adaptations. In fact, *Majorera* goats are similar to breeds from the Sahelian region, showing short hair, tall legs and slender body. In contrast, *Palmera* goats are more similar to breeds from the North of the Iberian Peninsula, with long hair and bulky body. Since the two breeds have a common ancestry they constitute a valuable and unique model for tolerance to SWL studies.

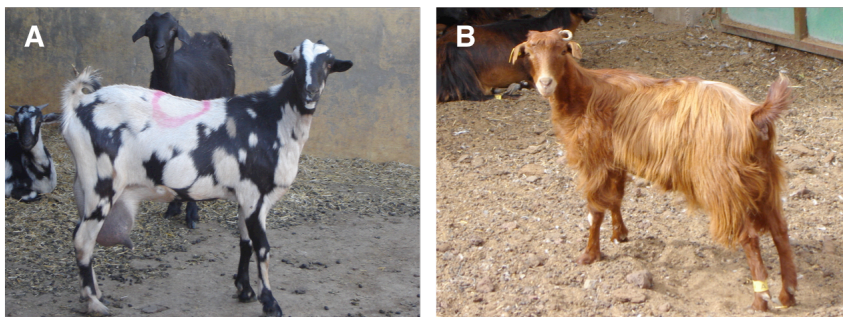


Figure 3.1. Representative female specimens of *Palmera* (A) and *Majorera* (B) goat breeds.

As in the Canary Islands, *Palmera* and *Majorera* goat breeds are mainly raised for milk production, we have targeted the milk and the mammary gland as study samples. The aim of this work was to characterize the metabolome and the fatty acid profile of the mammary gland and milk, and how they are affected by feed-restriction.

This work complements recently studies on the effect of feed-restriction in live weight and milk production in the same animals⁷ and on the blood metabolites and hormonal profiles⁸. Results will be of importance in animal selection in drought-prone regions. Moreover, since fat is the milk component with higher influence in organoleptic qualities and the more affected by environmental and physiological conditions⁹, outcomes will also help define strategies in dairy products optimization and selection, with application to other ruminant species in drought-prone regions.

3.3 MATERIAL AND METHODS

Animals

In this experiment, we used 19 of the animals already described in a companion paper (see details in Lérias *et al.*⁷). Herein we used nine *Majorera* breed and ten *Palmera* breed adult dairy goats from the experimental flock of Pico research station (ICIA, Valle Guerra, Tenerife, Spain). Animals were divided in two sets per breed: a restricted-fed group (*Majorera* n=5, *Palmera* n=4) and a control group (*Majorera* n=4, *Palmera* n=6). Animals were clinically healthy during the study.

Study

The study was conducted at the Faculty of Veterinary Medicine of the Universidad de Las Palmas de Gran Canaria (Arucas, Gran Canaria, Spain) during 23 days, in May-June 2012. As described by Martínez-De La Puente *et al.*¹⁰, animals from control groups were fed following the guidelines recommended by the Institut National de la Recherche Agronomique (INRA). This diet was composed by maize, soy 44 (44% crude protein), dehydrated lucerne, dehydrated beetroot, lucerne hay, and a vitamin-mineral supplement. Animals were also fed *ad-libitum* with standard wheat straw. Goats from restricted-fed groups were fed *ad-libitum* with standard wheat straw (low-level of crude protein, high-level of fiber and low energy)¹¹ and vitamin-mineral supplement. This diet restriction was optimized to reduce 15 – 20% of the initial body weight (day 0) at the end of the experimental period (day 23). Animals of each

nutritional group were housed together. For more details, kindly refer to Lérias *et al.*^{7,8}.

Sample collection

Mammary gland biopsies (27-268 mg fresh tissue) were collected from the left half udder. Before biopsy collection, the whole udder was cleaned and disinfected using povidone-iodine. Local analgesia and anaesthesia was induced by intramuscular injection of Xylazine (Xilagesic 20%, Calier, Barcelona, Spain). A 2 mm incision was performed, allowing the exposition of the secretory tissue. Then biopsies were collected using a scalpel blade. After sample collection, mammary gland was sutured using Safil 2/0 (Braun, Barcelona, Spain) and then sprayed with Terramicin (Terramicina spray, Pfizer, Madrid, Spain, containing oxytetracycline HCl and patent blue). Finally, prophylactic treatment was administered by intramuscular injection of Terramicin (Terramicina L.A., Pfizer, Madrid, Spain). Goats were milked daily following the milking parameters described by Torres *et al.*¹² and Hernández-Castellano *et al.*¹³. Milk samples were collected from the available milk of the last day of experiment before biopsy collection. All samples were immediately frozen in liquid nitrogen and stored at -80°C until use.

3.3.1 NMR-Metabolomics

Sample processing

Frozen mammary gland samples were powdered with porcelain pestle and mortar containing liquid nitrogen. Metabolites were then extracted

following Bligh and Dyer method with some modifications^{14–17}. Briefly, to every mammary gland sample, 1.2 ml cold chloroform/methanol mixture (1:2, v/v) were added and mixed. An additional volume of 0.4 ml of cold chloroform was added and mixed again. Then 0.4 ml of cold water was added and mixed by vortexing. The homogenate was centrifuged at 1935 *g* for 20 minutes at 4°C in a Beckman Coulter Avanti J25I (Pasadena, CA, USA) centrifuge. The chloroform and methanol/water fraction was separated by pipetting and dried in a vacuum concentrator Labconco CentriVap Concentrator (Kansas City, MO, USA). The water-soluble residue was dissolved in 600 μ l phosphate buffer (150 mM, pH 7.0, with 1 mM sodium-2,2-dimethyl-2-silapentane-5-sulfonate (DSS), in 90% H₂O and 10% D₂O) and transferred into a 5 mm NMR tube.

Milk samples were thawed on ice, centrifuged and filtered following the procedure described by Lu *et al.*¹⁸ and Sundekilde *et al.*¹⁹ with some modifications. Samples were ultracentrifuged at 110.000 *g* for 75 minutes at 4°C. Protein pellet and the lipidic fraction were removed and the milk serum was further centrifuged at 21.600 *g* for 20 minutes at 4°C. Both centrifugations were conducted in a Beckman TL-100 ultracentrifuge (Pasadena, USA). Milk serum was filtered using a Vivaspin2 3-kDa cut-off spin filter (Sartorius Stedium Biotech GmbH, Goettingen, Germany) centrifuged at 3.220 *g* for 2 hours at 4°C in an Eppendorf 5810R centrifuge (Hamburg, Germany). 300 μ l of the milk serum were mixed with 295 μ l phosphate buffer (300 mM, pH 7.0, in deuterium oxide) and 5 μ l 3-(trimethylsilyl)-2,2',3,3'-tetradeuteriopropionic acid (TSP) (0.05% wt in D₂O) and transferred to a 5 mm NMR tube.

NMR Spectroscopy

Proton (^1H) NMR spectroscopy was performed on an 800 MHz Bruker Avancell+ (Ettlingen, Germany) spectrometer equipped with a room temperature triple resonance HCN Z-gradient probe, at 298 K.

^1H 1D-NOESY spectra (spectral width: 20 ppm; mixing time: 0.01 s; relaxation delay: 4 s; acquisition time: 4 s) were collected for each mammary gland sample using the “noesygppr1d” pulse sequence. For the most concentrated sample of each group, additional spectra were collected to assist with assignment, namely J-Resolved and ^1H - ^1H COSY. The spectra acquisition conditions used were different from those suggested by Chenomx, however, comparison of the collected data with tests acquired under reference conditions revealed no significant differences and thus were used without corrections. Spectra were processed and analysed using TopSpin2.1 software (Bruker, Ettlingen, Germany).

The same set of experiments were used to collect the spectra from the milk serum samples as follows: ^1H 1D-NOESY (spectral width: 12 ppm; mixing time: 0.1 s; relaxation delay: 1 s; acquisition time: 4 s), J-Resolved and ^1H -COSY. Spectra were processed and analysed using TopSpin3.2 (Bruker, Ettlingen, Germany).

Metabolite Profiling

Metabolite identification and quantification was performed using Chenomx NMR Suite 8.0 software (Chenomx Inc., Edmonton, Canada),

using the internal reference library (Version 9), and by comparison with published literature for other animals^{20,21}.

Data Analysis

The sets of concentrations of each metabolite of each animal were analyzed using SIMCA 13.0.3.0 software (Umetrics AB, Umeå, Sweden). Statistical significances were tested through univariate analysis, using t-test with 2 tails and test type 3, considering $p < 0.05$ to reject null-hypothesis, using Microsoft Excel. To evaluate any clustering behaviour through multivariate analysis, an unsupervised Principal Components Analysis (PCA) was applied to all the sets of data for each sample type (mammary gland and milk serum) to: 1) cluster the samples (scores), and 2) identify metabolites responsible for the group clustering (loadings). Furthermore, when PCA did not reveal any clustering between groups, a supervised Partial Least Squares Discriminant Analysis (PLS-DA) and Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA) were performed. These tests evaluate repeated variables between groups and highlight differences between groups as previously used in other metabolomics approaches^{19,22–24}. The small sample size that is typical in such studies and the inherent large number of variables obtained may affect the consistency of the multivariate analysis used. To evaluate the consistency of the results, SIMCA-P performs a number of tests and reports the parameters Q^2 , $p_{CV-ANOVA}$ and R^2 as quality parameters of the models. Q^2 indicates the predictive ability of the model, while R^2 is the indicator of the goodness of the fit. Both should be as close to 1 as possible. $p_{CV-ANOVA}$ indicates the significance of PLS/OPLS and can help in the choice of the optimal number of

components. However, this value can vary during the validation process²⁴, so we will present it but consider only Q^2 and R^2 as quality parameters. The ellipses in the scores plots are drawn at the 95% confidence level.

3.3.2 Fatty Acids Profile

Sample processing

Lipids from mammary gland were extracted as described²⁵ but using dichloromethane and methanol (2:1, vol/vol). Total lipids were measured gravimetrically, weighing the residue after evaporation of solvents at 37°C. Extracted lipids were converted to fatty acid methyl esters (FAME) using sodium methoxide in anhydrous methanol (0.5N) followed by hydrochloric acid in methanol (1:1, vol/vol) and using 1 mg of nonadecanoic acid as internal standard. FAME from lyophilized milk fat samples were prepared by direct trans-esterification using KOH in methanol (2N) and extracted with hexane²⁶.

FAME from mammary gland and milk samples were then analyzed by gas chromatography using a Shimadzu 2010Plus (Shimadzu, Kyoto, Japan), equipped with a flame-ionization detector a fused silica capillary column (SP-2560, 100 m, 0.2 mm internal diameter, and 0.20 μ m film thickness; Supelco Inc., Bellefonte, PA, USA). Initial oven temperature of 50°C was held for 1 min, increased at 50°C/min to 150°C and held for 20 min, increased at 1°C/min to 190°C and then increased at 2°C/min to 220°C and held for 18 min. The injector and detector temperatures were maintained at 250°C. Helium was used as carrier gas at a flow rate of 1 mL/min and 1 μ L of sample was injected. Identification of FAME was

achieved by comparison of the FAME retention times with those of commercial standard mixtures (FAME mix 37 components from Supelco Inc., Bellefonte, PA, USA) and by electron impact mass spectrometry using a Shimadzu GC-MS QP2010 Plus (Shimadzu, Kyoto, Japan).

Data Analysis

For univariate analysis, data from both samples were evaluated using Proc MIXED of SAS (SAS Inst., Cary, NC, USA) with a model that included the treatment (control vs. restricted) and the breed and their interaction as fixed effects. Significance was considered for $p < 0.05$.

Multivariate analysis was performed to mammary gland and milk samples, using SIMCA 13.0.3.0 software (Umetrics AB, Umeå, Sweden). To evaluate sample clustering was applied the unsupervised Principal Component Analysis (PCA).

Animal Welfare Disclaimer

Spanish and European Union guidelines and legislation on care, use and handling of experimental farm animals were followed. This experiment was authorized by the scientific committee of the faculty of veterinary medicine of the Universidad de Las Palmas de Gran Canaria (Gran Canaria, Spain). Author AM Almeida holds a FELASA grade C certificate, enabling the design and conduction of animal experimentation in the European Union.

3.4 RESULTS

3.4.1 NMR-Metabolomics

Mammary Gland

A representative ^1H 1D NOESY spectrum of the mammary gland tissue, with examples of identified metabolites is shown in Figure 3.2. In this specific example a spectrum of the *Majorera* breed is presented (control group).

Metabolite concentrations in the mammary gland from the four experimental groups are shown in Table 3.1. A total of 46 metabolites were identified in the aqueous fraction of the mammary gland extract. The major constituents of the mammary gland in all groups are lactose, glutamate, glycine, lactate and glucose. Creatinine is the only metabolite whose concentration is significantly different between breeds ($p < 0.01$), being two times higher in *Majorera* breed control group than in *Palmera* breed control group. For the other metabolites, no statistically significant differences were found between breeds neither for control nor for restricted-fed groups. Additionally, some differences in metabolite concentrations were observed between control and underfed groups within each breed: acetate, AMP/ADP/ATP, IMP and phosphocholine.

Changes in different metabolite concentrations are highlighted in Table 3.1. Among these, the most evident variation occurred in adenosine-phosphates group (AMP/ADP/ATP), which decreased about 19 times from the control to the restricted-fed group in *Majorera*, without changes in *Palmera*.

PCA of the metabolite concentrations in all groups was performed. The scores plot did not show any clear grouping of the samples (Fig. 3.3A), but a slight discrimination between control and restricted-fed groups (in both breeds) was observed on the second principal component. PLS-DA was performed in paired groups: *Majorera* control versus *Palmera* control, *Majorera* control versus *Majorera* restricted-fed, and *Palmera* control versus *Palmera* restricted-fed. This test was able to separate *Majorera* control and *Palmera* control (Fig. 3.3B), *Majorera* control and *Majorera* restricted-fed groups (Fig. 3.3C), with good quality parameters, with only two components, in each test. On the other hand, it was only possible to separate *Palmera* control and *Palmera* groups applying the OPLS-DA model (Fig. 3.3D). These data indicate that animals of different groups have different responses to the weight loss induction. Loadings plots of the PLS-DA of *Majorera* breed (control versus restricted-fed) and the OPLS-DA of *Palmera* breed (control versus restricted-fed) are presented as supplementary material (Supplementary Figure S3.1).

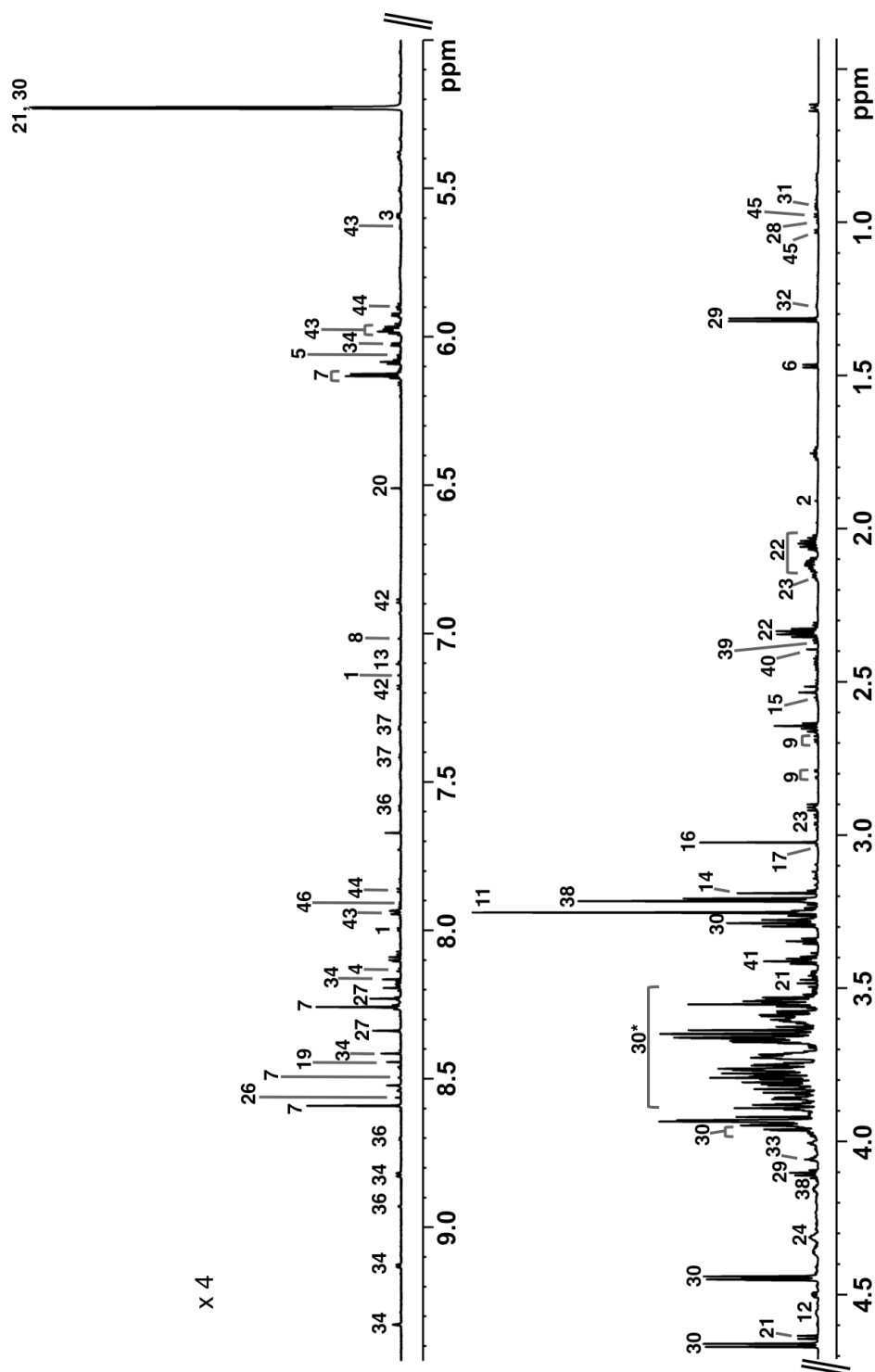


Figure 3.2. Representative 800 MHz ^1H 1D NOESY spectrum of mammary gland aqueous fraction in *Majorera* breed, control group. Key: (1) 3-methylhistidine; (2) acetate; (3) acetyl-L-carnitine; (4) adenine; (5) adenosylhomocysteine; (6) alanine; (7) AMP/ADP/ATP; (8) anserine; (9) aspartate; (10) benzoate; (11) betaine; (12) carnitine; (13) carnosine; (14) choline; (15) citrate; (16) creatine; (17) creatine phosphate; (18) creatinine; (19) formate; (20) fumarate; (21) glucose; (22) glutamate; (23) glutathione; (24) glycerophosphocholine; (25) glycine; (26) IMP; (27) inosine; (28) isoleucine; (29) lactate; (30) lactose; (31) leucine; (32) methylmalonate; (33) myo-inositol; (34) NAD $^{+}$; (35) NADP $^{+}$; (36) nicotinurate; (37) phenylalanine; (38) phosphocholine; (39) pyruvate; (40) succinate; (41) taurine; (42) tyrosine; (43) UDP-glucose; (44) uridine; (45) valine; (46) xanthine.

Table 3.1. Identified metabolites in aqueous fraction of mammary gland of *Majorera* and *Palmera* breeds (restricted and control groups). Average concentration (mmol/g tissue) and standard deviation are shown for each experimental group. In these samples the limit for compound identification in Chenomx software was 0.0145mM.

	<i>Majorera</i> control	<i>Majorera</i> restricted	<i>Palmera</i> control	<i>Palmera</i> restricted
3-methylhistidine	1.6E-04 ± 4.3E-05	2.1E-04 ± 1.2E-04	1.7E-04 ± 6.7E-05	2.3E-04 ± 1.2E-04
Acetate ^a	1.4E-04 ± 3.9E-05	2.9E-04 ± 9.6E-05*	2.3E-04 ± 1.1E-04	2.5E-03 ± 3.5E-03
Acetyl-L-carnitine	1.1E-04 ± 5.3E-05	7.7E-05 ± 4.9E-05	1.1E-04 ± 3.7E-05	1.5E-04 ± 9.4E-05
Adenine	3.1E-04 ± 4.2E-04	1.5E-04 ± 8.2E-05	1.9E-04 ± 1.2E-04	4.0E-04 ± 4.8E-04
Adenosylhomocysteine	7.4E-05 ± 3.2E-05	8.1E-05 ± 8.5E-05	1.1E-04 ± 8.6E-05	1.9E-04 ± 2.0E-04
Alanine	1.0E-03 ± 1.9E-04	8.0E-04 ± 3.5E-04	9.2E-04 ± 4.9E-04	8.6E-04 ± 7.1E-04
AMP/ADP/ATP ^a	1.2E-03 ± 5.7E-04	6.2E-05 ± 3.2E-05*	1.1E-03 ± 4.9E-04	7.8E-05 ± 2.7E-05
Anserine	1.6E-04 ± 3.7E-05	2.0E-04 ± 2.1E-04	1.9E-04 ± 7.1E-05	2.2E-04 ± 9.8E-05
Aspartate	8.2E-04 ± 4.1E-04	9.5E-04 ± 4.4E-04	8.8E-04 ± 3.7E-04	6.8E-04 ± 3.8E-04
Benzoate (moiety)	7.7E-05 ± 4.7E-05	5.4E-05 ± 3.8E-05	6.7E-05 ± 5.5E-05	5.0E-05 ± 3.3E-05
Betaine	2.4E-03 ± 1.2E-03	1.7E-03 ± 9.9E-04	1.3E-03 ± 9.7E-04	1.9E-03 ± 1.6E-03
Carnitine	6.8E-04 ± 1.2E-04	3.3E-04 ± 2.3E-04	4.9E-04 ± 1.8E-04	2.9E-04 ± 6.4E-04
Carnosine	1.6E-04 ± 4.7E-05	1.2E-04 ± 8.7E-05	1.1E-04 ± 4.4E-05	1.6E-04 ± 1.2E-04
Choline	9.9E-04 ± 1.2E-04	1.6E-03 ± 1.1E-03	7.4E-04 ± 2.6E-04	1.4E-03 ± 1.0E-03
Citrate	1.1E-04 ± 7.2E-05	1.8E-04 ± 1.3E-04	2.0E-04 ± 2.6E-04	1.6E-04 ± 1.3E-04
Creatine	2.2E-03 ± 5.3E-04	1.9E-03 ± 1.2E-03	1.9E-03 ± 5.9E-04	1.9E-03 ± 1.2E-03
Creatine phosphate	2.0E-04 ± 1.9E-04	1.5E-04 ± 8.0E-05	1.0E-04 ± 3.6E-05	1.4E-04 ± 4.0E-05
Creatinine	6.4E-05 ± 1.1E-05	3.8E-04 ± 5.8E-04	3.0E-05 ± 1.8E-05	2.2E-04 ± 3.7E-04
Formate	7.6E-04 ± 8.9E-04	4.0E-04 ± 1.9E-04	8.2E-04 ± 7.2E-04	3.4E-04 ± 6.4E-05
Fumarate	1.3E-04 ± 3.5E-05	6.0E-05 ± 4.2E-05	1.1E-04 ± 5.4E-05	3.2E-05 ± 6.9E-05
Glucose	3.8E-03 ± 1.5E-03	4.4E-03 ± 6.0E-04	3.4E-03 ± 1.2E-03	3.6E-03 ± 2.4E-03
Glutamate	7.2E-03 ± 1.8E-03	4.2E-03 ± 2.3E-03	6.3E-03 ± 2.9E-03	4.8E-03 ± 3.0E-03
Glutathione	8.0E-04 ± 3.2E-04	4.0E-04 ± 2.7E-04	8.6E-04 ± 3.7E-04	3.8E-04 ± 2.2E-04
Glycerophosphocholine	1.6E-03 ± 5.5E-04	1.5E-03 ± 1.2E-03	2.5E-03 ± 1.3E-03	1.4E-03 ± 9.6E-04
Glycine	3.2E-03 ± 1.2E-03	4.7E-03 ± 2.7E-03	5.0E-03 ± 3.0E-03	5.1E-03 ± 2.7E-03
IMP ^a	1.5E-04 ± 6.2E-05	2.0E-05 ± 2.1E-05*	1.7E-04 ± 1.0E-04	4.4E-05 ± 1.9E-05*
Inosine	6.8E-04 ± 2.0E-04	9.6E-04 ± 6.6E-04	5.3E-04 ± 1.5E-04	7.4E-04 ± 4.7E-04
Isoleucine	1.7E-04 ± 3.2E-05	1.4E-04 ± 6.5E-05	1.3E-04 ± 4.3E-05	1.3E-04 ± 1.3E-04
Lactate	3.9E-03 ± 8.6E-04	4.6E-03 ± 3.0E-03	4.2E-03 ± 2.2E-03	5.1E-03 ± 4.5E-03
Lactose	2.5E-02 ± 1.0E-02	9.8E-03 ± 7.1E-03	2.3E-02 ± 1.5E-02	1.2E-02 ± 8.0E-03
Leucine	3.1E-04 ± 5.1E-05	2.6E-04 ± 1.4E-04	2.6E-04 ± 8.8E-05	2.6E-04 ± 2.2E-04
Methylmalonate	5.9E-05 ± 7.3E-05	5.6E-04 ± 1.0E-03	5.7E-05 ± 6.8E-05	3.7E-05 ± 4.5E-05
Myo-inositol	1.5E-03 ± 5.5E-04	1.1E-03 ± 7.6E-04	1.6E-03 ± 6.2E-04	2.0E-03 ± 1.1E-03
NAD ⁺	8.4E-05 ± 7.9E-05	6.9E-05 ± 6.3E-05	5.9E-05 ± 3.2E-05	6.9E-05 ± 7.6E-05
NADP ⁺	4.2E-05 ± 1.4E-05	2.7E-05 ± 2.1E-05	3.2E-05 ± 1.1E-05	2.4E-05 ± 1.2E-05
Nicotinurate	2.5E-04 ± 1.6E-04	2.1E-04 ± 1.2E-04	1.8E-04 ± 5.3E-05	1.6E-04 ± 1.0E-04
Phenylalanine	1.1E-04 ± 3.9E-05	9.3E-05 ± 4.4E-05	9.0E-05 ± 2.6E-05	9.8E-05 ± 8.8E-05
Phosphocholine ^a	1.2E-03 ± 5.7E-04	1.6E-04 ± 1.2E-04*	1.1E-03 ± 3.7E-04	3.0E-04 ± 3.8E-04*
Pyruvate	2.0E-04 ± 8.1E-05	1.7E-04 ± 1.1E-04	1.6E-04 ± 1.3E-04	1.4E-04 ± 5.4E-05
Succinate	1.5E-04 ± 7.5E-05	2.3E-04 ± 1.2E-04	2.0E-04 ± 1.0E-04	2.8E-04 ± 1.7E-04
Taurine	3.2E-03 ± 1.4E-03	2.4E-03 ± 1.6E-03	1.8E-03 ± 1.0E-03	2.6E-03 ± 1.6E-03
Tyrosine	1.4E-04 ± 2.2E-05	1.0E-04 ± 5.2E-05	1.1E-04 ± 4.2E-05	1.0E-04 ± 8.8E-05
UDP-glucose	2.8E-04 ± 8.5E-05	1.9E-04 ± 1.4E-04	2.5E-04 ± 9.0E-05	2.3E-04 ± 9.9E-05
Uridine	1.5E-04 ± 1.0E-04	3.7E-04 ± 2.1E-04	1.2E-04 ± 2.7E-05	2.6E-04 ± 2.4E-04
Valine	2.8E-04 ± 3.9E-05	2.0E-04 ± 1.1E-04	2.5E-04 ± 6.7E-05	1.8E-04 ± 1.7E-04
Xanthine	1.5E-04 ± 6.0E-05	1.9E-04 ± 7.5E-05	1.3E-04 ± 6.2E-05	1.3E-04 ± 9.6E-05

Key: ^(a) metabolites with significant differences between control and restricted groups in one/both breeds; (moiety) only the aromatic part of the molecule was confirm; (*) $p < 0.05$ when compared with control of the same breed.

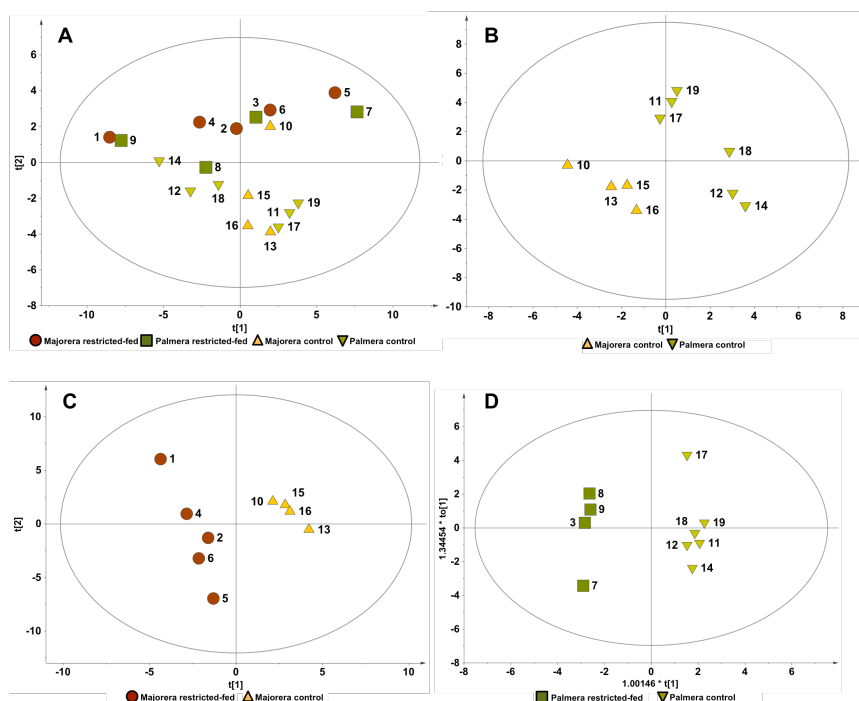


Figure 3.3. Multivariate analysis of mammary gland (aqueous fraction) metabolites concentrations. The ellipses in the scores plots are drawn at the 95% confidence level. (A) PCA scores plot for the 4 experimental groups (NC= 3, PC1=39.5%, PC2=13.9%); (B) PLS scores plot for *Majorera* control and *Palmera* control groups (NC=3, $Q^2=0.66$, $R^2=0.64$, $\rho_{CV-ANOVA}=0.89$, PC1=22.4%, PC2=21.3%); (C) PLS scores plot for *Majorera* control and *Majorera* restricted groups (NC=2, $Q^2=0.81$, $R^2=0.56$, $\rho_{CV-ANOVA}=0.37$, PC1=23.5%, PC2=32.1%); (D) OPLS scores plot for *Palmera* control and *Palmera* restricted groups (NC=4, $Q^2=0.63$, $R^2=0.74$, $\rho_{CV-ANOVA}=1.01$, PC1=12.3%, PC2=10.5%).

Milk Serum

A characteristic ^1H 1D NOESY spectrum of milk serum, with examples of identified metabolites, of *Palmera* breed (control groups) is shown in Figure 3.4.

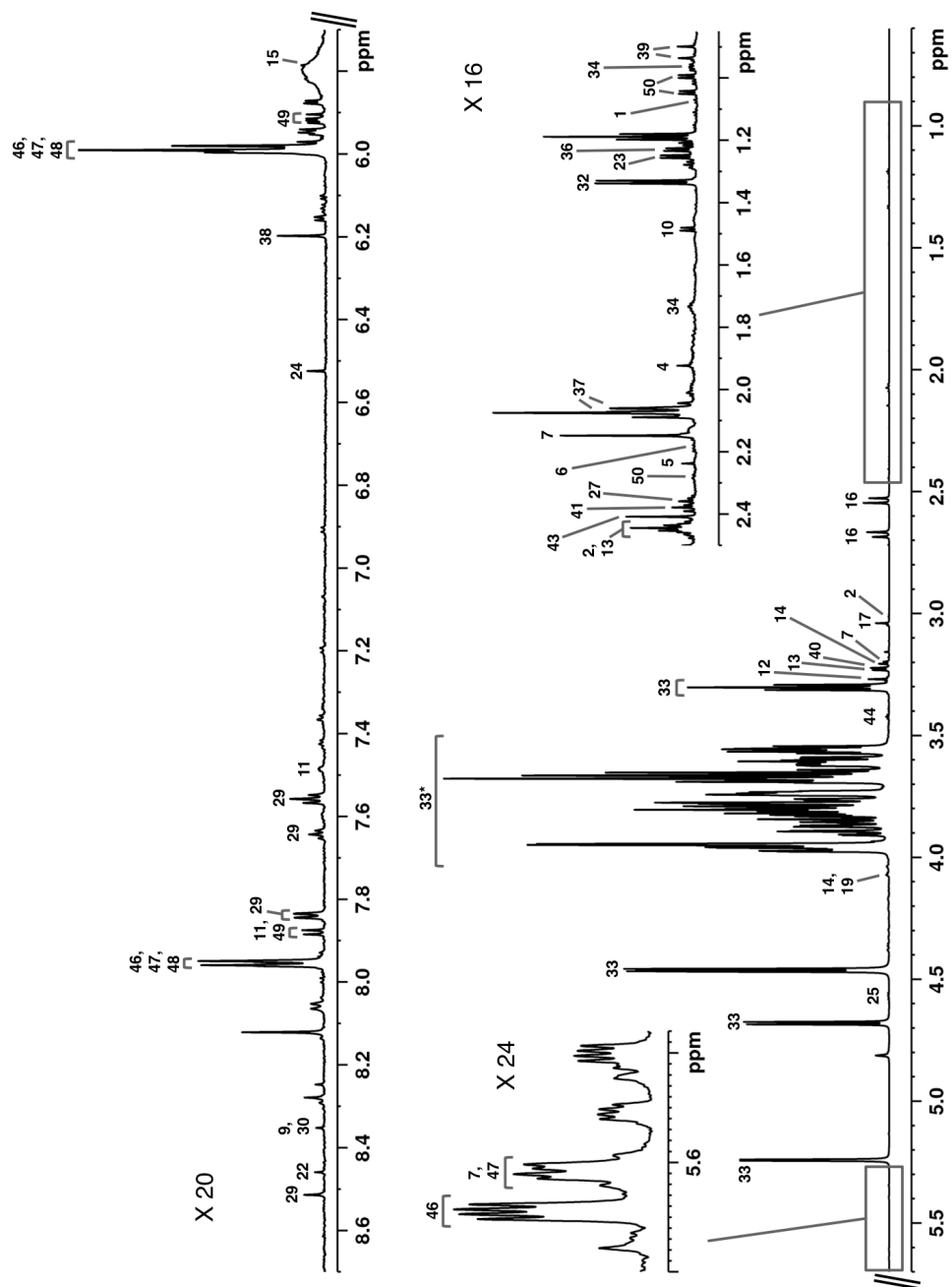


Figure 3.4. Representative 800 MHz ^1H 1D NOESY spectrum of milk serum in *Palmera* breed, control group. Key: (1) 2-oxobuturate; (2) 2-oxoglutarate; (3) β -hydroxybutyrate; (4) acetate; (5) acetone; (6) acetylcholine; (7) acetyl-L-carnitine; (8) adenine; (9) adenosine; (10) alanine; (11) benzoate; (12) betaine; (13) carnitine; (14) choline; (15) cis-aconitate; (16) citrate; (17) creatine; (18) creatine phosphate; (19) creatinine; (20) dimethylglycine; (21) ethanolamine; (22) formate; (23) fucose; (24) fumarate; (25) galactose; (26) glucose; (27) glutamate; (28) glycine; (29) hippurate; (30) inosine; (31) isoleucine; (32) lactate; (33) lactose; (33*) region strongly dominated by lactose peaks, but with several less concentrated metabolites underneath them; (34) leucine; (35) malonate; (36) methylmalonate; (37) N-acetylglucosamine; (38) orotate; (39) pantothenate; (40) phosphocholine; (41) pyruvate; (42) riboflavin; (43) succinate; (44) taurine; (45) trimethylamine oxide; (46) UDP-galactose; (47) UDP-glucose; (48) UDP-glucuronate; (49) uridine; (50) valine.

Similarly to mammary gland samples, identified metabolites from the four experimental groups are shown in Table 3.2. A total of 50 metabolites were identified in milk serum. No differences were observed between breeds, either when comparing control or when comparing restricted-fed groups. In control groups the first five most concentrated metabolites are the same: lactose, citrate, phosphocholine, taurine and glycine. In the restricted-fed groups, only the first three of the relative most concentrated metabolites are common: lactose, citrate and creatine. Although with only significant difference in citrate and creatine, these results suggest higher variation in relative concentration of metabolites in restricted-fed groups than in the control ones. Differences in metabolites concentration between control and restricted-fed groups for each breed are shown in Table 3.2. Several metabolites showed variation between experimental groups such as acetyl-L-carnitine, adenosine, alanine, carnitine, citrate, creatine, dimethylglycine, fucose, fumarate, hippurate, lactate, methylmalonate, N-aceylglucosamine, succinate, trimethylamine oxide (TMAO), UDP-galactose and UDP-glucose. Among these, the higher variation occurred in fumarate that decreased 15 fold in *Majorera* and approximately 7 times in case of *Palmera* breed.

Table 3.2. Identified metabolites in milk serum of *Majorera* and *Palmera* breeds (restricted and control groups). Average concentration (mM) and standard deviation are shown for each experimental group. In these samples the limit for compound identification in Chenomx software was 0.0004 mM.

	Majorera control	Majorera restricted	Palmera control	Palmera restricted
2-Oxobutyrate	3.9E-03 ± 2.0E-03	5.6E-03 ± 1.9E-03	3.9E-03 ± 2.0E-03	5.6E-03 ± 1.9E-03
2-Oxoglutarate	1.6E-01 ± 3.8E-02	3.3E-02 ± 2.3E-02	1.2E-01 ± 3.0E-02	3.9E-02 ± 3.6E-02
β-Hydroxybutyrate	4.4E-03 ± 0.0	5.9E-03 ± 2.2E-03	4.3E-03 ± 2.4E-04	4.4E-03 ± 0.0
Acetate	1.7E-02 ± 3.3E-03	1.9E-02 ± 2.7E-03	1.7E-02 ± 1.6E-03	2.6E-02 ± 7.3E-03
Acetone	6.9E-03 ± 1.5E-03	6.8E-03 ± 1.2E-03	6.7E-03 ± 1.3E-03	7.7E-03 ± 2.3E-03
Acetylcholine	4.8E-03 ± 8.4E-04	8.4E-03 ± 6.8E-03	8.0E-03 ± 4.3E-03	1.0E-02 ± 1.9E-03
Acetyl-L-carnitine ^a	4.7E-02 ± 1.1E-02	2.4E-01 ± 3.4E-02**	5.8E-02 ± 3.0E-02	2.4E-01 ± 7.7E-02*
Adenine	1.1E-02 ± 8.7E-03	4.3E-03 ± 3.0E-03	4.2E-03 ± 2.9E-03	8.7E-03 ± 6.8E-03
Adenosine (moiety) ^a	5.0E-03 ± 1.4E-03	1.9E-03 ± 3.6E-04*	4.1E-03 ± 2.5E-03	4.4E-03 ± 2.7E-03
Alanine ^a	2.3E-02 ± 1.1E-02	9.4E-03 ± 2.9E-03	3.8E-02 ± 2.1E-02	9.0E-03 ± 2.5E-03*
Benzoate (moiety)	8.0E-03 ± 3.5E-03	7.5E-03 ± 3.2E-03	5.9E-03 ± 3.0E-03	8.2E-03 ± 4.2E-03
Betaine	4.0E-01 ± 1.2E-01	5.2E-01 ± 1.9E-01	2.3E-01 ± 8.9E-02	5.4E-01 ± 2.0E-01
Carnitine ^a	9.6E-02 ± 1.4E-02	3.8E-02 ± 3.1E-02**	1.2E-01 ± 4.3E-02	4.7E-02 ± 2.0E-02**
Choline	1.5E-02 ± 4.3E-03	3.4E-01 ± 2.7E-01	7.8E-02 ± 8.7E-02	2.2E-01 ± 2.3E-01
Cis-Aconitate	6.2E-03 ± 5.3E-03	6.9E-03 ± 1.5E-03	1.6E-02 ± 1.1E-02	1.1E-02 ± 3.3E-03
Citrate ^a	4.0 ± 1.3	8.7E-01 ± 8.2E-01**	4.6 ± 1.7	1.7 ± 1.0**
Creatine ^a	4.4E-01 ± 6.2E-02	1.1 ± 4.2E-01*	3.8E-01 ± 1.4E-01	8.6E-01 ± 2.3E-01*
Creatine phosphate	4.6E-01 ± 1.3E-01	2.9E-01 ± 2.0E-01	2.7E-01 ± 9.9E-02	1.5E-01 ± 2.2E-01
Creatinine	5.1E-02 ± 3.1E-02	7.4E-02 ± 3.4E-02	6.6E-02 ± 1.7E-02	6.9E-02 ± 7.2E-02
Dimethylglycine ^a	3.3E-03 ± 6.8E-04	1.6E-03 ± 7.9E-04*	2.0E-03 ± 7.3E-04	1.3E-03 ± 6.8E-04
Ethanolamine	5.2E-02 ± 8.7E-03	2.8E-01 ± 2.1E-01	7.0E-02 ± 3.1E-02	2.2E-01 ± 1.1E-01
Formate	2.5E-02 ± 5.8E-03	2.2E-02 ± 5.6E-03	1.8E-02 ± 5.4E-03	3.0E-02 ± 7.4E-03
Fucose ^a	2.5E-02 ± 6.2E-03	1.8E-01 ± 9.8E-02*	3.7E-02 ± 2.9E-02	1.4E-01 ± 6.5E-02*
Fumarate ^a	1.4E-02 ± 7.6E-03	9.2E-04 ± 6.7E-04**	9.9E-03 ± 4.0E-03	1.3E-03 ± 1.3E-03**
Galactose	1.7E-01 ± 4.1E-02	2.9E-01 ± 7.0E-02	2.3E-01 ± 6.0E-02	2.0E-01 ± 3.3E-02
Glucose	1.3E-01 ± 1.1E-01	8.2E-02 ± 5.6E-02	8.0E-02 ± 1.2E-02	1.1E-01 ± 5.2E-02
Glutamate	3.3E-02 ± 1.1E-02	1.8E-02 ± 8.2E-03	4.5E-02 ± 1.3E-02	2.8E-02 ± 5.9E-03
Glycine	5.5E-01 ± 0.0	5.2E-01 ± 7.6E-02	5.5E-01 ± 9.4E-02	5.1E-01 ± 8.5E-02
Hippurate ^a	5.0E-02 ± 9.5E-03	9.0E-02 ± 7.9E-03**	5.1E-02 ± 1.4E-02	1.1E-01 ± 5.8E-02
Inosine	2.1E-02 ± 6.9E-03	9.3E-03 ± 1.0E-02	9.8E-03 ± 1.1E-02	1.3E-02 ± 1.4E-02
Isoleucine	2.0E-04 ± 0.0	2.0E-04 ± 0.0	2.0E-04 ± 0.0	7.0E-04 ± 1.0E-03
Lactate ^a	5.5E-02 ± 3.3E-02	3.0E-01 ± 2.5E-01	7.5E-02 ± 3.7E-02	2.5E-01 ± 8.3E-02*
Lactose	1.2E+02 ± 8.2	1.0E+02 ± 8.6	1.2E+02 ± 1.9E+01	9.8E+01 ± 1.3E+01
Leucine	9.2E-03 ± 5.1E-03	6.6E-03 ± 2.4E-03	1.0E-02 ± 3.2E-03	5.8E-03 ± 1.7E-03
Malonate	4.1E-02 ± 1.0E-02	4.6E-02 ± 3.1E-02	2.8E-02 ± 1.4E-02	2.3E-02 ± 7.0E-03
Methylmalonate ^a	4.8E-02 ± 5.9E-03	8.1E-02 ± 2.9E-02	4.0E-02 ± 9.6E-03	7.7E-02 ± 2.4E-02*
N-Acetylglucosamine ^a	2.4E-01 ± 1.4E-02	8.3E-02 ± 4.0E-02**	1.8E-01 ± 2.8E-02	8.0E-02 ± 2.1E-02**
Orotate	6.5E-04 ± 3.0E-04	2.8E-04 ± 3.0E-04	5.7E-04 ± 2.3E-04	7.0E-04 ± 3.8E-04
Pantothenate	1.7E-02 ± 5.0E-03	1.5E-02 ± 3.0E-03	1.6E-02 ± 5.2E-03	1.5E-02 ± 4.2E-03
Phosphocholine	1.0 ± 2.7E-01	6.1E-01 ± 4.6E-01	6.3E-01 ± 2.1E-01	2.7E-01 ± 4.4E-01
Pyruvate	1.7E-02 ± 9.6E-03	1.5E-02 ± 7.2E-03	1.7E-02 ± 7.2E-03	1.7E-02 ± 4.5E-03
Riboflavin	1.0E-03 ± 0.0	1.0E-03 ± 0.0	1.0E-03 ± 0.0	1.0E-03 ± 0.0
Succinate ^a	4.4E-02 ± 1.4E-02	1.2E-02 ± 7.9E-03*	4.0E-02 ± 1.6E-02	1.5E-02 ± 8.2E-03*
Taurine	5.6E-01 ± 9.1E-02	3.3E-01 ± 1.5E-01	3.4E-01 ± 1.7E-01	3.5E-01 ± 7.3E-02
TMAO ^a	2.9E-02 ± 2.9E-03	1.2E-02 ± 5.9E-03**	1.9E-02 ± 7.0E-03	2.2E-02 ± 1.8E-02
UDP-galactose ^a	3.5E-01 ± 9.4E-02	8.7E-02 ± 9.7E-02*	2.5E-01 ± 1.2E-01	5.4E-02 ± 3.8E-02**
UDP-glucose ^a	1.9E-01 ± 5.7E-02	4.4E-02 ± 4.2E-02**	1.1E-01 ± 7.3E-02	3.2E-02 ± 2.2E-02*
UDP-glucuronate	2.3E-02 ± 1.0E-02	6.2E-03 ± 4.5E-03	3.6E-02 ± 5.5E-02	1.7E-02 ± 1.5E-02
Uridine	5.2E-02 ± 1.7E-02	2.3E-02 ± 1.6E-02	3.8E-02 ± 5.1E-03	2.2E-02 ± 1.5E-02
Valine	2.2E-02 ± 1.1E-02	9.8E-03 ± 2.8E-03	2.1E-02 ± 6.1E-03	1.1E-02 ± 6.8E-03

Key: (^a) metabolites with significant differences between control and restricted groups in one/both breeds; (moiety) only the aromatic part of the molecule was confirm; (TMAO) trimethylamine oxide; (*) $p < 0.05$ when compared with control of the same breed; (**) $p < 0.01$ when compared with control of the same breed.

PCA performed with metabolite concentrations of samples from all groups, revealed an existing separation between control and restricted-fed groups. PLS-DA models were conducted to clarify this separation between groups in the following sets of data: *Majorera* control and *Palmera* control, *Majorera* control and *Majorera* restricted-fed, and *Palmera* control and *Palmera* restricted-fed. This test was able to separate the three data sets, as displayed in Figure 3.5, with good quality parameters.

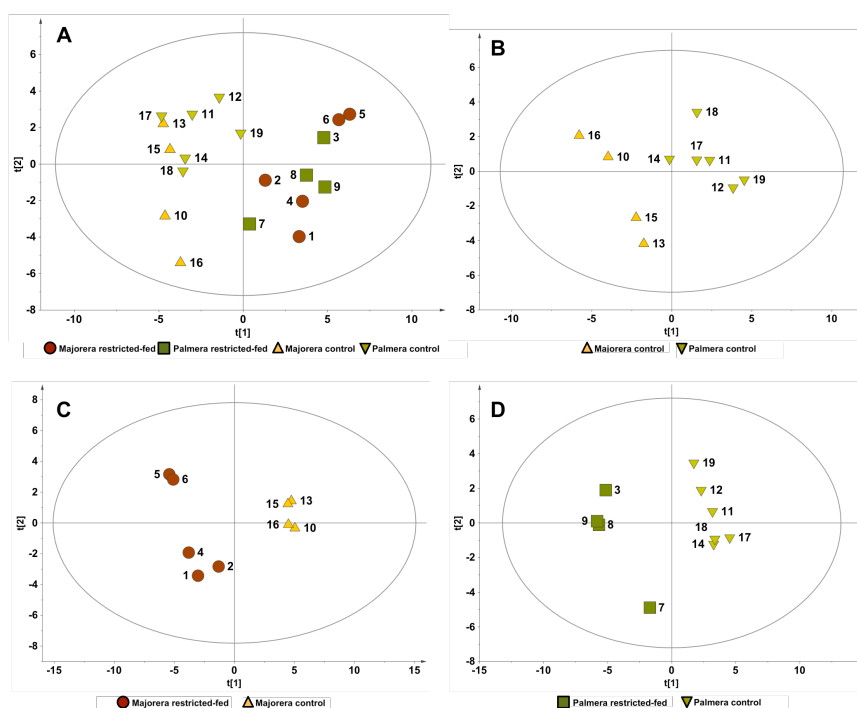


Figure 3.5. Multivariate analysis of milk serum metabolites concentrations. The ellipses in the scores plots are drawn at the 95% confidence level.. (A) PCA scores plot for the 4 experimental groups (NC= 3, PC1=32.8%, PC2=13.5%); (B) PLS scores plot for *Majorera* control and *Palmera* control groups (NC=3, $Q^2=0.68$, $R^2=0.52$, $\rho_{CV-ANOVA}=0.48$, PC1=25.3%, PC2=14.6%); (C) PLS scores plot for *Majorera* control and *Majorera* restricted-fed groups (NC=2, $Q^2=0.91$, $R^2=0.59$, $\rho_{CV-ANOVA}=0.03$, PC1=44.4%, PC2=14.2%); (D) PLS scores plot for *Palmera* control and *Palmera* restricted-fed groups (NC=2, $Q^2=0.82$, $R^2=0.48$, $\rho_{CV-ANOVA}=0.62$, PC1=35.7%, PC2=12.5%).

It is noteworthy to mention that only two components were needed to separate control and restricted-fed groups, in both breeds, with good quality parameters for the model. *Majorera* control and *Palmera* control groups were separated by three components.

Loadings plots of the PLS-DA of *Majorera* breed (control versus restricted-fed) and *Palmera* breed (control versus restricted-fed) are presented in supplementary material (Supplementary Figure S3.2).

3.4.2 Fatty Acids Profile

The lipid and fatty acid content (mg/g dry tissue) and fatty acid composition of mammary gland tissue, expressed in percentage of the total fatty acids, is presented in Supplementary Table S3.1.

The most representative fatty acids of mammary gland tissue in all experimental groups were oleic acid (18:1*cis*-9), palmitic acid (16:0) and stearic acid (18:0), representing 69% of the total fatty acids in *Palmera* control group and 77% in *Palmera* restricted-fed group. It is noteworthy that 16:0 and 18:1*cis*-9 were the only two fatty acid that presented significant ($p \leq 0.05$) interactions between restricted-fed and breed. Although the proportions of 16:0 and 18:1*cis*-9 numerically decreased and increased respectively with feed restriction in both breeds, only in *Palmera* the changes were significant. In the mammary gland, most of the differences observed were due to the restricted-fed-effect and no breed-effect differences ($p > 0.05$) were observed besides 16:0 and 18:1*cis*-9. Concerning the effect of feed restriction, it was observed that the proportions of 12:0, 14:0, 14:1*cis*-9, 15:0, iso-16:0, 16:0, 18:3n-3, 18:2 *cis*-9,*trans*-11 and a few 18:1-*trans* were lower, and only the

17:1*cis*-9, 18:1*cis*-9 and 20:4n-6 were higher in restricted-fed group compared to control. Thus, the effect of feed restriction was also observed in SFA and MUFA sums, with the *cis*-MUFA presenting also greater proportions in restricted-fed group than in control. However, looking to the indexes used to estimate the SCD activity only SCD-17 (17:1*cis*-9/(17:1*cis*-9+17:0) showed a tendency ($p=0.078$) to present higher values for the restricted-fed group compared to control.

The PCA of the four experimental groups did not reveal any clear group separation (Figure 3.6). Although a slight tendency to sample clustering by breed along the PC1, results are not robust to discuss this separation. Loadings list of this model is presented in Supplementary Table S3.2.

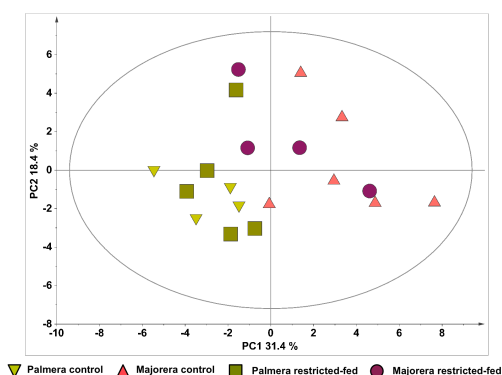


Figure 3.6. Multivariate analysis of mammary gland fatty acids percentages: Principal Component Analysis scores of the four experimental groups (NC = 4; PC1 = 31.4%; PC2 = 18.4%; ellipse: Hostelling's T2 (95%)).

The fatty acid composition of milk, expressed in percentage of the total fatty acids, is presented in Supplementary Table S3.3. From the ten more abundant fatty acids in this sample, eight are saturated fatty acids representing around 75% of the total fatty acids. Oleic acid (18:1*cis*-9)

and linoleic acid (18:2n-6) are the other two compounds in this bulk, noting that 18:1*cis*-9 is the second most abundant fatty acid in the sample. Milk presented only two minor fatty acids with significant interaction ($p < 0.05$) between breed and treatment, namely margaric acid (17:0) and heptadecenoic acid (17:1*cis*-9). In both breeds the proportions of 17:0 and 17:1*cis*-9 were higher in restricted-fed groups compared to control groups, however *Majorera* showed the greatest increase in both fatty acids. The majority of the 46 identified fatty acids presented differences due to feed restriction, while 10 fatty acids did not show any ($p > 0.05$) effect with treatment. Some minor fatty acids also showed significant differences ($p < 0.05$) between breeds, with the short chain-fatty acids, iso-14:0, 18:0, 20:0 and 22:0 presenting the highest proportions in the *Palmera* breed and the 14:0, 14:1*cis*-9, 16:1*cis*-9 and 18:2 isomers the highest proportions in the *Majorera* breed. A decrease in the total saturated fatty acids (SFA) from control to restricted-fed groups was also observed. Concerning the *cis*-monounsaturated fatty acids (*cis*-MUFA), restricted-fed groups presented higher values than control groups. The opposite response was observed in the total *trans*-MUFA restricted-fed groups presented a lower percentage of total *trans*-MUFA than control groups. No significant differences were observed between treatments in the total polyunsaturated fatty acids (PUFA). Regarding the indexes that estimate SCD activity, except SCD₁-14, all indexes were higher ($p < 0.001$) in restricted-fed animals compared to control. In addition, *Majorera* breeds showed higher ($p < 0.05$) indexes compared to *Palmera* breed. No interaction ($p > 0.05$) between breed and feed restriction was detected in any of the estimated indexes.

A PCA of the four experimental groups revealed separation by treatment, along to the first principal component (PC1) (Figure 3.7). Analysing the

loadings values for this model (Supplementary Table S3.4), it was possible to identify that major differences were due to variations in 18:1*cis*-9, 17:1*cis*-9, 15:0 and 10:0 between control and restricted groups. It is noteworthy to mention that the variables related as relevant in loadings were all fatty acids with significant variation due to feed restriction (18:1*cis*-9 and 10:0) and due to interaction between breed and feed restriction (17:1*cis*-9 and 17:0) in the univariate analysis.

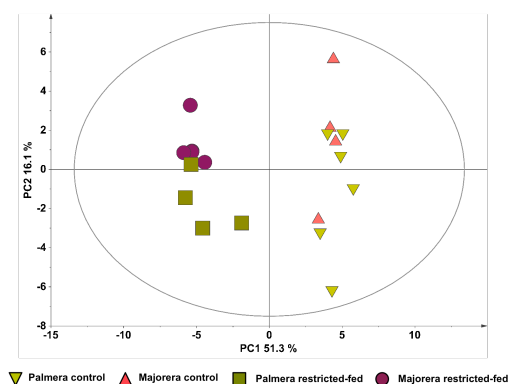


Figure 3.7. Multivariate analysis of milk fatty acids percentages: Principal Component Analysis scores of the four experimental groups (NC = 3; PC1 = 51.3%; PC2 = 16.1%; ellipse: Hostelling's T2 (95%)).

3.5 DISCUSSION

3.5.1 NMR-Metabolomics

NMR-based metabolomics proved to be a straightforward approach to investigate the responses to feed-restriction in mammary gland and milk in dairy goats. A simple 1D spectrum (Fig. 3.2) enabled the identification of metabolites, and a preliminary pattern recognition analysis. In this work, NMR also met other important advantages for metabolomics studies: simple sample preparation, quick spectra acquisition and the non-destructive nature of the method, allowing samples to be used with other techniques, if necessary.

Since it is the first time that mammary gland and milk is studied in these goat breeds, we followed both the univariate and the multivariate methods to analyse the data, as suggested in a review about data analysis in metabolomics²⁷ and followed by some authors in specific metabolomics studies^{19,23}. Given the exploratory character of the work it is not possible to preview if the studied condition (SWL) will express though differences in variations of single metabolites or in the relation of multiple metabolites. Statistical analysis revealed differences between control and restricted-fed groups and also the breed response-tendency to the treatment. Concerning univariate analysis, we observed differences in metabolites between control and restricted-fed groups, in both mammary gland and milk. In most of the cases, the variation in univariate analysis was confirmed by the loadings plot of the multivariate analysis (PCA).

In the mammary gland, PCA was not able to separate the four study groups. In fact, control and restricted-fed groups, were only separated

using PLS in the *Majorera* breed and OPLS in the *Palmera* breed. Given the nature of these tests, that over-fit models to data, analysis requires special attention. For this reason these models need validation²⁸. In our study, we accepted the results of PLS and OPLS when the quality parameters for these tests (Q^2) was above 0.6, although some authors have considered 0.541 and 0.430 as acceptable values. Nevertheless, this behaviour in PLS and OPLS tests suggests that the metabolome of the individuals from the *Palmera* breed (control and restricted-fed groups) are similar, reflecting a slower or less expressive response to feed restriction on the metabolite composition of this tissue. In the *Majorera* breed, restricted-fed and control groups were easily discriminated suggesting that, in the same experimental time, these animals had a sharper, and prompt response to the treatment. This fact could be a possible indication of an adaptive reaction via a stronger response to SWL from this breed. This indication however should be confirmed with more metabolomics experiments or with complementary transcriptomic and proteomic analysis.

PCA analysis of serum milk was able to discriminate control and restricted-fed groups, but not the breeds, which by itself, could mean that milk is a sample with more marked variations than the mammary gland. Moreover PLS was capable to separate breeds between control animals (both breeds), and control and restricted-fed groups in each breed. PLS analysis guided by the previous PCA discrimination (first grouping) is expected to produce more relevant results²⁸. Therefore, it seems that the metabolite content of the milk is more sensitive to feed restriction than those from the mammary tissue, or that the variation in milk is sharper and prompt, considering the experimental period.

Mammary gland

Most of the identified compounds in the aqueous fraction of mammary gland extract have been previously described as constituents of the mammary gland^{29,30}, as well as for other animal tissues, such as goat plasma, red meat, squirrels liver and fish muscle^{14,31–33}.

Creatinine is the only metabolite that significantly varies between control groups of the two breeds, being two times higher in *Majorera* than in the *Palmera* breed. Creatinine is constantly produced in the body and, although its concentration in blood depend of some factors (diet, gender), in general varies along with muscle mass³⁴. Lérias et al.⁷ measured the live body weight of the study animals, and observed a significant higher body weight in *Majorera* control group than in *Palmera* control group during the whole experimental period (23 days). This fact could explain the higher concentration of creatinine in this group. Another publication with the same animals⁸, revealed significant differences between creatinine concentration in blood plasma, although in the cited study, *Palmera* breed had higher creatinine concentration than *Majorera* breed (control groups). These variations with apparently opposite trends could be due to the milking process and its subsequent nutrients mobilization and synthesis, or some breed-related factors, especially when considering their different milk production yields⁷.

Significant differences were observed between control and restricted-fed groups in acetate, adenosine-phosphates (AMP/ADP/ATP), IMP and phosphocholine. Increased concentration of acetate was observed in the *Majorera* restricted-fed group. In mammals, acetate can be produced by the body, but can also be introduced by exogenous mechanisms such as bacterial fermentation in the digestive tract³⁵. For ruminants, gut

microbiota is essential for their digestion process^{35–37}. High acetate concentration is related to high fibre contents in food-intake³⁸, which was indeed one of the characteristics of the diet of the two restricted-fed groups. This difference in acetate concentration was only observed in *Majorera* breed, which was two times higher in restricted-fed than in control groups. This fact could suggest the existence of different microbiota bulks between breeds. It was referred that ruminants have a general rumen community which equilibrium is affected by diet composition^{39,40}. These differences in microbiota could be an explanation to the observed levels of acetate, since each microorganism has a different metabolism releasing different compounds to the rumen. Considering that the restricted-fed regime was used as study of SWL, this result can suggest that a similar behaviour could occur during the dry season. This could be determinant for the success of each breed in its specific environment⁴¹. Indeed, it is referred that ruminants in tropical and sub-tropical regions could have some adaptations in their digestive system that facilitate their adaptation to these environments^{42,43}, particularly to high fibrous diets^{44,45}. Acetate and β -hydroxybutyrate (identified in milk serum) are important sources of energy in ruminants⁴⁶. Increased acetate levels are also associated with higher lipid catabolic rate during fasting periods⁴⁷, which can be related with the restricted-fed diet in this group. Moreover, higher acetate levels are associated with higher fat levels in milk, since acetate is the major precursor of milk fat^{38,46}. This observation suggests that milk from restricted-fed groups could have a higher fat content than the control groups. It was described before that milk fat content varies with the characteristics and content of the diet, mediated by rumen⁴⁸. Lérias *et al.*⁷ reported a significant decrease in milk yield in both restricted-fed groups in the end of the

experiment, which corroborates this idea. However, it is important to highlight that the analysis was performed in milk serum and the lipidic fraction had been previously removed, so this interpretation should only be seen as an indication of what might have changed in that fraction. In this study, no differences were observed in β -hydroxybutyrate. However, Lérias *et al.*⁷ reported significant differences in β -hydroxybutyrate levels in blood plasma, between *Majorera* groups in the beginning and in the end of the experimental time.

Decrease in the energy-related molecules: AMP/ADP/ATP and IMP, in restricted-fed groups could be related to the feed-restriction in these animals that can lead to energy reduction in some tissues. However, Lérias *et al.*⁸ reported no changes in glucose and insulin in blood of the same animals. Adenosine-phosphates showed the sharper variation, decreased 19 times from control to restricted-fed group in *Majorera* breed with no significant variation in the *Palmera* breed. Feed restriction led to reduction in energy spending, that is greater the higher the metabolic rate of the organ⁴⁹. During lactation, the mammary gland is a very active tissue which could explain the reduction of these energy-related molecules. These compounds are also nucleosides, nucleotides precursors and considered to be essential components of milk for feeding newborn animals^{50,51}. The lower concentration of this compound in restricted-fed groups could be related to the lower nutrition value of the diet of these groups, given the fact that food-intake is one of the major sources of nucleotides in animals. Phosphocholine is one of the precursors of membrane phospholipid, phosphatyl choline and other membrane fractions of milk fat globule membrane^{30,52} and one of the storage forms of choline, a ubiquitously distributed and essential component of cells and an essential nutrient for most mammals²⁹. Since

phosphocholine is a storage form of a nutrient, the lower values in restricted-fed groups could be a direct consequence of the lower nutritional value of the diet of these groups.

Differences observed were consistent in both breeds (IMP and phosphocholine), or occurred in a single breed (acetate and AMP/ADP/ATP). However, *Majorera* breed seems to be more sensitive to the treatment (feed restriction) showing in general, sharper responses than the *Palmera* breed. Antagonistic variations between breeds were not observed for any of the metabolites studied. The same variation in a metabolite implies that both breeds respond in the same way to diet restriction but in different ranges. On the other hand, the single-breed variation suggest an exclusive breed response for a specific metabolite, or, a feed restriction not enough to reveal variations in one of the breeds.

Milk serum

The majority of the compounds identified in milk serum in this study have been previously described in milk samples^{20,21,53–55}. Other compounds are related to Krebs cycle, energy production as well as amino acid and carbohydrate metabolisms. Milk composition is affected by the breed, the genetic variation within breed, health state, the environment, the management practices and diet^{56,57}. Moreover, constituents of milk could arise from five main pathways⁵⁸, as presented in general introduction. Therefore, these several sources of possible variation should be considered for the interpretation of milk serum results.

Significant differences were observed between control and restricted-fed groups in acetyl-L-carnitine, adenosine, alanine, carnitine, citrate,

creatine, dimethylglycine, fucose, fumarate, hippurate, lactate, methylmalonate, N-acetylglucosamine, succinate, TMAO, UDP-galactose and UDP-glucose.

Significant differences were observed in some Krebs cycle intermediates, like citrate, fumarate and succinate, and their concentration decreased in restricted-fed groups. The correlation between the variations of these compounds could be confirmed by the PLS loadings plot of both breeds (Supplementary Information, Fig. S3.2), suggesting a variation in the regulation of this pathway. Fumarate and succinate, together with lactate and methylmalonate, are also intermediates in fermentation processes in the rumen^{46,59,60}. Variations of these compounds in restricted-fed groups may be related to different microbiota adaptations as a consequence of the diet change, once different diet composition determine different microbiota percentages³⁶. Noteworthy in this group of compounds, is the variation of fumarate that was 15 times decreased in *Majorera* breed and about 8 times decreased in *Palmera* breed. The other two metabolites varied in more similar ways in both breeds.

We found some metabolites whose differences between control and restricted-fed groups could also be related with variations in energy metabolism. Carnitine and acetyl-L-carnitine are related to fatty acids metabolism. Acetyl-L-carnitine, derives from carnitine, is the last transporter of long-chain fatty acids into the inner membrane of mitochondria for β -oxidation. During fasting periods, fatty acids become the major energy production substrate⁶¹, which could justify the increased concentration of acetyl-L-carnitine. Other authors mention changes in these metabolites in milk during different physiological conditions⁶². Both metabolites varied in the same way in both breeds. Alanine is an amino

acid and besides its nutritional value in milk it is also an antioxidant of milk fat⁶³ and intermediate in glucose metabolism, working as signalling molecule between muscle and liver in gluconeogenesis⁶⁴. Its decrease in milk of restricted-fed groups could be related to the regulation of some amino acids metabolic pathways⁴⁷ or glucose metabolism as consequence of variations in blood. In meat, creatine concentration has been related to the crude proteins content⁶⁵. The increased concentration of creatine in restricted-fed groups (2 to 3 times higher than the control groups) could indicate metabolism changes, to the use of stored proteins as energy source, due to the low energetic diets in these groups. All these variation could be indicative of changes in energy production due to diet restriction that could also be related to the observed variations in Krebs cycle intermediates. Blood profiles of the same animals indicated an increased level of non-esterified fatty acids in restricted-fed animals due to fat mobilization for energy production. These results corroborate the hypothesis of variations in energy production metabolism.

Adenosine is a nucleoside and a precursor of energy molecules, as explained before for other nucleosides in mammary gland^{50,51}. Similarly, its lower concentration in *Majoreira* restricted-fed group (around 3 times less than control group) could be related to the lower nutrition value of the diet that in this case is decreasing the nutritional value of the milk.

Dymethylglycine, together with choline and betaine (identified in these milk samples), share a pathway of glycine production and consecutive glutathione synthesis⁶⁶. Although without any observable correlation between them in this study, this variation could suggest some disturbance in oxidative stress regulation in restricted-fed groups, where dymethylglycine varies. Choline, as shown before, is an essential nutrient

for mammals with high demands in newborn animals, however, in ruminants it is highly degraded in rumen, so these animals synthesize it in their body⁶⁷. Choline is synthesized endogenously using metabolites from maternal blood and its storage as glycerophosphocholine and phosphocholine^{68,69}. Although we cannot correlate directly results from mammary gland and milk, it is worth mentioning that phosphocholine concentration in mammary gland tissue is lower in both restricted-fed groups, while choline concentration shows a tendency to be higher in milk serum from *Majorera* restricted-fed group, albeit with no significant difference. Given the relation between choline and the lipid metabolism, variations in choline concentration could be indicative of alterations in fat distribution in restricted-fed groups⁶⁸.

UDP-galactose and UDP-glucose were decreased in both restricted-fed groups (*Majorera* and *Palmera* breed). It is well known the importance of these two metabolites for lactose production⁷⁰. As a consequence, the lactose production in these restricted-fed groups should be decreased compared to controls, although no differences in lactose concentration were found. A relation between variations of these metabolites can be evaluated in the loading plots (Supplementary Information, Fig. S3.2) where they lay in the same region of the graphic, especially in *Majorera* breed.

Fucose and N-acetylglucosamine are components of gastrointestinal mucus. Variations in these components could determine the microbiota colonization of the gut⁷¹ and therefore affect the digestion process. It is supposed that variations in these compounds in restricted-fed groups (3-5 times lower than in control groups) are directly caused by the diet change or a side effect of some presumed changes in rumen microbiota.

Indeed it was reported that changes in rumen microbiota could affect the gastrointestinal community, since a large population in rumen easily deplete sugars and nitrogen-compounds from feedstuffs⁷¹. However, variation in fucose in the *Majorera* breed (7 times higher than control) is more marked than in *Palmera* breed (4 times higher than control), what could be indicative of different adaptive strategies between the two breeds.

TMAO and hippurate, among other compounds, were referred recently as a product of gut microbiota activity^{72,73} in humans. Although they were already described in milk²⁰, their variations between control and restricted-fed groups could be a consequence of adaptation of the microbiota to the new diet. As mentioned before, microbiota communities could be affected by several factors such as weight and diet and the combination of gut flora in each individual create a unique metabolome⁷². Indeed, the contribution of microbiota to the whole metabolome dynamics is gaining importance, and it is now considered as an essential factor in physiologic interpretation^{72,74}. In this study, these metabolites only varied in the *Majorera* breed. This fact could be perceived as a different and quicker adaptation to the restricted-fed condition by the *Majorera* breed compared to the *Palmera* breed. *Majorera* breed shown a higher number of metabolites with significant variations (between control and restricted-fed groups) than *Palmera*. As explained above, most of these variations may be related to different microbiota bulks, or to microbiota communities with different adaptability to diet changes. As seen in mammary gland, all variations were consistent in both breeds, suggesting that indeed, the *Palmera* breed could have a different metabolic reaction when exposed to diet restriction.

Differences in milk composition might not always be related to differences in mammary gland composition, suggesting that the animal tends to maintain its regular function during lactation, adjusting the milk composition to the new diet conditions. Milk serum showed more metabolites' variation than mammary gland, supporting the idea that milk is not only more susceptible to change, but also can reflect these changes quickly than secretory tissue. Also, considering that we used the secretory tissue of mammary gland, changes in milk composition could be related to differences in secretion regulation not affecting the composition of the gland itself. Moreover, some compounds could pass directly from blood to milk without any regulation at the mammary gland level. This effect of diet composition in small ruminant milk composition, as previously mentioned^{57,75,76}, could be further explored to diet optimization for milk production, especially in drought-prone regions.

Concerning the biomarker identification, no metabolite seems to be a clear biomarker candidate of SWL tolerance, as significant differences were observed when comparing restricted-fed groups of both breeds. Although without significant differences between breeds, some metabolites such as AMP/ADP/ATP in mammary gland and fumarate in milk serum, could be correlated with an adaptive response to SWL factors.

3.5.2 Fatty Acids Profile

Mammary gland

Mammary gland presented significant interactions between breed and feed restriction for 18:1*cis*-9 and 16:0, the two major fatty acids of this

tissue. Apparently, *Majorera* goats were able to maintain the fatty acid composition of mammary tissue when subjected to feed restriction, whereas the *Palmera* breed goats clearly increased the concentration of 18:1 *cis*-9 and decreased the concentration of 16:0 in the same situation. Due to the importance of the mammary gland to new-born survival⁹, it is likely that this tissue has significant tolerance to external influence, keeping its integrity in order to preserve functions, namely lactopoiesis. Thus, the larger ability of *Majorera* goats to maintain the fatty acid composition of mammary tissues when compared to *Palmera* goats might be a reflex of its higher tolerance to SWL. On the contrary, because *Palmera* goats are more susceptible to SWL, the decrease in 16:0 and increase of 18:1 *cis*-9 found in feed restricted *Palmera* breed could result from extensive fat mobilization in order to compensate the feed restriction and maintain milk production as previously suggested⁷⁷.

Milk

The milk fatty acid composition seems to be largely affected by feed-restriction irrespective of the breed. In our experiment, an interaction between breed and feed restriction was only observed for two minor fatty acids (17:0 and 17:1 *cis*-9). These two fatty acids represent only about 2% of the total fatty acids identified, indicating the small influence of the breed in milk variations. In contrast, the main fatty acids present in milk such as 10:0, 12:0, 14:0, 16:0, 18:0 and 18:1 *cis*-9 were only affected by feed restriction. These results indicate that milk fatty acid composition seems to be easily affected by feed-restriction but that both breeds respond similarly as also observed in the multivariate analysis of milk fat percentages (Figure 3.7). Milk fat can arise from two sources, *de novo*

synthesis in mammary gland (mainly the short and medium chain fatty acids including about 50% of the 16:0) and uptake of exogenous fatty acids from circulation (mainly 16:0 and C18 fatty acids). Supply of exogenous fatty acids to the mammary gland comprise mostly the circulating lipoproteins, derived either from digestive tract or from hepatic reassembly of non-esterified fatty acids mobilized from the body fat reserves. Utilization of fat depots are especially important in early lactation given that up to 40% of the milk fat is derived from mobilized fatty acids⁷⁸. During early lactation and undernutrition periods, adipose tissues not only contribute to milk fat secretion, but also to supply energy to other tissues and spare glucose and amino acids for use in the mammary gland⁷⁷. Thus, milk fatty acid composition is quite susceptible to the energy balance status⁹. In particular, when the energy balance is negative, animals mobilize lipids stored in adipose tissues to be used for energy production, mainly the 16:0, 18:0 and 18:1*cis*-9⁷⁹. In our study, the large decrease in the proportions of *de novo* synthesized fatty acids including the 16:0 and the increased proportions of 18:1*cis*-9 and 18:0 in milk of restricted-fed group compared to controls are consistent with an extensive fat tissue mobilization and lack of dietary derived precursors for the *de novo* fatty acid synthesis. Previous studies on the same animals, analysed the influence of feed restriction in blood metabolites and protein expression in the mammary gland secretory tissue. Both studies revealed results that support these conclusions. Namely, it was observed an increase in non-esterified FA in blood, from control to restricted-fed groups due to higher metabolization of fatty acids depots⁸. Concerning protein expression, both breeds exhibited a decrease in expression of proteins related with fat biosynthesis⁸⁰.

We also observed an increase in *cis*-MUFA values in milk, from control to restricted-fed groups, in particular in the 16:1 *cis*-9, 17:1 *cis*-9 and 18:1 *cis*-9. This suggests a high stearoyl-CoA desaturase (SCD) activity, which is responsible for synthesizing mostly the *cis*-9 MUFA from their respective fatty acid. In fact, the SCD product/substrate ratio computed with the fatty acid present in milk used to estimate the overall SCD activity supports these findings. Moreover, the SCD seems to be more active in *Majorera* than in the *Palmera* breed. Observed values of linoleic acid (18:2n-6) and 18:1 *cis*-9 were significantly higher in restricted-fed groups of both breeds. This result is of particularly interest for milk production management and product optimization, due to its importance in human nutrition and health^{9,81}.

In summary, the differential response pattern observed between mammary gland tissue and milk could be due to the high level of organization of the mammary epithelium, whose cells are specialized in converting circulating nutrients in milk components, and the several secretory and regulatory pathways present in this tissue^{9,58}. Indeed, we observed an influence of breed in feed-restriction conditions in mammary gland fatty acid composition, with *Majorera* goats, an observation which might indicate a higher tolerance to SWL. In milk, the influence of breed in restricted-fed conditions was not significant, affecting less than 2% of the total FA in the sample. Milk fat composition presented several significant responses to feed restriction in both breeds. Considering that fat can influence milk characteristics and even cheese quality and properties, it opens some possibilities for product optimization. The *Majorera* breed shows higher tolerance to feed restriction appearing to be then the better breed to work in a product management context.

3.6 CONCLUSIONS

In general, the two breeds had more similar results in milk than for the mammary gland. Milk composition is more affected by the feed-restriction than by the breed. On the other hand, results from mammary gland revealed breed specific responses. *Majorera* breed presented more variations due to feed-restriction in the metabolites profile than in the fatty acids profile. Indeed, it was observed influence of the breed with the feed-restriction in the fatty acids. Results suggest that, during the time of experiment, *Majorera*, response to feed-restriction with some changes in the metabolic pathways related to energy production, with no significant use of body fat depots. The *Palmera* breed presented fewer variations in the small metabolites but higher interaction of breed with feed-restriction in the fatty acids profile. This breed seems to start using the body fat resources as energy source, earlier than *Majorera* breed. These results could confirm that the *Majorera* breed can cope with feed-restriction conditions during longer periods than *Palmera* breed, being then more tolerant to this condition.

Milk fat composition presented several significant responses to feed restriction in both breeds. Considering that fat can influence milk characteristics and even cheese quality and properties, it opens some possibilities for product optimization. *Majorera* breed shows higher tolerance to feed restriction being then the better breed to work in the product management context.

3.7 ABBREVIATIONS LIST

ADP adenosine diphosphate

AMP adenosine monophosphate

ATP adenosine triphosphate

DSS sodium-2,2-dimethyl-2-silapentane-5-sulfonate

FA fatty acid(s)

IMP inosine monophosphate

MUFA monounsaturated fatty acids

MW molecular weight

NMR nuclear magnetic resonance

OPLS-DA orthogonal partial least squares discriminant analysis

PCA principal component analysis

PLS partial least squares

PUFA polyunsaturated fatty acids

SFA saturated fatty acids

SWL seasonal weigh loss

TMAO trimethylamine oxide

TSP 3-(trimethylsilyl)-2,2',3,3'-tetrauteropropionic acid

UDP-(...) uridine diphosphate (...)

1D-COSY one dimension correlation spectroscopy

1D-NOESY one dimension nuclear Overhauser effect spectroscopy

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4

NMR METABOLOMICS AND FEED-RESTRICTION EFFECTS IN SHEEP

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- Palma M, Scanlon T, Kilminster T, Milton J, Oldham C, Greeff J, Matzapetakis M, Almeida AM. The hepatic and skeletal muscle ovine metabolomes as affected by weight loss: a study in three sheep breeds using NMR-metabolomics. *Scientific Reports*. (2016) doi: 10.1038/srep39120.

Mariana Palma designed and performed all the experiments, except field work and sample collection.

4.1 SUMMARY

Sheep are a valuable resource especially for meat and wool production. During the dry summer and autumn period, pastures are scarce and animals face Seasonal Weight Loss (SWL), which decreases production yields. The study of breeds tolerant to SWL is important to understand the physiological mechanisms of tolerance to nutritional scarcity, and define breeding strategies. *Merino*, *Damara* and *Dorper* sheep breeds have been described as having different levels of tolerance to SWL. In this work, we assess their liver and muscle metabolomes, and compare the responses to feed restriction. Ram lambs from each breed were divided into growth and feed restricted groups, over 42 days. Tissue metabolomes were assessed by ^1H -NMR, and univariate and multivariate approaches were used for statistical analysis. The *Dorper* restricted group showed few changes in both tissues, suggesting higher tolerance to nutritional scarcity. The *Merinos* presented more differences between treatment groups. Major differences were related to fat and protein mobilization, and antioxidant activity. Between the *Damara* groups, the main differences between groups were observed in amino acid composition in muscle and in energy-related pathways in the liver. Integration of present results and previous data on the same animals support the hypothesis that, *Dorper* and *Damara* breeds are more tolerant to SWL conditions and thus, more suitable breeds for harsh environmental conditions.

4.2 INTRODUCTION

The *Australian Merino* breed is the basis of the wool, ovine meat, and live animal export markets for that country. The *Merino* (Fig. 4.1A) has a long history in Australia after being introduced in 1797¹, but in the last two decades, market changes and animal welfare policies have led to an increased interest for breeds with other characteristics, particularly regarding the absence of wool (shedding hair sheep), heat tolerance and a natural adaptation to Seasonal Weight Loss (SWL)². Recently, two breeds from South Africa, the *Damara* (Fig. 4.1B) and the *Dorper* (Fig.4.1C), have been introduced to Australia. The *Damara* is a large fat-tailed, hair sheep breed, native to the fringes of the Kalahari Desert in Namibia and South Africa. This breed is well adapted to arid climatic conditions and water scarcity¹. The *Dorper* is also a hair sheep breed native to Southern Africa. It was selected by combining the hardiness of the *Blackhead Persian* indigenous breed with the carcass and meat traits of *British Dorset Horn* breed¹. These sheep breeds have been reviewed by Almeida *et al.* (2011)¹.

In a previous work a productive characterization of these breeds and their reaction to SWL was conducted. SWL effects on live weight³, carcass and meat characteristics^{2,4}, in gene expression of regulatory enzymes in liver⁵, and more recently on the skeletal muscle proteome⁶, and fatty acid composition of muscle⁷ and the *Damara* fat tail adipose tissue⁸ were studied. However, no broad characterization of the muscle and liver metabolomes of these three breeds has ever been conducted.



Figure 4.1. Representative male specimens of *Merino* (A), *Dorper* (B) and *Damara* (C) sheep breeds.

The aim of this work was to characterize the metabolome of the muscle and liver of *Merino*, *Damara* and *Dorper* sheep breeds, and study the effect of feed restriction in these tissues, which are important from the productive and metabolic perspectives. We used an NMR-metabolomics based approach, which, to the best of our knowledge was for the first time applied to these breeds. The results will be of importance to understand which biochemical pathways are associated with SWL tolerance in sheep and that may benefit breeding programs.

4.3 MATERIAL AND METHODS

Animal experiment

The trial was carried out at the Merredin Research Station in Western Australia, following the experimental design and nutritional treatments previously described³. Briefly, a total of 72 six-month-old ram lambs from each of the *Merino*, *Dorper* and *Damara* breeds were divided into the experimental diet groups (12 animals per group: *Merino* growth, *Merino* restricted, *Dorper* growth, *Dorper* restricted, *Damara* growth and *Damara* restricted). All animals were fed on commercial pellets and had free

access to drinking water as described³. Individual nutritional treatments were calculated so that animals in the growth groups gained weight (100g/day) and animals in the restricted groups lost weight (100g/day). The trial lasted 42 days, after which animals were slaughtered in a commercial abattoir, following commercial practices. For further information, kindly refer to Scanlon *et al.* (2013)³ and Almeida *et al.* (2013)⁹.

By the end of the nutrition trial, gastrocnemius muscle and liver tissues were sampled and preserved at -80°C for further analysis.

NMR-Metabolomics

Sample processing

For muscle tissue we analysed 11 samples in all experimental groups, with the exception of the *Merino* Restricted group where 10 samples were used. For the liver tissue we used 12 samples for all groups, with the exception of the *Merino* Restricted group where 11 samples were used. Frozen tissues were powdered individually with porcelain mortar and pestle with liquid nitrogen. Metabolites were extracted following the Bligh and Dyer method¹⁰ with adaptations. Muscle samples were processed as previously described for goat mammary gland samples¹¹. For liver samples the solvent volumes used were modified as follows: an initial 3 ml cold chloroform/methanol mixture (1:2, v/v) was added to the tissue and mixed, followed by the addition and mixing of 2 ml of cold chloroform. Then 1 ml of cold water was added and mixed by vortexing. For both tissues, the mixture was finally centrifuged and the

methanol/water fraction was separated and dried as previously described¹¹.

NMR Spectroscopy

The aqueous fraction of the muscle samples was re-suspended in 600 μ l phosphate buffer (150 mM, pH 7.0/ pD 7.4, with 1 mM sodium-2,2-dimethyl-2-silapentane-5-sulfone (DSS), in D₂O), while the water-soluble fraction of the liver samples was dissolved in 800 μ l phosphate buffer (100 mM, pH 7.4/pD 7.8, with 0.5 mM DSS, in D₂O). Samples were transferred into 5 mm NMR tubes.

Proton (¹H) NMR spectroscopy was conducted on an 800 MHz Bruker Avancell⁺ (Ettlingen, Germany) spectrometer, with a triple resonance HCN Z-gradient probe, at 298 K.

¹H 1D-NOESY spectra were collected for each sample using the “noesypr1d” pulse sequence (spectral width: 12 ppm; mixing time: 0.1 s; relaxation delay: 1 s; acquisition time: 4 s), following the parameters for profiling recommended from Chenomx NMR Suite software (Chenomx Inc., Edmonton, Canada). All spectra were processed with a line broadening (lb) of 0.5 Hz and a final number of 128K points. Additional J-resolved spectra were collected to assist with assignment. All spectra were acquired, processed and analysed using TopSpin 3.2 (Bruker, Ettlingen, Germany).

Metabolite Profiling

Metabolite identification and quantification was carried out using Chenomx NMR Suite 8.12 software (Chenomx Inc., Edmonton, Canada), using the internal reference library (Version 10), and with support of published data for other animals^{12–14}.

Data Analysis

Both univariate and multivariate analysis were performed for the obtained metabolite concentrations, following the approach previously described¹¹. Briefly, for univariate analysis we performed a t-test with 2 tails and test type 3, considering $p < 0.05$ to reject null-hypothesis (equal means between groups), using Microsoft Excel. Multivariate analysis was performed using the SIMCA 13.0.3.0 software (Umetrics AB, Umeå, Sweden) for unsupervised Principal Components Analysis (PCA) and supervised Partial Least Squares Discriminant Analysis (PLS-DA). In PLS analysis, Q^2 (predictive ability of the model) and R^2 (goodness of the fit) were considered as quality parameters of the model. Results were accepted for Q^2 above 0.5¹⁵. For PLS models, a permutation test was additionally performed, using 100 permutations and accepting the model as “valid” when $R^2Y\text{-intercept} < 0.4$ and $Q^2Y\text{-intercept} < 0.05$. All ellipses in the scores plots were drawn at the 95% confidence level.

Animal Welfare Disclaimer

All work involving animals was conducted according to relevant international guidelines (European Union procedures on animal

experimentation—Directive 2010/63/EU) that regulate the use of production animals in animal experimentation. These define that in the case of experiments carried out under standard production conditions, no approval from an ethics committee is required. Nevertheless, this experiment was conducted with the approval of the Ethics Committee of the Department of Agriculture and Food Western Australia (DAFWA, Perth, WA, Australia) registered as process 07ME06. The entire trial was conducted under the supervision of the veterinary authority in the State of Western Australia. Author AM Almeida holds a FELASA (Federation of European Laboratory Animal Society Associations) grade C certificate that enables designing and carrying out animal experimentation under European Union regulations. Animal management, handling, transport and slaughter were all conducted replicating approved standard commercial practices in the Commonwealth of Australia and in the State of Western Australia.

4.4 RESULTS

NMR-Metabolomics

Muscle tissue

A representative ^1H 1D NOESY spectrum of the sheep gastrocnemius muscle (Merino breed, growth group) is shown in Figure 4.2.

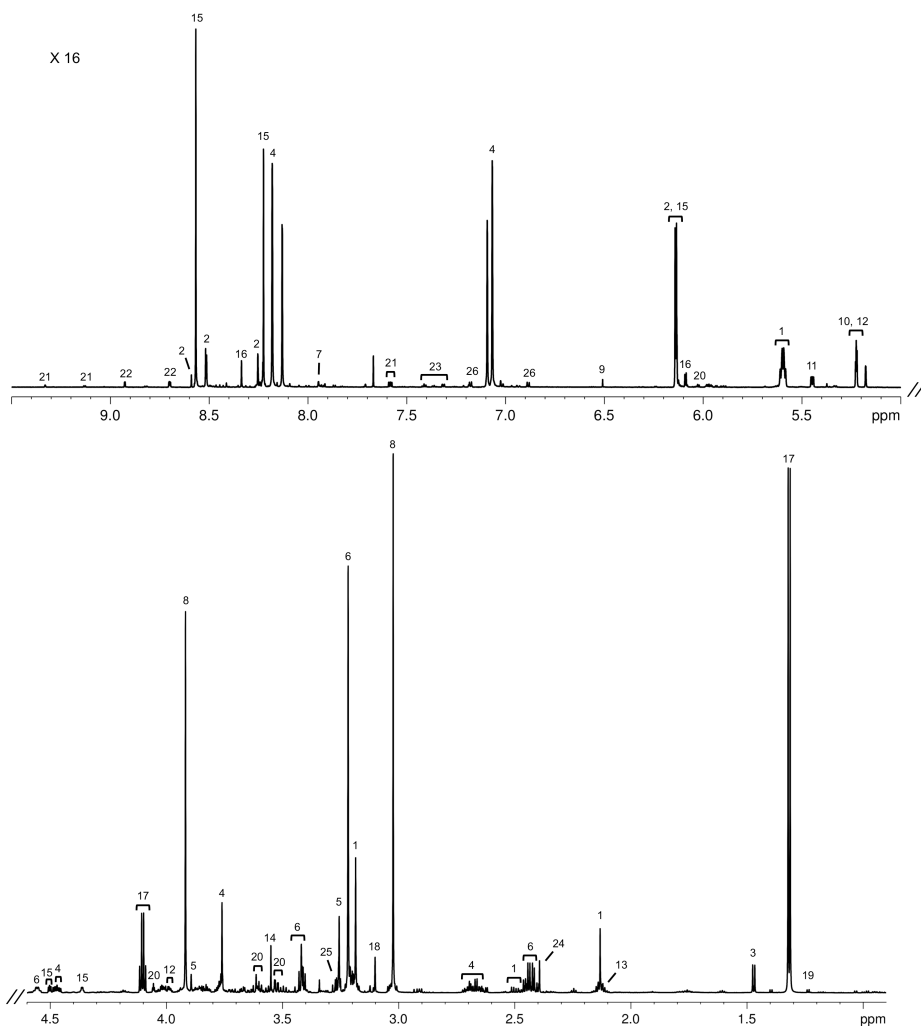


Figure 4.2. Representative 800 MHz ^1H 1D NOESY spectrum of gastrocnemius muscle aqueous fraction in *Merino* breed, growth group. Key: (1) acetyl-L-carnitine; (2) ADP/AMP/ATP; (3) alanine; (4) anserine; (5) betaine; (6) carnitine; (7) carnosine; (8) creatine/creatine phosphate; (9) fumarate; (10) glucose; (11) glucose-1-phosphate; (12) glucose-6-phosphate; (13) glutamine; (14) glycine; (15) IMP; (16) inosine; (17) lactate; (18) malonate; (19) methylmalonate; (20) myo-inositol; (21) $\text{NAD}^+/\text{NADP}^+$; (22) nicotinurate; (23) phenylalanine; (24) succinate; (25) taurine; (26) tyrosine.

A total of 51 metabolites were identified in the aqueous fractions of the muscle, and the metabolite concentrations from the six experimental groups are presented in Supplementary Table S4.1. The most abundant metabolites are lactate and creatine/creatine-phosphate in all breeds, followed by taurine, anserine, carnitine and glutamine in the *Dorper* and *Damara* breeds; and carnitine, malonate, taurine and anserine in the *Merino* breed.

Significant differences ($p < 0.05$) between growth and restricted groups for each breed are presented in Table 4.1. Among these, the more marked differences were observed in glycerophosphocholine in the *Merino* breed, which decreased 4.1 times between growth and restricted groups; and adenine in the *Dorper* breed, which increased 2.5 times from growth to restricted groups. In the *Merino* breed, we identified differences between growth and restricted groups in citrate, glucose-6-phosphate, glutathione, glycerophosphocholine, glycine, acetyl-L-carnitine, taurine and tyrosine. In the *Damara* breed, differences between growth and restricted groups were observed in glucose-1-phosphate, inosine monophosphate (IMP), isoleucine, leucine, tyrosine, valine, phenylalanine and taurine. In the *Dorper* breed only four metabolites show significant differences between groups: adenine, formate, glycine and taurine.

Table 4.1. Metabolites in gastrocnemius muscle of *Merino*, *Dorper* and *Damara* sheep breeds, with significant differences between growth and restricted groups, in at least one breed. Average concentration (mmol/ g tissue) and standard deviation are shown for each experimental group.

	Merino		Dorper		Damara	
	growth	restricted	growth	restricted	growth	restricted
Acetyl-L-carnitine	2.6 E-03 ± 1.2 E-03	4.6 E-03* ± 1.9 E-03	1.6 E-03 ± 5.8 E-04	1.6 E-03 ± 1.0 E-03	2.2 E-03 ± 3.8 E-04	2.2 E-03 ± 7.6 E-04
Adenine	8.3 E-05 ± 7.5 E-05	7.2 E-05 ± 5.0 E-05	2.1 E-05 ± 1.1 E-05	5.3 E-05* ± 4.1 E-05	8.5 E-05 ± 4.3 E-05	7.4 E-05 ± 6.5 E-05
Citrate	1.7 E-04 ± 8.6 E-05	2.7 E-04* ± 1.1 E-04	1.5 E-04 ± 7.5 E-05	1.5 E-04 ± 1.2 E-04	1.9 E-04 ± 7.9 E-05	2.2 E-04 ± 6.8 E-05
Formate	5.3 E-05 ± 2.0 E-05	4.0 E-05 ± 7.1 E-06	4.8 E-05 ± 1.7 E-05	3.5 E-05* ± 8.2 E-06	4.7 E-05 ± 1.4 E-05	5.6 E-05 ± 1.1 E-05
Glucose-1-phosphate	6.3 E-04 ± 2.7 E-04	5.2 E-04 ± 2.0 E-04	5.0 E-04 ± 1.5 E-04	4.6 E-04 ± 3.2 E-04	7.0 E-04 ± 2.1 E-04	4.8 E-04* ± 2.7 E-04
Glucose-6-phosphate	2.9 E-03 ± 1.2 E-03	1.6 E-03** ± 7.3 E-04	2.7 E-03 ± 6.3 E-04	1.9 E-03 ± 1.2 E-03	3.1 E-03 ± 1.1 E-03	2.2 E-03 ± 9.7 E-04
Glutathione	1.7 E-04 ± 1.0 E-04	2.8 E-04* ± 1.3 E-04	2.2 E-04 ± 6.6 E-05	2.5 E-04 ± 1.3 E-04	2.3 E-04 ± 9.6 E-05	3.1 E-04 ± 1.6 E-04
Glycerophosphocholine	3.1 E-04 ± 1.1 E-04	7.7 E-05** ± 2.4 E-05	3.8 E-04 ± 1.9 E-04	5.7 E-04 ± 3.0 E-04	4.0 E-04 ± 1.7 E-04	5.5 E-04 ± 1.8 E-04
Glycine	3.5 E-03 ± 1.7 E-03	1.9 E-03* ± 5.4 E-04	2.8 E-03 ± 7.7 E-04	1.9 E-03* ± 1.3 E-03	2.2 E-03 ± 4.4 E-04	1.8 E-03 ± 7.0 E-04
IMP	2.5 E-03 ± 1.1 E-03	1.9 E-03 ± 7.4 E-04	1.5 E-03 ± 6.5 E-04	1.7 E-03 ± 8.6 E-04	2.5 E-03 ± 7.0 E-04	1.7 E-03* ± 7.7 E-04
Isoleucine	9.3 E-05 ± 3.9 E-05	7.8 E-05 ± 2.2 E-05	8.6 E-05 ± 2.4 E-05	8.2 E-05 ± 4.8 E-05	1.1 E-04 ± 1.4 E-05	9.1 E-05* ± 2.5 E-05
Leucine	1.4 E-04 ± 5.6 E-05	1.1 E-04 ± 2.6 E-05	1.3 E-04 ± 3.7 E-05	1.2 E-04 ± 5.3 E-05	1.6 E-04 ± 2.2 E-05	1.3 E-04* ± 3.1 E-05
Phenylalanine	4.9 E-05 ± 1.6 E-05	3.9 E-05 ± 1.1 E-05	4.4 E-05 ± 9.5 E-06	3.5 E-05 ± 1.3 E-05	5.3 E-05 ± 8.2 E-06	4.0 E-05** ± 8.6 E-06
Taurine	5.4 E-03 ± 3.3 E-03	3.0 E-03* ± 1.1 E-03	6.3 E-03 ± 2.8 E-03	3.9 E-03* ± 2.4 E-03	1.4 E-02 ± 4.4 E-03	8.8 E-03** ± 2.0 E-03
Tyrosine	5.5 E-05 ± 2.7 E-05	3.5 E-05* ± 1.3 E-05	5.0 E-05 ± 1.7 E-05	3.8 E-05 ± 1.6 E-05	6.3 E-05 ± 1.6 E-05	4.6 E-05* ± 1.2 E-05
Valine	2.1 E-04 ± 10.0 E-05	1.5 E-04 ± 3.5 E-05	2.0 E-04 ± 5.7 E-05	1.9 E-04 ± 1.1 E-04	2.3 E-04 ± 4.0 E-05	1.9 E-04* ± 3.7 E-05

Key: (*) $p < 0.05$ when compared with growth groups of the same breed; (**) $p < 0.01$ when compared with growth groups of the same breed.

Multivariate analysis was applied to metabolite concentrations. PCA scores plot of all groups does not show any specific separation by group (Supplementary Figure S4.1A), however it is possible to see some distinction by breed. In the PCA scores plot of the growth groups (Fig.

4.3A) it is possible to see some separation between breeds, especially *Merino* and *Dorper*. On the other hand, the PCA scores plot of the restricted groups revealed no specific separation between breeds (Supplementary Figure S4.1B). However, it is noteworthy that the *Damara* restricted groups show a less broad distribution when compared to the distributions of the other two breeds. Multivariate analysis was also applied per breed. PCA scores of *Merino* growth and restricted groups revealed the two groups could be separated by the second principal component (PC2) (Fig. 4.3B). Analysis of the loadings (Supplementary Table S4.2) indicates that the metabolites that contribute more to this separation were glycerophosphocholine, citrate, acetyl-L-carnitine, myo-inositol, glutathione and glucose-6-phosphate. For *Damara* and *Dorper* breeds it was only possible to separate their growth and restricted groups applying PLS analysis. *Damara* growth and restricted groups were separated by the first principal component (PC1) with acceptable quality parameters (Supplementary Figure 4.1C). However, the permutation test (Supplementary Figure S4.1D) failed the model validation. Concerning the *Dorper* growth and restricted groups, PLS was not able to separate the groups with acceptable quality parameters (Supplementary Figure 4.1E). Furthermore, permutation test (Supplementary Figure S4.1F) do not validate the model.

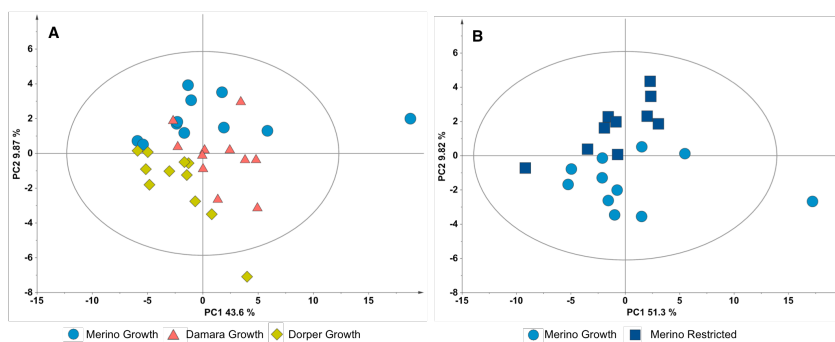


Figure 4.3. Multivariate analysis of gastrocnemius muscle (aqueous fraction) metabolites concentration. (A) PCA scores plot for the three growth groups (NC = 2, PC1 = 43.6 %, PC2 = 9.87 %); (B) PCA scores plot for *Merino* growth and restricted groups (NC = 2, PC1 = 51.3 %, PC2 = 9.82 %). Ellipse Hotelling's T2 (95%).

Liver tissue

A representative ^1H 1D NOESY spectrum of the sheep liver (*Merino* breed, growth group) was selected and is shown in Figure 4.4, with examples of some of the identified metabolites.

A total of 46 metabolites were identified in the aqueous fraction of sheep liver and the metabolite concentration of all experimental groups are presented in Supplementary Table S4.3. The most abundant metabolites are glucose, lactate, glycerophosphocholine and glutamate in the three breeds, followed by taurine, glycine, glutathione and alanine in different orders depending on the breed.

In the *Damara* breed, differences between growth and restricted groups are identified in acetate, adenine, alanine, ascorbate, choline/ acetylcholine/ phosphocholine, citrate, fomite, lactate and UDP-glucose/ UDP-glucuronate. It is noteworthy to mention that in the *Dorper* breed

only two metabolites: carnitine and sarcosine, show differences between growth and restricted groups.

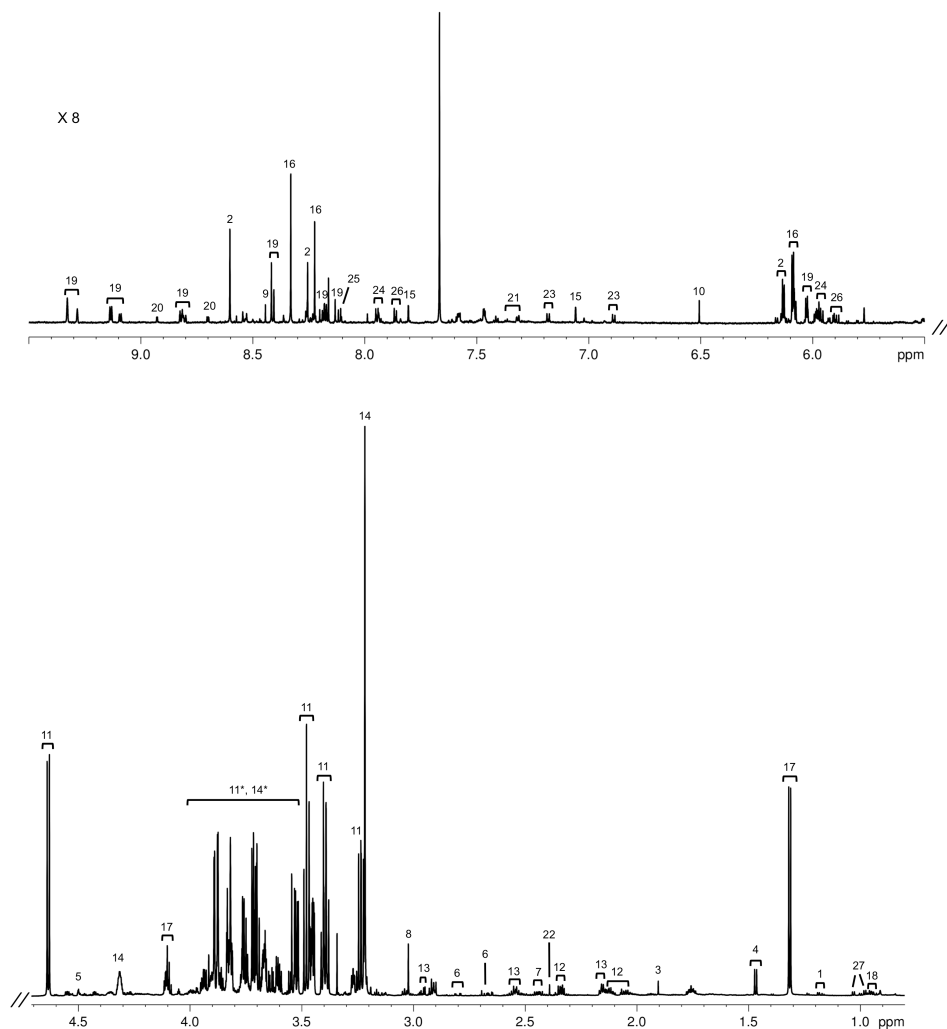


Figure 4.4. Representative 800 MHz ^1H 1D NOESY spectrum of liver aqueous fraction in *Merino* breed, growth group. Key: (1) 3-hydroxybutyrate; (2) ADP/AMP/ATP; (3) acetate; (4) alanine; (5) ascorbate; (6) aspartate; (7) carnitine; (8) creatine/creatine phosphate; (9) formate; (10) fumarate; (11) glucose; (12) glutamate; (13) glutathione; (14) glycerophosphocholine; (15) histamine; (16) inosine; (17) lactate; (18) leucine; (19) $\text{NAD}^+/\text{NADP}^+/\text{NADPH}$; (20) nicotinurate; (21) phenylalanine; (22) succinate; (23) tyrosine; (24) UDP-glucose/UDP-glucuronate; (25) UMP; (26) uridine; (27) valine; (11*, 14*) region strongly dominated by glucose and glycerophosphocholine peaks, but with some less concentrated metabolites underneath them.

Table 4.2.. Metabolites in liver of *Merino*, *Dorper* and *Damara* sheep breeds, with significant differences between growth and restricted groups, in at least one breed. Average concentration (mmol/ g tissue) and standard deviation are shown for each experimental group

	Merino		Dorper		Damara	
	growth	restricted	growth	restricted	growth	restricted
3-Hydroxybutyrate	3.3 E-04 ± 1.0 E-04	4.7 E-04* ± 1.4 E-04	2.4 E-04 ± 10.0 E-05	3.2 E-04 ± 1.4 E-04	2.9 E-04 ± 7.9 E-05	2.7 E-04 ± 1.4 E-04
Acetate	5.9 E-04 ± 1.3 E-04	8.3 E-04** ± 1.7 E-04	4.9 E-04 ± 2.3 E-04	5.8 E-04 ± 2.5 E-04	6.1 E-04 ± 1.3 E-04	4.3 E-04* ± 2.3 E-04
Adenine	3.3 E-04 ± 1.5 E-04	3.0 E-04 ± 1.6 E-04	1.6 E-04 ± 9.7 E-05	1.9 E-04 ± 1.0 E-04	3.0 E-04 ± 4.5 E-05	1.6 E-04** ± 1.2 E-04
Alanine	2.0 E-03 ± 4.9 E-04	1.4 E-03** ± 4.2 E-04	1.8 E-03 ± 8.8 E-04	1.4 E-03 ± 4.6 E-04	2.4 E-03 ± 2.8 E-04	1.5 E-03** ± 7.2 E-04
Ascorbate	1.2 E-03 ± 3.7 E-04	7.7 E-04** ± 3.5 E-04	9.2 E-04 ± 5.0 E-04	7.2 E-04 ± 3.2 E-04	1.2 E-03 ± 2.5 E-04	8.2 E-04* ± 4.1 E-04
Carnitine	5.7 E-04 ± 3.2 E-04	5.8 E-04 ± 3.5 E-04	6.4 E-04 ± 3.5 E-04	9.7 E-04* ± 4.3 E-04	7.0 E-04 ± 2.6 E-04	7.1 E-04 ± 3.8 E-04
Choline/ Acetylcholine/ Phosphocholine	6.0 E-04 ± 3.0 E-04	8.8 E-04 ± 6.6 E-04	4.6 E-04 ± 3.6 E-04	5.4 E-04 ± 2.7 E-04	6.2 E-04 ± 2.6 E-04	3.4 E-04* ± 2.3 E-04
Citrate	3.6 E-04 ± 1.2 E-04	2.6 E-04 ± 1.3 E-04	3.2 E-04 ± 1.4 E-04	2.5 E-04 ± 1.1 E-04	4.9 E-04 ± 1.2 E-04	2.9 E-04** ± 1.8 E-04
Creatine/ Creatine phosphate	1.7 E-03 ± 5.2 E-04	2.6 E-03** ± 7.2 E-04	1.5 E-03 ± 6.8 E-04	1.8 E-03 ± 8.3 E-04	1.6 E-03 ± 3.3 E-04	1.7 E-03 ± 1.0 E-03
Formate	1.2 E-04 ± 7.6 E-05	1.3 E-04 ± 5.6 E-05	3.9 E-05 ± 3.4 E-05	5.0 E-05 ± 5.0 E-05	1.2 E-04 ± 5.6 E-05	4.8 E-05** ± 2.5 E-05
Glutathione	2.5 E-03 ± 7.4 E-04	1.2 E-03** ± 4.3 E-04	2.2 E-03 ± 1.4 E-03	1.5 E-03 ± 6.4 E-04	2.5 E-03 ± 4.8 E-04	1.9 E-03 ± 1.3 E-03
Lactate	2.3 E-02 ± 4.4 E-03	2.0 E-02* ± 3.0 E-03	1.7 E-02 ± 7.8 E-03	1.5 E-02 ± 5.3 E-03	2.1 E-02 ± 2.7 E-03	1.5 E-02** ± 5.3 E-03
Sarcosine	2.5 E-05 ± 1.1 E-05	6.6 E-05* ± 4.7 E-05	1.9 E-05 ± 9.9 E-06	5.0 E-05** ± 3.3 E-05	2.5 E-05 ± 9.9 E-06	2.8 E-05 ± 1.9 E-05
Succinate	1.9 E-03 ± 7.0 E-04	1.1 E-03* ± 7.4 E-04	1.4 E-03 ± 9.4 E-04	1.0 E-03 ± 5.2 E-04	1.6 E-03 ± 6.4 E-04	1.4 E-03 ± 6.2 E-04
UDP-glucose/ UDP-glucuronate	2.4 E-04 ± 6.0 E-05	1.7 E-04* ± 6.7 E-05	1.8 E-04 ± 8.4 E-05	2.0 E-04 ± 8.6 E-05	1.8 E-04 ± 4.4 E-05	1.4 E-04* ± 5.2 E-05

Key: (*) $p < 0.05$ when compared with growth groups of the same breed; (**) $p < 0.01$ when compared with growth groups of the same breed.

Concerning the multivariate analysis, albeit the PCA scores plot of the six experimental groups (Fig. S4.5A) does not reveal a clear separation of the groups, some tendencies may be noted. *Dorper* growth and restricted group have indistinguishable distribution, whereas the *Merino* groups seem to be separated into clusters influenced by the PC2. This separation of the *Merino* groups from the other groups could be due to variations in benzoate, glycine and succinate, as shown in loadings list (Supplementary Table S4.4). PCA scores plot of the three restricted groups (Fig. 4.5B) show a distinct clustering of the *Merino* restricted samples away from the other two restricted groups of *Damara* and *Dorper* breeds. Loadings list of this model indicates that separation is due to variations in alanine, formate, benzoate and glycine (Supplementary Table S4.5). *Merino* growth and restricted groups show separation in the PCA scores plot by the PC2 (Fig. 4.5C). Loading list of this model (Supplementary Table S4.6) indicate variations in succinate, formate and xanthine. PLS analysis for this breed (*Merino* growth and restricted groups) has good quality parameters but the permutation test does not validate the model. PCA scores plot of the *Damara* growth and restricted groups (Fig. 4.5D) show a slight separation in the PC1. The growth group has less disperse distributions whereas the restricted group is more spread-out along the plot area. Separation of the two groups could be due to differences in glucose, tyrosine, isoleucine, inosine and leucine, as shown in the loadings list (Supplementary Table S4.7). PLS analysis for the two groups of *Damara* breed has unacceptable quality parameters and cannot be validate by the permutation test. The *Dorper* growth and restricted groups (Supplementary Figure 4.2A) were only separated by the PLS analysis. Although the quality parameters are

acceptable, the permutation test does not validate the model (Supplementary Figure S4.2B).

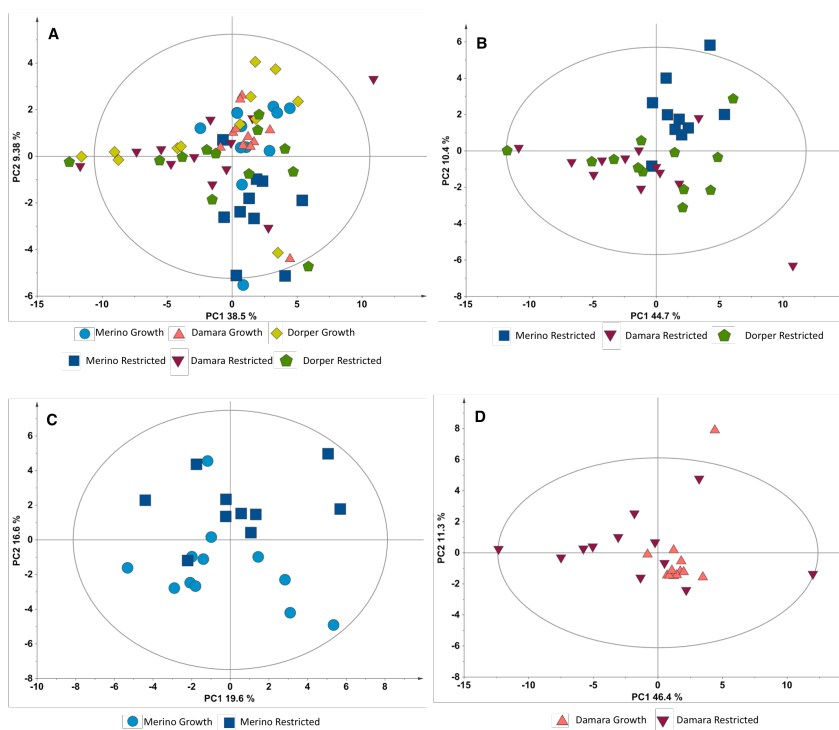


Figure 4.5. Multivariate analysis of liver (aqueous fraction) metabolites concentration. (A) PCA scores plot for the six experimental groups (NC = 4, PC1 = 38.5%, PC2 = 9.38%); (B) PCA scores plot for the three restricted groups (NC = 5, PC1 = 44.7%, PC2 = 10.4%); (C) PCA scores plot for *Merino* growth and restricted groups (NC = 2, PC1 = 19.6%, PC2 = 16.6%); (D) PCA scores plot of *Damara* growth and restricted groups (NC = 3, PC1 = 46.4%, PC2 = 11.3%). The ellipses in the scores plots are drawn at the 95% confidence level. Ellipse Hotelling's T2 (95%).

4.5 DISCUSSION

To the best of our knowledge, this work is the first to apply NMR technique to studies on the effects of SWL in muscle and liver metabolome on the *Merino*, *Damara* and *Dorper* sheep breeds. The approach proved to be adequate for the study, allowing the identification and quantification of 51 metabolites in muscle, and 46 in liver.

As suggested for other metabolomics studies¹⁶, we analysed the data using both univariate and multivariate analyses. PCA of the different groups did not reveal a clear separation between groups, neither for muscle nor for liver, although the muscle samples could be discriminated by breed. In the per-breed analysis, some tendencies are noted, especially in the *Dorper* breed, where it was impossible to discriminate between the two nutritional treatment groups with good quality parameters, in both liver and muscle tissues. Such separation was possible for the *Merino* and *Damara* in both tissue samples. These results could be an indicator of a more pronounced reaction of the muscle to feed-restriction in *Merino* and *Damara*, and a general different response in the *Dorper* breed.

Univariate analysis results of the *Dorper* growth and restricted groups revealed that few metabolite variations were found in both tissues, four in muscle and two in liver. In the *Merino* and *Damara* breeds, the extent of metabolites differences in both tissues was comparable, with half of them coinciding in both breeds. The *Merino* had eight metabolites with variations in muscle and 10 in liver tissue, while the *Damara* had eight in muscle and nine in liver.

The distinct variations among breeds, identified by both statistical approaches, could be indicative of breed-specific response to the feed-restriction treatment. Interestingly, we observed that some muscle metabolites could explain differences between treatment groups in both statistical analyses in *Merino* and *Damara*. In the liver, the same was observed in *Merino* breed. However, a more detailed analysis of these variations is needed to understand the physiological significance of these observations. Figure 4.6 summarises the major results for each breed and includes some results from previous studies on these animals, to help integrate and evaluate all the information.

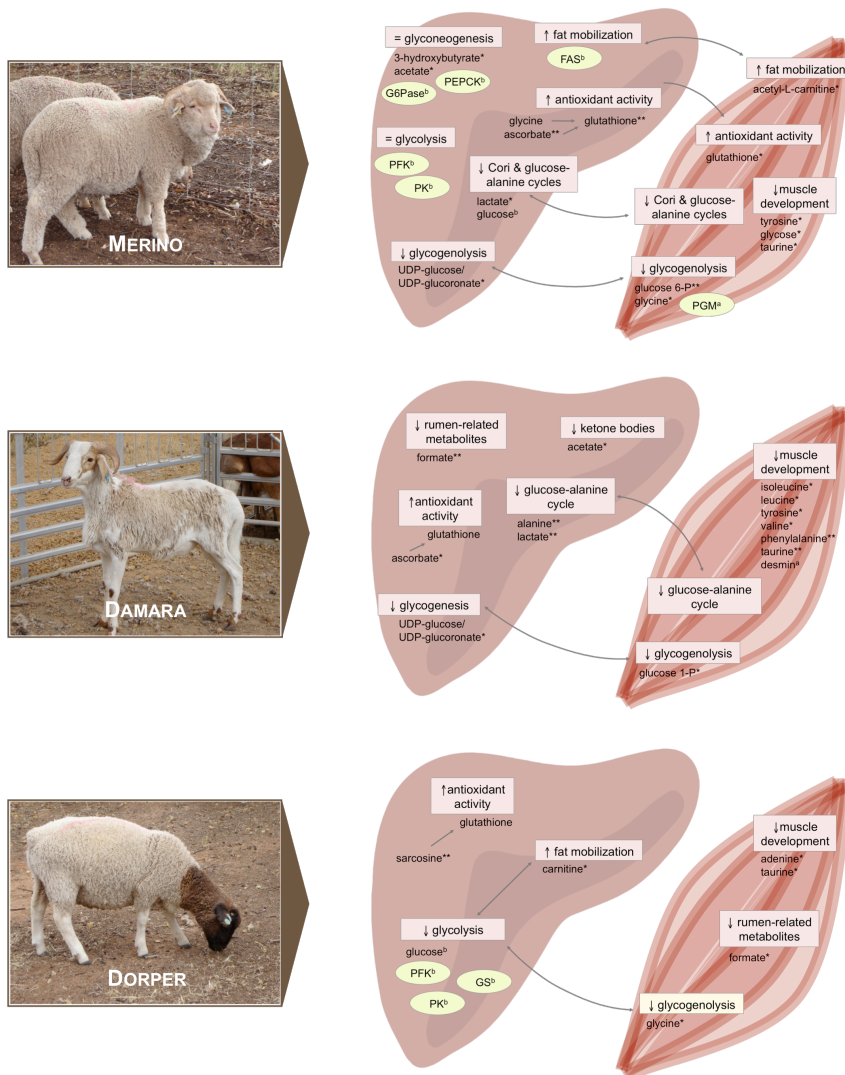


Figure 4.6. Schematic representation of major results in muscle and liver metabolome of restricted groups, of *Merino*, *Damara* and *Dorper* breeds, when compared with the respective growth group. Results from previous work in the same animals are also shown, and marked with uppercase letters (a / b). Metabolites with significant variation are marked with stars (* / **). Key: (a) Almeida *et al.*, 2016. PLoS One. 11(2):e0146367; (b) van Harten *et al.*, 2013. Animal. 7:439-445; (*) $p < 0.05$; (**) $p < 0.01$; (FAS) fatty acid synthase; (G6Pase) glucose 6-phosphatase; (PEPCK) phosphoenolpyruvate carboxykinase; (PFK) phosphofructokinase; (PK) pyruvate kinase; (PGM) phosphoglucomutase; (GS) glycogen synthase; (=) no variation in the pathway; (↑) pathway increased; (↓) pathway decreased.

4.5.1 Merino breed metabolomes

In muscle samples, decreased levels of amino acids like tyrosine, glycine and taurine during restriction could be an indication of a reduction of muscle growth. Taurine levels can also be affected by a reduction in dietary cysteine levels¹⁷, making it difficult to separate the effect of diet restriction and the inner response of the animal.

Glycine also has an additional role as a precursor of glucagon in glycogenolysis¹⁸, and can affect glutathione production^{19,20} in the antioxidant defence mechanism. However, glutathione synthesis can also be limited by diet related cysteine intake²¹, mixing the effects of the restricted feed to those of the metabolomics response. Since glutathione is produced in the liver and released to the muscle²², its decreased levels in the liver could be related with its increase in muscle of the same animals. The lower levels of ascorbate (Vitamin C) in the liver could also be related to glutathione levels²³. However, ruminants are able to synthesize it from glucose, and its reduction could also be related to the diet restriction. Low levels of glutathione and ascorbate could be indicative of oxidative stress in the tissue²⁴ likely as a consequence of weight loss.

Sarcosine is a precursor of creatine, so variations in their concentrations in liver could be related with this pathway^{25,26}. Levels of creatine/creatine-phosphate, two metabolites related to energy production in muscle, are in fact increased in the restricted merino group as well as in the other breeds. As previously mentioned, glycine is a precursor of glucagon, which, during low glucose levels and under stress conditions, promotes gluconeogenesis and glycogenolysis. Indeed, significant differences in some metabolites related with such these metabolic pathways were

observed. Glucose-6-phosphate and UDP-glucose/UDP-glucuronate are intermediate products in the glycogenolysis pathway. Their lower levels in muscle of the restricted group could be due to the depletion of glucose and glycogen stock in these animals, due to the diet limitation. Previous proteome analysis of the same muscle samples revealed an increased expression of phosphoglucomutase, an enzyme involved in glycogenolysis and glycogenesis⁶. The over expression occurs only in the *Merino* breed and not in the SWL tolerant breeds, confirming that this breed is more susceptible to feed restriction and has a specific response to this treatment. van Harten *et al.* (2013)⁵ determined the gene expression of regulatory enzymes in the liver of the same animals (*Dorper* and *Merino*), and no changes were observed in the enzymes of the gluconeogenesis (phosphoenolpyruvate carboxylase) and glycolysis (phosphofructokinase and pyruvate kinase) pathways. The expression level of glucose-6-phosphatase, essential in glucose supply during feed-restriction, was determined and its value decreased in restricted groups. During diet restriction animals tend to reduce the glycolytic pathway and promote gluconeogenesis⁵. These results could then be indicative of a minor adaptation to the feed-restriction.

In the livers of the restricted group we also observed lower levels of alanine and lactate. These two metabolites are related in both muscle and liver to the Cori and the Alanine-Glucose Cycles. A decrease on their concentrations could be an indirect consequence of the lower levels of glucose in both tissues. Alanine is also a structural amino acid and its concentration is usually low during diet restriction when muscle breakdown occurs. Glucose levels in the liver of the same animals were determined in a previous study and was observed a significant decrease between growth and restricted animals⁵, supporting this hypothesis.

Energy from nutrients in the diet could be obtained through processes other than glycolysis, especially during feed-restriction periods. Variations in some metabolites are indicative of such process. The increased levels of acetyl-L-carnitine in the muscle of the restricted merino group are an indication of fat mobilization. This metabolite is responsible for the transport of fatty acids into mitochondria to be oxidized and used as energy sources during high energy demanding or glucose starvation periods. Previous results showed lower expression levels of fatty acid synthase, an enzyme responsible for fatty acids synthesis, in the liver of restricted animals⁵. These results suggest that restricted-fed animals are using fatty acids as an energy source.

During fasting periods and low carbohydrate diets, ketone bodies (acetoacetate and 3-hydroxybutyrate) are produced in liver. We have indications of ketone body production in the liver of the *Merino* fed-restricted animals, as 3-hydroxybutyrate level is higher in the restricted group than in the control group. However, in ruminant both ketone compounds could be a result of rumen activity^{11,27} due either to changes in microbiota profile or in the diet composition.

Succinate and citrate show variations between groups in liver and muscle, respectively. Both metabolites are intermediates in the Krebs cycle with associations to other metabolic pathways. These variations could be indicative of variations in the Krebs cycle or regulation of other secondary pathway, as the inhibition of glycolysis and promotion of gluconeogenesis by elevated levels of citrate. The increased levels of glycerophosphocholine in the muscle could also be related with some regulatory process, since this metabolite is a storage form of choline in cytosol, with functions as muscle control and source of methyl group²⁸.

It is noteworthy that, as wool producers these animals will use important nutritional resources for wool production, making them unavailable to be used in other pathways in harsh conditions. Also, the wool production continued during the trial, channelling nutritional resources to it and influencing the general pathways.

4.5.2 *Damara* breed metabolomes

Levels of isoleucine, leucine, tyrosine, valine, phenylalanine and taurine decreased in the muscle of the restricted group. All these amino acids are related to muscle development and their lower concentrations could be directly linked to muscle production decrease. However, a previous study on muscle of these animals revealed a unique individual response of this breed, when compared with the *Merino* and *Dorper* breeds⁶. In the *Damara* breed the levels of desmin, a muscle-specific protein responsible for cell architecture, increased, ensuring the structure and function of the muscle even if some tissue mobilization occurs⁶. Isoleucine and leucine are also related with other metabolic pathways, related to ketonic bodies production and cell growth regulator respectively, that can influence their concentrations. The reduction of UDP-glucose/UDP-gucoronate and glucose-1-phosphate in liver and muscle respectively, as observed in the *Merino* breed, is indicative of changes in the glycogenolysis/glycogenesis pathway.

IMP and adenine levels are both lower in restricted group, in muscle and liver respectively. IMP is a nucleoside and an intermediate in purine metabolism, from which adenine is one of the examples. This result could be a consequence of a slower muscle development or an imbalance in

these tissues. Choline could also be connected with cell development and balance²⁸, and it is also reduced in the liver of the restricted-fed animals. Variations in Krebs cycle intermediates (citrate), in rumen-related metabolite (formate), and in ascorbate were observed in this breed, similarly to the *Merino* breed.

Considering the special adaptation of this breed to store fat in the tail, it is interesting that changes observed in the metabolism are in general not directly related to fat metabolism. Previous studies with the same animal groups suggest that the *Damara* breed has a unique lipid metabolism, mostly due to the putative contribution of the fat tail as supplier of odd and branched-chain fatty acids (BCFA) to the muscle²⁹. However, it is also suggested that this tolerance to feed restriction could also be due to some kind of peculiarities in rumen activity²⁹. Specifically, if the *Damara* breed has some digestive adaptation that can increase the efficiency of fibre digestion, the acetate-propionate ratio will be affected^{29,30}. Indeed, in the present study, levels of acetate in the liver of the restricted group were lower than in the growth group.

4.5.3 *Dorper* breed metabolomes

Variations in glycine and sarcosine in this breed show the same pattern as observed in *Merino* breed. Higher levels of sarcosine in liver of the restricted groups could be indicative of an increase glutathione production. At the same time, lower levels of glycine in muscle of restricted groups suggest a decrease of glycogenolysis. Previous results⁵ on the enzyme expression levels show that enzymes related with glycolysis (phosphofructokinase and pyruvate kinase) have lower

expression in the restricted group. Simultaneously, enzyme related to glycogenesis (glycogen synthase) did not vary between treatments. Moreover, the same study showed that levels of glucose in liver of restricted group did not differ from the values of growth group⁵, suggesting an efficient response of this breed to feed restriction condition.

Since carnitine is essential for fat mobilization and energy production during fasting periods and feed restriction, higher levels of this metabolite in the liver could help explain the glucose homeostasis of this breed. Previous results on the enzymes related with fatty acid synthesis (fatty acid synthase)⁵ revealed a decrease on its expression in the restricted group, reinforcing the hypothesis of fatty acids request for energy production.

As observed in the other two breeds, taurine levels are lower in the muscle of the restricted group, being indicative of lower muscle production and development. However, previous results on enzymes involved in protein catabolism (glutamate dehydrogenase) show a decrease in restricted groups. These results suggest that in the *Damara*, muscle production could be reduced due to feed restriction, while the *Dorper* breed can maintain tissue function and structure. Adenine concentration was higher in muscle of the restricted group, opposite to what was observed in the liver of the *Damara* breed. This metabolite is a nucleoside with functions in protein synthesis and energy production.

The *Dorper* breed also showed differences in rumen-related metabolites (formate) between the restricted and growth groups that could be indicative of adaptations in rumen microbiota as response to the restriction-fed regime.

4.5.4 Metabolomics results, growth, carcass traits, leptin and insulin concentrations

In general, we observed a decrease in muscle development, an increase in antioxidant activity and differences in energy production pathways between the different breeds as each breed cope with feed restriction. *Dorper* and *Damara* breeds seem to be more tolerant to feed restriction. Previous studies on these animals^{2,6} (Supplementary Table S4.8) already suggested this tendency. Variations in the carcass weight and yields and in the dimensions of eye muscle were similar in *Dorper* and *Damara* breed, and both different of what was observed in *Merino*. Concerning plasma parameters, *Damara* breed presented differences in leptin and insulin concentrations, when compared with the other breeds. Higher levels of leptin and insulin in *Damara* could be justified by the existence of the tail fat depot. Leptin is produced by adipose cells and is highly correlated with body fat, whereas insulin stimulates lipogenesis and fatty acids esterification³¹. In ruminants, insulin stimulates the leptin expression³², which together with the body fat content, could explain the higher level of leptin concentrations in this breed.

In the context of the breed selection towards SWL tolerance, our results finally confirm that the *Dorper* and *Damara* breeds have performed better under SWL conditions.

4.6 ABBREVIATIONS LIST

DSS sodium-2,2-dimethyl-2-silapentane-5-sulfonate

IMP inosine monophosphate

MW molecular weight

NC number of components

NMR nuclear magnetic resonance

PCA principal component analysis

PC1/PC2 principal component 1/2

PLS partial least squares

SWL seasonal weigh loss

UDP(...) uridine diphosphate (...)

1D-NOESY one dimension nuclear Overhauser effect spectroscopy

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GENERAL DISCUSSION

The goal of this work was to study the effect of feed-restriction, as the major promoter of SWL in small ruminant breeds with different levels of adaptation to SWL. To accomplish that, we studied two dairy goat breeds (*Palmera* and *Majorera*) focusing on the mammary gland and milk and three meat-producing sheep breeds (*Merino*, *Dorper* and *Damara*) focusing on skeletal muscle and liver. We followed an NMR-metabolomics approach to profile the aqueous metabolome of the mammary gland and milk of the goats, and the liver and muscle of the sheep. To complement this analysis, we profiled also the fatty acid composition of goat mammary gland and milk.

5.1 NMR-METABOLOMICS AND FARM ANIMAL EXPERIMENTATION

The NMR-Metabolomics approach has been applied to farm animals¹⁻⁸, but its use in small ruminants⁹⁻¹⁷ is relatively recent. Although literature highlights the proficiency and reproducible character of the presented methods¹⁸⁻²⁰ it is the first time that such samples (goat mammary gland and sheep muscle and liver) were tested and evaluated altogether. In this study, the NMR-based technique used proved to be adequate to assess the metabolite profile of these samples. Regarding data analysis and considering that it was a new approach to these specific samples, the

targeted approach (profiling) provided more complete information. This first approach can now be used as a starting point for future studies in the same type of samples. Based on this information, it is now possible to conduct other works following the untargeted approach (chemometrics), especially if concerned with presence/absence of a given metabolite. This type of information is of utmost importance for biomarker identification studies, which in turn may have important implications for areas such as quality control, or authenticity tests that are important for instance for the meat and dairy industries, governing bodies, consumers and the society.

5.2 GOAT EXPERIMENTS

Concerning the dairy small ruminants and their response to feed-restriction, we studied two breeds with different levels of tolerance to SWL: the *Palmera* and *Majorera* breeds.

Differences observed in the mammary gland and milk small metabolite composition revealed a strong effect of diet restriction, albeit with no differences between breeds. Results from both samples were consistent between breeds and shown variations in metabolic pathways related to adaptation to a low-energy diet. We also observed variations in metabolites related with microbiota profile or rumen kinetics. The *Majorera* breed showed more variations in small metabolites, however no interaction of breed and treatment was observed in fatty acids profile. The opposite response was observed in the *Palmera* breed. This could be an indication that *Majorera* breed has a higher tolerance to feed-

restriction, showing only variations in metabolites without using the body fat depots.

In general, milk presented more variations than the mammary gland tissue. Accordingly, this fluid seems to be more susceptible to feed-restriction and changes in diet composition. Milk is therefore a good indicator of variations in the animal metabolism and could be a prime sample in other diet-related and biomarker identification studies. Milk has also the advantage of being collected in a simple and non-invasive way, meeting the strongly recommended directions for dairy animal production²¹. Moreover, milk collection does not require significant changes in the usual routine in farms nor specialized professionals to do it, enabling farmers to easily collaborate in the field work. In the context of dairies production and optimization for different market products, *Majorera* breed seems to be a more workable breed. Changes in diet can be used to adjust the fat content of milk and of dairy products.

5.3 SHEEP EXPERIMENTS

To study the response of meat-producer ruminants to feed-restriction, we studied three breeds with different levels of tolerance to SWL: *Merino*, *Damara* and *Dorper*, and two tissues, muscle and liver.

In general, the *Dorper* breed seems to be more adapted to SWL, showing few changes in both tissues when subjected to feed restriction. This tolerance could be a result of the breed-selection history. The *Merino*, probably due to its selection for wool production, showed more marked changes both tissues and seems to be the less adapted to SWL. The *Damara* showed a specific set of adaptations, reflecting the

physiology of its major body characteristic, the fat-tail. In the context of the breed selection towards SWL tolerance, our results confirm that the *Dorper* and *Damara* breeds performed better under SWL conditions. Their adaptation seems to be linked to a more efficient metabolic adaptation to feed-restriction, which changes the nutritional energy source without compromising the overall muscle structure. A possible adaptation at the rumen level should also be considered in these breeds, since they presented some variations related with rumen microbiota composition and activity.

5.4 SMALL RUMINANTS AND SEASONAL WEIGHT LOSS

Seasonal weight loss has an important influence in small ruminant breeding, either for milk or meat production. Different species and different breeds seem to respond in different ways to this condition. Dairy goats tend to keep the mammary gland structure and function to ensure milk production despite the conditions. Milk reflects readily the adaptation mechanisms and the variations in diet composition, being therefore an excellent sample to be used in studies of this nature. Meat-producing sheep, namely the breeds tolerant to SWL, tend to keep the muscle structure, appealing to alternative energy sources but their structural proteins.

Interestingly, the rumen microbiota seems to be involved and has an important role in adaptation process in both species and almost all breeds. Changes in some metabolites are indicative of different microbiota profile or kinetics, revealing critical variations in rumen. It was already described²² that ruminants could take advantage of the quicker

adaptation capacity of the rumen microbiota to adapt to several conditions. This capacity is also referred as essential to small ruminant establishment in different climate regions, and to adaptation to the changing environment during migrations (climate, habitat and pasture botanical composition). Individual variability within groups (herds and breeds) seems to have been essential to these processes²². Our results, mainly on the goats, reflect this individual variability within groups, even in control groups. Several ruminant species show some capacity to select and sort food according to availability and nutritional needs that confer them a wider range of opportunities²².

5.5 FUTURE PERSPECTIVES

According to the main objective of this work, we determined the major effects of feed-restriction in small ruminants. The adaptive process to an environment depends not only on the animal tolerance capacity but also on the available resources. Along the work development some questions and hypothesis arise, opening the opportunity to continue and expand the study:

- In future studies, an increase in the time of experiment, especially in the goat study could be considered. This could allow to identify additional variations between breeds. It is however a perspective with certain limitations, particularly from the animal welfare point of view. Furthermore, and particularly for the goat experiment, increasing the experimental period could lead to an anticipated lactation end.
- Metabolomic analysis of tissues related with body fat depots as adipose tissue, liver and muscle should be considered, especially in goats. This

information could be helpful to complete a systematic approach in this specie. As shown in the *Damara* breed, specific adaptations are specifically related to fat tail that in turn is essential to cope with periods of feed restriction.

- Some indications that the studied differences could be related to rumen kinetics and profile were found in both species. The study of the rumen microbiome profile could further elucidate mechanisms of tolerance to seasonal weight loss. Fistulated animals could be used to accomplish that. Application of Omics approaches, such as genomics and metabolomics could also allow the study the whole microbiome and its dynamics during the digestive process.
- Characterization of the environments where the breeds evolved could give some clues about the adaptive process. The characteristics of the endemic plants, as chemical composition and the water retention capacity, could also be determinant to the evolutionary success of small ruminants.
- Considering the most recent guidelines in animal science, non invasive samples should be preferred, not only for physiological studies but also for biomarkers identification. In this context, further metabolomics studies on milk, and saliva could also be considered.
- Given the complementary nature of NMR and MS, a follow up analysis by MS could be considered. A useful approach could be the use of LC-MS/MS to identify peptides that could be residually present in this fraction after extraction.

- Implementation of NMR tasks as complementary to other Omics approaches could be essential to complement analysis, and allow an integrated and broader systematic review.
- The present methods and results could be a useful starting point to explore SWL in other livestock species. There are different dairy and beef producing breeds to which a similar approach could be further applied.

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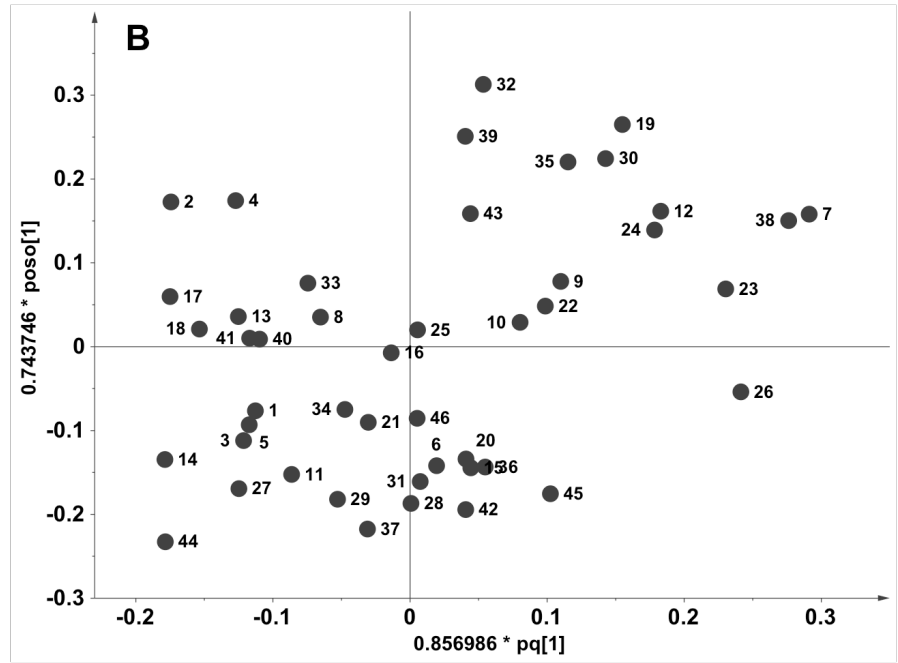
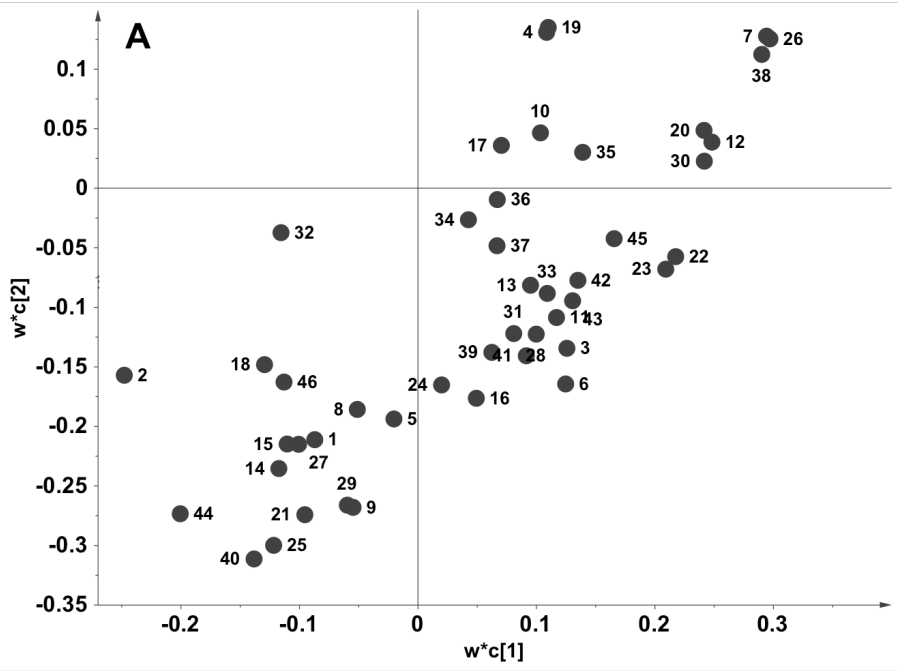
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APPENDIX

SUPPLEMENTARY MATERIAL CHAPTER 3

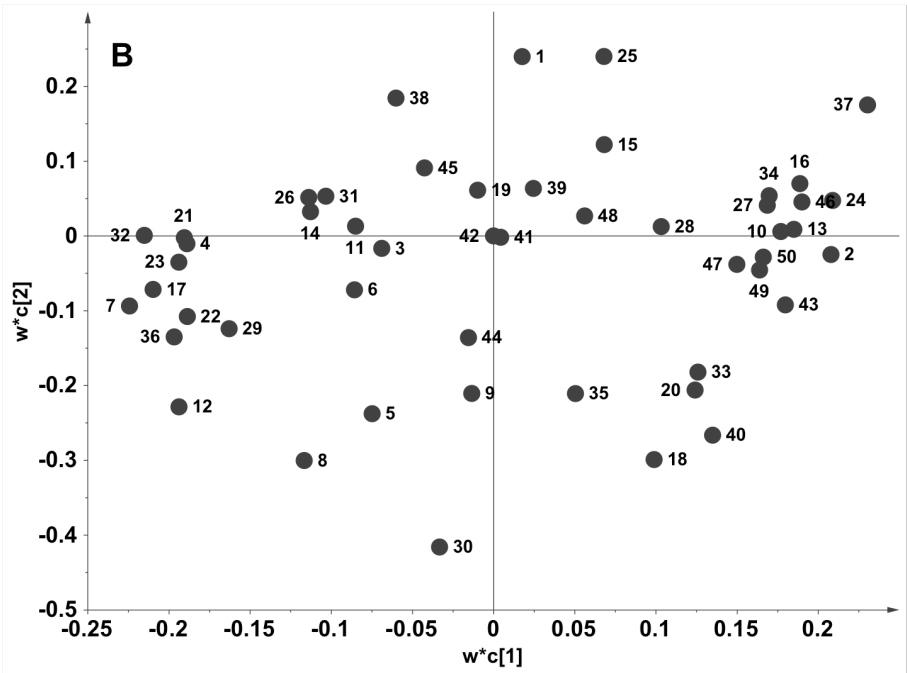
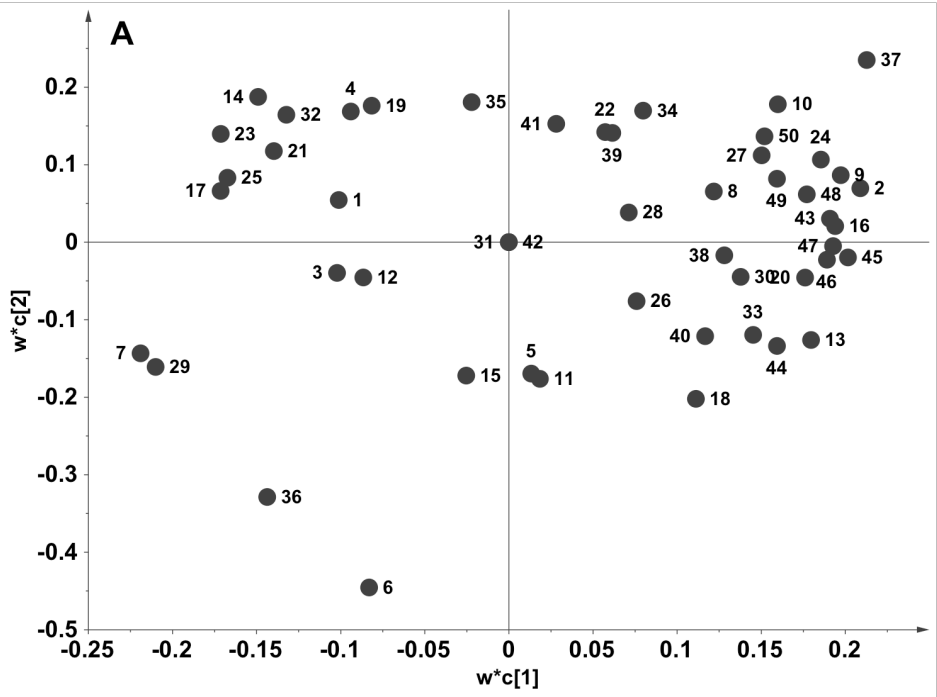
Supplementary Figure S3.1

Loadings from mammary gland aqueous fraction: (A) PLS loadings plot for *Majorera* control and *Majorera* restricted group; (B) OPLS loadings plot for *Palmera* control and *Palmera* restricted group. Key: (1) 3-methylhistidine; (2) acetate; (3) acetyl-L-carnitine; (4) adenine; (5) adenosylhomocysteine; (6) alanine; (7) AMP/ADP/ATP; (8) anserine; (9) aspartate; (10) benzoate; (11) betaine; (12) carnitine; (13) carnosine; (14) choline; (15) citrate; (16) creatine; (17) creatine phosphate; (18) creatinine; (19) formate; (20) fumarate; (21) glucose; (22) glutamate; (23) glutathione; (24) glycerophosphocholine; (25) glycine; (26) IMP; (27) inosine; (28) isoleucine; (29) lactate; (30) lactose; (31) leucine; (32) methylmalonate; (33) myo-inositol; (34) NAD⁺; (35) NADP⁺; (36) nicotinurate; (37) phenylalanine; (38) phosphocholine; (39) pyruvate; (40) succinate; (41) taurine; (42) tyrosine; (43) UDP-glucose; (44) uridine; (45) valine; (46) xanthine.



Supplementary Figure S3.2

Loadings from milk serum: (A) PLS loadings plot for *Majorera* control and *Majorera* restricted group; (B) PLS loadings plot for *Palmera* control and *Palmera* restricted group. Key: (1) 2-oxobutyrate; (2) 2-oxoglutarate; (3) β -hydroxybutyrate; (4) acetate; (5) acetone; (6) acetylcholine; (7) acetyl-L-carnitine; (8) adenine; (9) adenosine; (10) alanine; (11) benzoate; (12) betaine; (13) carnitine; (14) choline; (15) cis-aconitate; (16) citrate; (17) creatine; (18) creatine phosphate; (19) creatinine; (20) dimethylglycine; (21) ethanolamine; (22) formate; (23) fucose; (24) fumarate; (25) galactose; (26) glucose; (27) glutamate; (28) glycine; (29) hippurate; (30) inosine; (31) isoleucine; (32) lactate; (33) lactose; (33) lactose, but with several less concentrated metabolites underneath them; (34) leucine; (35) malonate; (36) methylmalonate; (37) N-acetylglucosamine; (38) orotate; (39) pantothenate; (40) phosphocholine; (41) pyruvate; (42) riboflavin; (43) succinate; (44) taurine; (45) trimethylamine oxide; (46) UDP-galactose; (47) UDP-glucose; (48) UDP-glucuronate; (49) uridine; (50) valine.



Supplementary Table S3.1

Lipid and fatty acid (FA) content (mg/g tissue DM) and fatty acid composition (% of total FA) in mammary gland of *Majorera* and *Palmera* goat breeds, in control and restricted feed groups.

	Majorera		Palmera		P		
	Control	Restricted	Control	Restricted	B	T	BxT
Lipids (mg/g MS)	280 ± 37.3	208 ± 33.4	256 ± 30.5	218 ± 37.3	0.854	0.132	0.645
FA (mg/g MS)	153 ± 20.3	85 ± 18.2	122 ± 16.6	80 ± 20.3	0.362	0.011	0.500
8:0	0.26 ± 0.091	0.14 ± 0.048	0.42 ± 0.209	0.28 ± 0.088	0.272	0.342	0.937
10:0	2.2 ± 0.87	1.1 ± 0.16	3.1 ± 0.96	1.6 ± 0.34	0.291	0.086	0.753
12:0	1.43 ± 0.564	0.71 ± 0.128	2.09 ± 0.460	0.76 ± 0.143	0.370	0.024	0.443
i-14:0	0.04 ± 0.013	0.04 ± 0.012	0.07 ± 0.011	0.04 ± 0.013	0.220	0.464	0.196
14:0	4.0 ± 1.06	3.1 ± 0.26	6.0 ± 0.86	2.6 ± 0.29	0.320	0.014	0.116
i-15:0	0.10 ± 0.018	0.10 ± 0.016	0.12 ± 0.015	0.11 ± 0.018	0.297	0.677	0.967
a-15:0	0.16 ± 0.034	0.13 ± 0.031	0.22 ± 0.028	0.12 ± 0.034	0.501	0.062	0.304
14:1 <i>cis</i> -9	0.12 ± 0.019	0.10 ± 0.017	0.13 ± 0.015	0.06 ± 0.019	0.309	0.014	0.184
15:0	0.53 ^{ab} ± 0.064	0.40 ^b ± 0.057	0.68 ^a ± 0.052	0.33 ^b ± 0.064	0.534	0.001	0.078
i-16:0	0.21 ± 0.031	0.17 ± 0.028	0.23 ± 0.025	0.13 ± 0.031	0.780	0.034	0.295
16:0	24.2 ^{ab} ± 1.09	21.4 ^b ± 0.98	27.8 ^a ± 0.89	20.6 ^b ± 1.09	0.213	<0.001	0.050
i-17:0	0.26 ^a ± 0.028	0.26 ^a ± 0.025	0.32 ^a ± 0.022	0.22 ^a ± 0.028	0.706	0.098	0.076
16:1 <i>cis</i> -7	0.27 ± 0.038	0.29 ± 0.034	0.28 ± 0.031	0.34 ± 0.038	0.457	0.221	0.611
16:1 <i>cis</i> -9	1.25 ± 0.314	1.18 ± 0.281	1.29 ± 0.256	0.90 ± 0.314	0.677	0.447	0.590
a-17:0	0.47 ± 0.050	0.49 ± 0.044	0.43 ± 0.041	0.36 ± 0.050	0.084	0.570	0.370
17:0	0.87 ± 0.133	1.02 ± 0.12	0.72 ± 0.109	0.80 ± 0.133	0.168	0.364	0.785
17:1 <i>cis</i> -9	0.42 ± 0.104	0.88 ± 0.093	0.45 ± 0.085	0.65 ± 0.104	0.308	0.004	0.204
18:0	13.8 ± 2.75	13.7 ± 2.46	11.8 ± 1.28	13.5 ± 1.57	0.614	0.668	0.699
18:1 <i>trans</i> -6/7/8	0.11 ± 0.017	0.07 ± 0.015	0.13 ± 0.014	0.08 ± 0.017	0.362	0.013	0.776
18:1 <i>trans</i> -9	0.19 ± 0.024	0.16 ± 0.021	0.19 ± 0.019	0.11 ± 0.024	0.409	0.023	0.256
18:1 <i>trans</i> -10	0.20 ± 0.029	0.16 ± 0.019	0.29 ± 0.109	0.09 ± 0.025	0.859	0.078	0.255
18:1 <i>trans</i> -11	0.49 ^a ± 0.067	0.35 ^a ± 0.041	0.89 ^a ± 0.174	0.32 ^a ± 0.074	0.107	0.008	0.066
18:1 <i>cis</i> -9	36.0 ^{ab} ± 2.2	41.8 ^a ± 1.9	30.9 ^b ± 1.8	44.0 ^a ± 2.2	0.312	0.001	0.022
18:1 <i>cis</i> -11	1.04 ± 0.074	1.04 ± 0.067	1.00 ± 0.061	0.93 ± 0.074	0.297	0.622	0.601
18:1 <i>cis</i> -12	0.33 ± 0.572	0.29 ± 0.051	0.33 ± 0.047	0.20 ± 0.057	0.427	0.124	0.463
18:2 isomers	0.33 ± 0.046	0.28 ± 0.042	0.27 ± 0.038	0.17 ± 0.046	0.064	0.098	0.577
18:2 n-6	4.1 ± 0.55	4.7 ± 0.49	4.3 ± 0.45	4.06 ± 0.55	0.724	0.732	0.441
20:0	0.13 ± 0.023	0.17 ± 0.061	0.23 ± 0.019	0.24 ± 0.069	0.120	0.548	0.780
18:3 n-3	0.39 ± 0.045	0.36 ± 0.040	0.46 ± 0.036	0.28 ± 0.045	0.855	0.024	0.121
18:2 <i>cis</i> -9, <i>trans</i> -11	0.52 ± 0.104	0.28 ± 0.093	0.72 ± 0.085	0.32 ± 0.10	0.237	0.005	0.436
20:4 n-6	1.2 ± 0.37	3.0 ± 0.33	1.5 ± 0.30	2.36 ± 0.37	0.658	0.001	0.178
22:4 n-6	0.24 ^a ± 0.060	0.42 ^a ± 0.054	0.32 ^a ± 0.049	0.25 ^a ± 0.373	0.500	0.427	0.098
22:5 n-3	0.76 ± 0.201	1.20 ± 0.180	0.93 ± 0.164	1.18 ± 0.201	0.706	0.085	0.633
Other	2.38 ± 0.389	1.43 ± 0.348	2.52 ± 0.318	1.89 ± 0.389	0.427	0.046	0.674
SFA	48 ± 2.5	43 ± 2.3	53 ± 2.1	41 ± 2.5	0.382	0.002	0.169
<i>cis</i> -MUFA	41 ± 2.5	45 ± 2.2	34 ± 2.1	48 ± 2.5	0.309	0.002	0.058
<i>trans</i> -MUFA	0.99 ± 0.225	0.74 ± 0.201	1.50 ± 0.184	0.61 ± 0.225	0.375	0.015	0.149
PUFA	6.9 ± 1.13	9.5 ± 1.01	7.8 ± 0.92	8.2 ± 1.13	0.859	0.191	0.311
SCD _i -14	3.1 ± 0.71	3.2 ± 0.638	2.6 ± 0.58	2.2 ± 0.71	0.272	0.846	0.697
SCD _i -16	4.9 ± 1.09	5.3 ± 0.97	4.5 ± 0.89	4.2 ± 1.09	0.476	0.999	0.755
SCD _i -17	34 ± 5.5	47 ± 5.0	37 ± 4.5	44 ± 5.5	0.994	0.078	0.588
SCD _i -18	74 ± 3.1	75 ± 2.8	73 ± 2.5	77 ± 3.1	0.972	0.343	0.569
SCD _i -t11	51 ± 3.7	44 ± 3.3	46 ± 3.0	49 ± 3.7	0.938	0.581	0.149

Key: (SEM) standard error of mean; (B) breed; (T) treatment; (FA) fatty acids; (DM) dry matter; (other) sum of other fatty acids; (SFA) saturated fatty acids; (MUFA) monounsaturated fatty acids; (PUFA) polyunsaturated fatty acids; (SCD) estimated stearyl-CoA desaturase activity indexes; SCD_i-14, (14:1/(14:1+14:0)*100); SCD_i-16, (16:1/(16:1+16:0)*100); SCD_i-17, (17:1/(17:1+17:0)*100); SCD_i-18, (18:1/(18:1+18:0)*100). (a, b) within a row, means without a common letter differ (p<0.05).

Supplementary Table S3.2

PCA loadings values of principal component 1 (p[1]) and principal component 2 (p[2]) of mammary gland from the four experimental groups. Highlighted values are the more representatives for the groups clustering along the principal component 1.

fatty acid	p[1]	p[2]
C18:1c9	-0.264668	-4.46E-05
C17:1c9	-0.146357	-0.0924077
C20:4n-6	-0.136248	-0.289984
C17:0	-0.10835	0.216588
C18:0	-0.084521	0.242516
DMA-C16:0	-0.0785919	-0.308417
C22:5n-3	-0.0565417	-0.308813
C16:1c9	-0.055461	0.185648
C16:1c7	-0.0252886	-0.128828
C18:1c11	-0.0068685	-0.0520676
LT (mg/g MS)	-0.0019512	0.279835
C20:0	0.0244574	-0.229331
aC17:0	0.0426691	0.146457
AGT(mg/g MS)	0.066277	0.294971
iC17:0	0.066802	0.0879935
C18:2n-6	0.0861685	-0.269561
C22:4n-6	0.0898652	-0.164366
C18:1t10	0.0990322	0.193492
C14:1c9	0.114154	0.102406
iC15:0	0.134289	-0.0968628
C18:2 isomers	0.167499	0.0322525
C8:0	0.175885	-0.154831
C16:0	0.18647	0.0781456
Rest	0.189043	-0.0955506
C18:1t9	0.189627	0.0692925
C18:1t11	0.195359	0.120596
C18:1t6/t7/t8	0.198336	0.114925
CLA	0.19838	0.0731203
iC16:0	0.204584	0.115011
iC14:0	0.221635	-0.0930905
C18:1c12	0.223882	-0.0154905
aC15:0	0.230073	-0.0062049
C14:0	0.234925	-0.0900802
C10:0	0.240799	-0.153335
C18:3n-3	0.243568	-0.0198951
C12:0	0.255677	-0.144151
C15:0	0.275824	0.0131943

SUPPLEMENTARY TABLE S3.3

Fatty acid (FA) composition (% of total FA) in milk of *Majorera* and *Palmera* goat breeds, in control and restricted feed groups.

	Majorera		Palmera		P		
	Control	Restricted	Control	Restricted	B	T	B x T
4:0	1.80 ± 0.080	1.37 ± 0.071	1.89 ± 0.065	1.66 ± 0.080	0.023	<0.001	0.203
6:0	2.27 ± 0.094	1.54 ± 0.084	2.49 ± 0.077	1.79 ± 0.094	0.016	<0.001	0.845
8:0	2.80 ± 0.135	1.77 ± 0.121	3.18 ± 0.110	2.02 ± 0.135	0.025	<0.001	0.599
10:0	11.41 ± 0.402	5.44 ± 0.359	11.97 ± 0.328	5.74 ± 0.402	0.269	<0.001	0.734
12:0	5.67 ± 0.433	2.01 ± 0.387	5.38 ± 0.353	1.96 ± 0.433	0.678	<0.001	0.767
14:0	0.06 ± 0.080	0.07 ± 0.080	0.08 ± 0.080	0.08 ± 0.080	0.308	0.669	0.545
14:1	11.44 ± 0.405	6.12 ± 0.362	10.08 ± 0.330	5.76 ± 0.405	0.038	<0.001	0.209
15:0	0.13 ± 0.013	0.14 ± 0.012	0.16 ± 0.011	0.15 ± 0.013	0.149	0.789	0.262
a-15:0	0.31 ± 0.024	0.20 ± 0.021	0.33 ± 0.019	0.23 ± 0.024	0.347	<0.001	0.704
14:1 cis-9	0.24 ± 0.019	0.14 ± 0.017	0.14 ± 0.015	0.09 ± 0.019	<0.001	<0.001	0.123
15:0	1.10 ± 0.040	0.56 ± 0.036	1.04 ± 0.032	0.51 ± 0.040	0.148	<0.001	0.850
16:0	0.22 ± 0.020	0.19 ± 0.018	0.23 ± 0.017	0.18 ± 0.020	0.811	0.047	0.678
16:0	32.6 ± 0.99	21.5 ± 0.89	29.9 ± 0.81	21.9 ± 0.99	0.247	<0.001	0.106
17:0	0.24 ± 0.019	0.39 ± 0.017	0.27 ± 0.016	0.37 ± 0.019	0.737	<0.001	0.149
16:1 cis-7	0.24 ± 0.019	0.27 ± 0.017	0.27 ± 0.016	0.26 ± 0.019	0.631	0.578	0.216
16:1 cis-9	0.75 ± 0.051	0.99 ± 0.046	0.57 ± 0.042	0.88 ± 0.051	0.009	<0.001	0.494
a-17:0	0.35 ± 0.027	0.46 ± 0.024	0.37 ± 0.022	0.41 ± 0.027	0.509	0.010	0.208
17:0	0.54 ^c ± 0.036	1.03 ^b ± 0.032	0.63 ^c ± 0.030	0.87 ^b ± 0.036	0.324	<0.001	0.003
17:1 cis-9	0.25 ^c ± 0.045	0.80 ^b ± 0.040	0.25 ^c ± 0.036	0.57 ^b ± 0.045	0.012	<0.001	0.015
18:0	5.84 ± 0.534	7.88 ± 0.478	7.81 ± 0.436	9.50 ± 0.534	0.003	0.002	0.733
18:1 trans-6/7/8	0.15 ± 0.014	0.09 ± 0.012	0.17 ± 0.011	0.09 ± 0.014	0.706	<0.001	0.318
18:1 trans-9	0.16 ± 0.014	0.16 ± 0.012	0.17 ± 0.011	0.14 ± 0.014	0.826	0.245	0.281
18:1 trans-10	0.24 ± 0.030	0.16 ± 0.027	0.23 ± 0.025	0.13 ± 0.030	0.437	0.004	0.692
18:1 trans-11	0.53 ± 0.101	0.29 ± 0.090	0.85 ± 0.083	0.29 ± 0.101	0.115	<0.001	0.116
18:1 trans-12	0.33 ± 0.026	0.16 ± 0.023	0.32 ± 0.021	0.13 ± 0.026	0.464	<0.001	0.708
18:1 cis-9	15.0 ± 1.06	39.8 ± 0.95	15.7 ± 0.87	38.9 ± 1.06	0.902	<0.001	0.412
18:1 trans-15	0.15 ± 0.009	0.08 ± 0.008	0.14 ± 0.007	0.06 ± 0.009	0.159	<0.001	0.673
18:1 cis-11	0.42 ± 0.034	0.64 ± 0.031	0.42 ± 0.028	0.59 ± 0.034	0.451	<0.001	0.551
18:1 cis-12	0.27 ± 0.030	0.26 ± 0.027	0.24 ± 0.024	0.19 ± 0.030	0.106	0.319	0.510
18:1 cis-13	0.04 ± 0.003	0.05 ± 0.003	0.04 ± 0.003	0.05 ± 0.003	0.548	<0.001	0.699
18:1 trans-16	0.25 ± 0.015	0.14 ± 0.013	0.25 ± 0.012	0.12 ± 0.015	0.373	<0.001	0.442
18:1 cis-15	0.07 ± 0.048	0.05 ± 0.043	0.07 ± 0.039	0.04 ± 0.048	0.417	<0.001	0.167
18:2 isomers	0.43 ± 0.038	0.42 ± 0.034	0.35 ± 0.031	0.29 ± 0.038	0.006	0.287	0.547
18:2 n-6	2.10 ± 0.182	3.18 ± 0.163	2.13 ± 0.149	2.57 ± 0.182	0.111	<0.001	0.079
20:0	0.13 ± 0.008	0.13 ± 0.007	0.17 ± 0.007	0.15 ± 0.008	0.001	0.107	0.189
18:3 n-6	0.03 ± 0.048	0.04 ± 0.048	0.03 ± 0.048	0.04 ± 0.048	0.711	0.003	0.606
18:3 n-3	0.45 ± 0.062	0.38 ± 0.055	0.54 ± 0.050	0.26 ± 0.062	0.062	0.004	0.209
20:1	0.04 ± 0.005	0.07 ± 0.004	0.04 ± 0.004	0.07 ± 0.005	0.717	<0.001	0.977
18:2 cis-9,trans-11	0.42 ± 0.063	0.35 ± 0.056	0.51 ± 0.051	0.27 ± 0.063	0.910	0.019	0.177
21:0	0.05 ± 0.006	0.04 ± 0.005	0.06 ± 0.005	0.04 ± 0.006	0.159	0.004	0.377
22:0	0.05 ± 0.006	0.05 ± 0.006	0.07 ± 0.005	0.06 ± 0.006	0.034	0.416	0.363
20:4 n-6	0.21 ± 0.022	0.38 ± 0.020	0.22 ± 0.018	0.33 ± 0.022	0.409	<0.001	0.141
20:5 n-3	0.05 ± 0.004	0.05 ± 0.004	0.05 ± 0.003	0.05 ± 0.004	0.233	0.914	0.855
22:4 n-6	0.02 ± 0.004	0.03 ± 0.004	0.04 ± 0.003	0.03 ± 0.004	0.283	0.453	0.114
22:5 n-3	0.09 ± 0.013	0.14 ± 0.011	0.11 ± 0.010	0.11 ± 0.013	0.869	0.037	0.071
22:6 n-3	0.01 ± 0.004	0.03 ± 0.004	0.02 ± 0.003	0.02 ± 0.004	0.541	0.025	0.187
SFA	77.0 ± 1.26	50.9 ± 1.13	76.1 ± 1.03	77.0 ± 1.26	0.489	<0.001	0.157
cis-MUFA	17.3 ± 1.14	43.1 ± 1.02	17.8 ± 0.93	41.6 ± 1.14	0.630	<0.001	0.383
trans-MUFA	1.80 ± 0.178	1.07 ± 0.159	2.12 ± 0.145	0.95 ± 0.178	0.577	<0.001	0.204
PUFA	3.84 ± 0.306	4.97 ± 0.273	4.01 ± 0.249	4.00 ± 0.306	0.179	0.067	0.064
SCD ₁ -14	2.03 ± 0.178	2.20 ± 0.159	1.34 ± 0.145	1.65 ± 0.178	0.002	0.172	0.675
SCD ₁ -16	2.26 ± 0.178	4.39 ± 0.159	1.90 ± 0.145	3.86 ± 0.178	0.054	<0.001	0.694
SCD ₁ -17	31.9 ± 1.52	43.6 ± 1.37	28.3 ± 1.25	39.3 ± 1.52	0.015	<0.001	0.799
SCD ₁ -18	72.0 ± 1.38	83.5 ± 1.23	66.9 ± 1.25	39.3 ± 1.13	0.006	<0.001	0.482
SCD ₁ -11	43.5 ± 1.82	54.2 ± 1.63	37.8 ± 1.49	48.9 ± 1.82	0.005	<0.001	0.909

Key: (SEM) standard error of mean; (B) breed; (T) treatment; (FA) fatty acids; (DM) dry matter; (other) sum of other fatty acids; (SFA) saturated fatty acids; (MUFA) monounsaturated fatty acids; (PUFA) polyunsaturated fatty acids; (SCD) estimated stearyl-CoA desaturase activity indexes; SCD₁-14, (14:1/(14:1+14:0)*100); SCD₁-16, (16:1/(16:1+16:0)*100); SCD₁-17, (17:1/(17:1+17:0)*100); SCD₁-18, (18:1/(18:1+18:0)*100). (a, b) within a row, means without a common letter differ (p<0.05).

Supplementary Table S3.4

PCA loadings values of principal component 1 (p[1]) and principal component 2 (p[2]) of milk from the four experimental groups. Highlighted values are the more representatives for the groups clustering along the principal component 1.

fatty acid	p[1]	p[2]
C18:1c9	-0.203997	0.00113312
C17:1c9	-0.191026	0.0391509
C17:0	-0.185802	-0.0378738
C20:1	-0.180149	-0.0487033
i-C17:0	-0.178427	-0.074843
C18:1c11	-0.177048	0.119201
C20:4n-6	-0.176448	0.0686584
C18:1c13	-0.173305	0.125073
C16:1c9	-0.168196	0.147227
C18:2n6	-0.150806	0.124703
C18:3n6	-0.138323	0.0865175
a-C17:0	-0.128543	-0.0679209
C22:6n-3	-0.115834	0.0466135
C22:5n-3	-0.104008	0.0331235
C18:0	-0.0902215	-0.165596
C16:1c7	-0.0251307	0.0906373
C20:5n-3	0.00518563	0.0545764
others C18:2	0.0228415	0.298377
C22:4n-6	0.0342531	0.00286681
i-C15:0	0.0378706	-0.272239
C18:1c12	0.0404287	0.318235
i-C14:0	0.0481355	-0.248753
C22:0	0.0625756	-0.253858
C18:1t9	0.0687373	0.260828
C20:0	0.0902031	-0.240486
C14:1c9	0.0931969	0.23141
i-C16:0	0.100867	-0.191957
CLAc9t11	0.116761	0.193466
C18:1t10	0.137381	0.187788
C18:3n3	0.140831	-0.032085
C21:0	0.141177	-0.149737
C18:1t11	0.153217	0.061876
C4:0	0.158126	-0.137611
a-C15:0	0.162483	-0.162738
C18:1c15	0.167896	0.172653
C18:1t6/t7/t8	0.17412	0.146804
C16:0	0.184924	-0.0163582
C12:0	0.185529	0.069672
C18:1t12	0.186297	0.130231
C18:1t15	0.186935	0.135485
C6:0	0.189518	-0.0806647
C14:0	0.189744	0.0330473
C8:0	0.190168	-0.0551646
C18:1t16+c14	0.191163	0.0914593
C15:0	0.196843	0.00628086
C10:0	0.202056	-0.0057705

SUPPLEMENTARY MATERIAL CHAPTER 4

Supplementary Table S4.1

Identified metabolites in gastrocnemius muscle of *Merino*, *Dorper* and *Damara* sheep breeds (growth and restricted groups). Average concentration (mmol/ g tissue) and standard deviation are shown for each experimental group.

	Merino		Dorper		Damara	
	growth	restricted	growth	restricted	growth	restricted
3-Methylhistidine	2.23E-05 ± 1.82E-05	2.76E-05 ± 2.98E-05	7.08E-06 ± 3.36E-06	6.36E-06 ± 4.28E-06	1.19E-05 ± 3.33E-06	1.49E-05 ± 1.91E-05
β-Alanine	1.21E-04 ± 4.02E-05	1.05E-04 ± 2.58E-05	1.00E-04 ± 5.65E-05	1.06E-04 ± 5.50E-05	1.20E-04 ± 5.31E-05	9.21E-05 ± 3.78E-05
Acetate	8.96E-05 ± 3.04E-05	5.31E-05 ± 2.18E-05	6.04E-05 ± 1.37E-05	6.58E-05 ± 3.16E-05	6.95E-05 ± 1.49E-05	6.98E-05 ± 1.14E-05
Acetyl-L-carnitine ^a	2.57E-03 ± 1.23E-03	4.58E-03* ± 1.94E-03	1.64E-03 ± 5.76E-04	1.59E-03 ± 1.03E-03	2.20E-03 ± 3.79E-04	2.17E-03 ± 7.57E-04
Adenine ^a	8.29E-05 ± 7.53E-05	7.22E-05 ± 4.99E-05	2.08E-05 ± 1.07E-05	5.26E-05* ± 4.11E-05	8.46E-05 ± 4.29E-05	7.35E-05 ± 6.46E-05
Adenosine	7.30E-06 ± 4.27E-06	7.68E-06 ± 1.75E-06	7.27E-06 ± 2.35E-06	5.84E-06 ± 3.65E-06	6.96E-06 ± 1.33E-06	6.63E-06 ± 2.54E-06
ADP/ AMP/ ATP	7.45E-04 ± 5.27E-04	8.81E-04 ± 4.55E-04	1.32E-03 ± 7.84E-04	1.07E-03 ± 9.58E-04	9.43E-04 ± 4.66E-04	1.29E-03 ± 6.91E-04
Alanine	2.36E-03 ± 1.17E-03	2.23E-03 ± 9.02E-04	1.62E-03 ± 7.56E-04	1.80E-03 ± 1.22E-03	3.19E-03 ± 1.12E-03	2.50E-03 ± 1.01E-03
Anserine	5.11E-03 ± 2.49E-03	4.46E-03 ± 2.13E-03	5.48E-03 ± 1.49E-03	5.42E-03 ± 3.08E-03	6.14E-03 ± 1.09E-03	6.66E-03 ± 1.58E-03
Aspartate	1.50E-04 ± 8.45E-05	1.29E-04 ± 6.25E-05	1.22E-04 ± 4.38E-05	1.75E-04 ± 1.23E-04	1.44E-04 ± 4.81E-05	1.61E-04 ± 5.43E-05
Betaine	1.54E-03 ± 5.48E-04	1.61E-03 ± 4.12E-04	8.75E-04 ± 2.80E-04	8.02E-04 ± 5.61E-04	9.88E-04 ± 2.44E-04	1.17E-03 ± 4.31E-04
Carnitine	8.66E-03 ± 4.13E-03	9.19E-03 ± 3.04E-03	4.59E-03 ± 1.82E-03	5.48E-03 ± 3.46E-03	5.98E-03 ± 8.71E-04	6.42E-03 ± 1.16E-03
Carnosine	1.83E-04 ± 3.64E-04	2.59E-04 ± 3.26E-04	4.95E-05 ± 3.02E-05	6.61E-05 ± 4.54E-05	1.32E-04 ± 8.76E-05	9.34E-05 ± 3.81E-05
Choline/ Phosphocholine	8.10E-04 ± 2.81E-04	7.73E-04 ± 2.48E-04	6.42E-04 ± 1.21E-04	7.78E-04 ± 3.33E-04	6.58E-04 ± 1.26E-04	7.56E-04 ± 1.34E-04
Citrate ^a	1.74E-04 ± 8.55E-05	2.69E-04* ± 1.07E-04	1.46E-04 ± 7.50E-05	1.53E-04 ± 1.20E-04	1.88E-04 ± 7.87E-05	2.23E-04 ± 6.82E-05
Creatine/ Creatine phosphate	2.80E-02 ± 1.03E-02	2.80E-02 ± 6.15E-03	2.46E-02 ± 5.06E-03	2.53E-02 ± 1.36E-02	2.59E-02 ± 4.08E-03	2.55E-02 ± 5.13E-03
Creatinine	1.51E-04 ± 7.20E-05	1.28E-04 ± 5.71E-05	1.16E-04 ± 2.99E-05	1.30E-04 ± 8.07E-05	1.56E-04 ± 4.86E-05	1.25E-04 ± 3.30E-05
Formate ^a	5.33E-05 ± 2.03E-05	3.98E-05 ± 7.12E-06	4.82E-05 ± 1.68E-05	3.48E-05* ± 8.20E-06	4.67E-05 ± 1.38E-05	5.58E-05 ± 1.11E-05

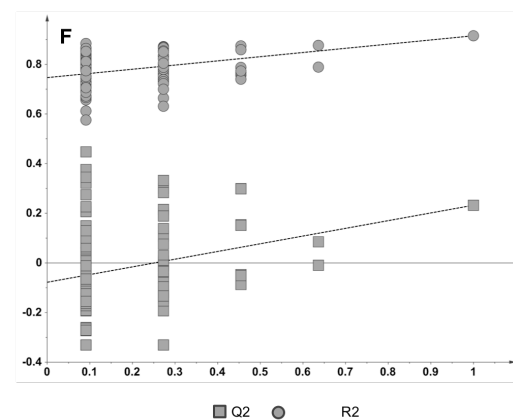
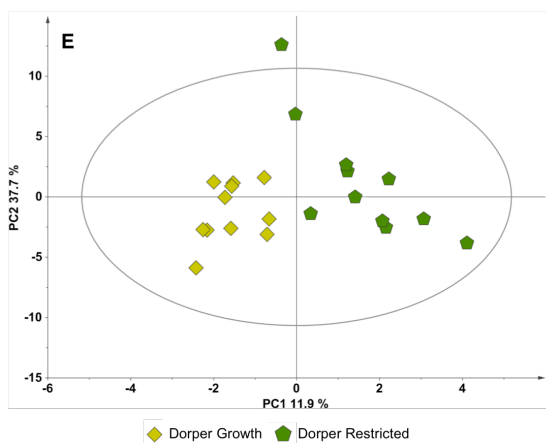
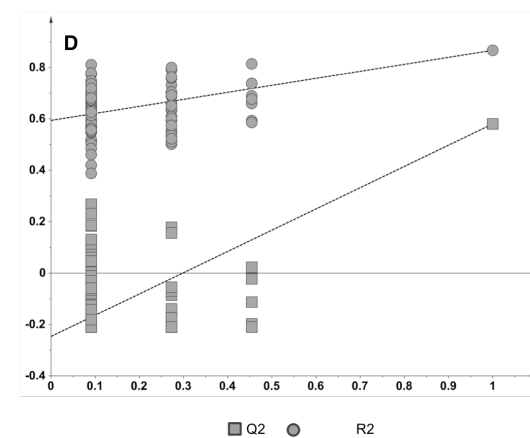
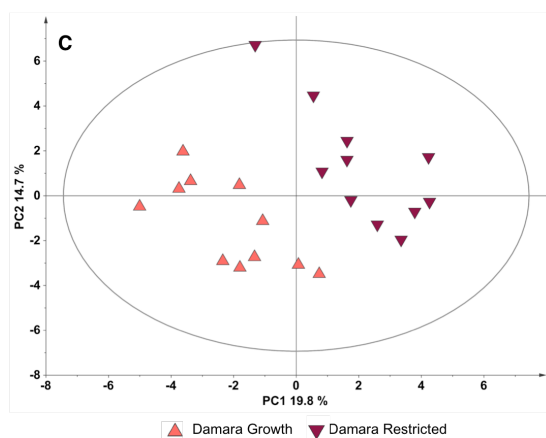
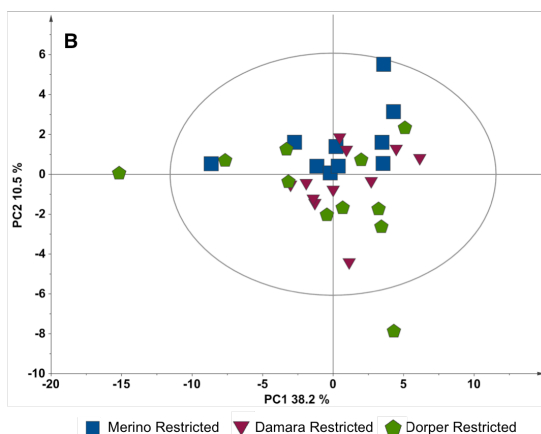
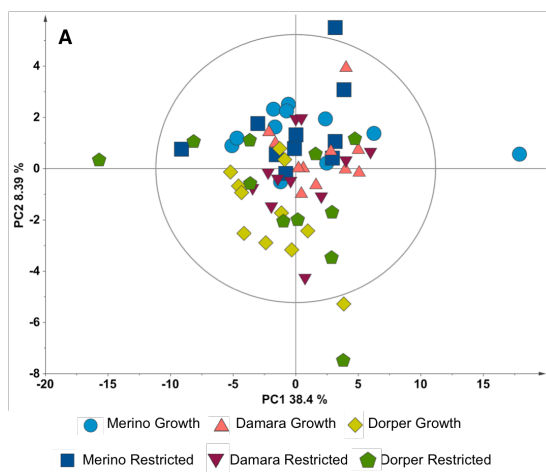
Fumarate	4.53E-05 ± 1.85E-05	4.30E-05 ± 1.57E-05	4.34E-05 ± 2.42E-05	3.79E-05 ± 3.05E-05	6.19E-05 ± 1.79E-05	5.75E-05 ± 2.20E-05
GTP	3.53E-05 ± 1.77E-05	3.29E-05 ± 1.05E-05	3.37E-05 ± 1.16E-05	3.57E-05 ± 1.87E-05	3.58E-05 ± 9.98E-06	3.49E-05 ± 9.17E-06
Glucose	1.52E-03 ± 5.24E-04	1.45E-03 ± 4.53E-04	1.22E-03 ± 2.77E-04	1.37E-03 ± 7.61E-04	1.78E-03 ± 3.04E-04	1.67E-03 ± 6.04E-04
Glucose-1-phosphate ^a	6.29E-04 ± 2.65E-04	5.22E-04 ± 2.01E-04	4.97E-04 ± 1.45E-04	4.61E-04 ± 3.15E-04	7.00E-04 ± 2.14E-04	4.80E-04* ± 2.71E-04
Glucose-6-phosphate ^a	2.90E-03 ± 1.21E-03	1.57E-03** ± 7.25E-04	2.69E-03 ± 6.31E-04	1.89E-03 ± 1.20E-03	3.13E-03 ± 1.05E-03	2.24E-03 ± 9.70E-04
Glutamate	4.10E-04 ± 2.26E-04	5.19E-04 ± 1.73E-04	4.80E-04 ± 1.69E-04	6.11E-04 ± 3.28E-04	3.83E-04 ± 9.56E-05	4.83E-04 ± 2.42E-04
Glutamine	4.01E-03 ± 1.85E-03	3.86E-03 ± 2.51E-03	3.53E-03 ± 1.51E-03	4.60E-03 ± 3.03E-03	5.11E-03 ± 1.83E-03	5.17E-03 ± 1.51E-03
Glutathione ^a	1.72E-04 ± 1.03E-04	2.79E-04* ± 1.26E-04	2.20E-04 ± 6.60E-05	2.52E-04 ± 1.29E-04	2.30E-04 ± 9.64E-05	3.13E-04 ± 1.59E-04
Glycerophosphocholine ^a	3.13E-04 ± 1.13E-04	7.71E-05** ± 2.43E-05	3.81E-04 ± 1.89E-04	5.67E-04 ± 2.97E-04	3.98E-04 ± 1.65E-04	5.45E-04 ± 1.82E-04
Glycine ^a	3.48E-03 ± 1.73E-03	1.90E-03* ± 5.35E-04	2.84E-03 ± 7.71E-04	1.87E-03* ± 1.31E-03	2.24E-03 ± 4.39E-04	1.76E-03 ± 7.04E-04
Histidine	7.53E-06 ± 2.96E-06	6.02E-06 ± 2.40E-06	3.06E-06 ± 1.03E-06	3.77E-06 ± 2.33E-06	5.40E-06 ± 2.01E-06	4.20E-06 ± 1.96E-06
Homocysteine	1.47E-03 ± 8.12E-04	1.36E-03 ± 5.47E-04	1.22E-03 ± 4.30E-04	1.41E-03 ± 6.20E-04	1.29E-03 ± 3.50E-04	1.41E-03 ± 4.36E-04
IMP ^a	2.46E-03 ± 1.05E-03	1.89E-03 ± 7.44E-04	1.46E-03 ± 6.49E-04	1.70E-03 ± 8.59E-04	2.45E-03 ± 7.02E-04	1.72E-03* ± 7.69E-04
Inosine	1.78E-04 ± 8.73E-05	1.27E-04 ± 5.61E-05	9.23E-05 ± 4.47E-05	9.90E-05 ± 7.30E-05	1.59E-04 ± 6.48E-05	1.39E-04 ± 8.86E-05
Isoleucine ^a	9.29E-05 ± 3.88E-05	7.76E-05 ± 2.02E-05	8.58E-05 ± 2.36E-05	8.18E-05 ± 4.78E-05	1.09E-04 ± 1.35E-05	9.05E-05* ± 2.47E-05
Lactate	4.50E-02 ± 1.83E-02	4.12E-02 ± 1.11E-02	3.87E-02 ± 3.96E-03	4.12E-02 ± 1.55E-02	4.92E-02 ± 7.93E-03	4.43E-02 ± 1.02E-02
Leucine ^a	1.40E-04 ± 5.55E-05	1.12E-04 ± 2.55E-05	1.27E-04 ± 3.67E-05	1.17E-04 ± 5.31E-05	1.64E-04 ± 2.20E-05	1.34E-04* ± 3.11E-05
Malonate	5.48E-03 ± 2.14E-03	5.72E-03 ± 2.33E-03	2.93E-03 ± 1.14E-03	3.75E-03 ± 1.71E-03	5.11E-03 ± 1.68E-03	5.85E-03 ± 1.90E-03
Methylmalonate	3.02E-04 ± 1.67E-04	2.70E-04 ± 6.69E-05	2.57E-04 ± 2.62E-05	2.68E-04 ± 9.76E-05	3.16E-04 ± 4.53E-05	2.85E-04 ± 6.91E-05
Myo-Inositol	1.21E-03 ± 7.57E-04	1.69E-03 ± 6.59E-04	1.25E-03 ± 7.87E-04	1.58E-03 ± 9.12E-04	1.15E-03 ± 7.34E-04	1.29E-03 ± 8.69E-04
NAD ⁺ /NADP ⁺	1.16E-04 ± 6.46E-05	8.57E-05 ± 2.90E-05	1.27E-04 ± 5.27E-05	1.00E-04 ± 5.21E-05	1.19E-04 ± 3.17E-05	1.14E-04 ± 5.84E-05
Nicotinurate	1.44E-04 ± 5.72E-05	1.26E-04 ± 2.96E-05	1.00E-04 ± 3.06E-05	1.16E-04 ± 4.09E-05	1.42E-04 ± 3.98E-05	1.19E-04 ± 3.22E-05
Phenylalanine ^a	4.90E-05 ± 1.62E-05	3.86E-05 ± 1.07E-05	4.39E-05 ± 9.47E-06	3.46E-05 ± 1.34E-05	5.34E-05 ± 8.22E-06	3.97E-05** ± 8.62E-06

Proline	2.42E-04 ± 1.08E-04	2.73E-04 ± 9.73E-05	2.62E-04 ± 8.01E-05	3.32E-04 ± 1.78E-04	2.52E-04 ± 5.58E-05	3.24E-04 ± 1.68E-04
Pyruvate	2.04E-04 ± 1.13E-04	2.10E-04 ± 1.43E-04	1.65E-04 ± 5.75E-05	1.97E-04 ± 9.99E-05	2.70E-04 ± 7.93E-05	2.37E-04 ± 1.08E-04
Riboflavin	1.42E-05 ± 4.41E-06	1.28E-05 ± 5.83E-06	1.71E-05 ± 5.87E-06	1.93E-05 ± 9.60E-06	1.59E-05 ± 3.76E-06	1.60E-05 ± 6.08E-06
Succinate	1.01E-03 ± 3.87E-04	1.01E-03 ± 3.72E-04	6.67E-04 ± 3.30E-04	6.43E-04 ± 3.80E-04	1.19E-03 ± 4.14E-04	1.08E-03 ± 3.87E-04
Taurine ^a	5.35E-03 ± 3.30E-03	2.96E-03* ± 1.13E-03	6.32E-03 ± 2.82E-03	3.88E-03* ± 2.35E-03	1.44E-02 ± 4.40E-03	8.77E-03** ± 2.02E-03
Tyrosine ^a	5.50E-05 ± 2.65E-05	3.52E-05* ± 1.34E-05	5.04E-05 ± 1.68E-05	3.83E-05 ± 1.55E-05	6.26E-05 ± 1.60E-05	4.59E-05* ± 1.17E-05
UDP-glucose	3.22E-05 ± 2.13E-05	3.14E-05 ± 1.11E-05	4.02E-05 ± 1.20E-05	3.47E-05 ± 2.26E-05	3.66E-05 ± 1.10E-05	3.59E-05 ± 2.15E-05
Uridine	1.94E-05 ± 6.83E-06	1.54E-05 ± 8.41E-06	1.53E-05 ± 5.28E-06	1.15E-05 ± 6.99E-06	1.89E-05 ± 4.49E-06	1.48E-05 ± 5.91E-06
Valine ^a	2.13E-04 ± 9.95E-05	1.48E-04 ± 3.51E-05	1.95E-04 ± 5.68E-05	1.86E-04 ± 1.07E-04	2.25E-04 ± 3.97E-05	1.87E-04* ± 3.74E-05
Xanthine	5.83E-05 ± 5.44E-05	2.63E-05 ± 2.33E-05	1.85E-05 ± 6.97E-06	2.59E-05 ± 3.26E-05	1.56E-05 ± 5.50E-06	3.66E-05 ± 5.63E-05

Key: (a) metabolites with significant differences between growth and restricted groups in at least one breed; (*) $p < 0.05$ when compared with growth groups of the same breed; (**) $p < 0.01$ when compared with growth groups of the same breed.

Supplementary Figure S4.1

Multivariate analysis of gastrocnemius muscle (aqueous fraction) metabolites concentration. (A) PCA scores plot for all the experimental groups (NC = 5, PC1 = 38.4 %, PC2 = 8.39 %); (B) PCA scores plot for the three restricted groups (NC = 4, PC1 = 38.2 %, PC2 = 10.5 %); (C) PLS scores plot of *Damara* growth and restricted groups (NC = 2, $R^2=0.346$, $Q^2=0.580$, $p_{CV-ANOVA} = 0.0063$); (D) Permutation test of the PLS obtained from the *Damara* growth and restricted groups (100 permutations; Intercepts: $R^2=(0.0, 0.588)$; $Q^2=(0.0, -0.244)$); (E) PLS scores plot of *Dorper* growth and restricted groups (NC = 3, $R^2=0.602$, $Q^2=0.232$, $p_{CV-ANOVA} = 1$); (F) Permutation test of the PLS obtained from the *Dorper* growth and restricted groups (100 permutations; Intercepts: $R^2=(0.0, 0.743)$; $Q^2=(0.0, -0.0635)$).



Supplementary Table S4.2

PCA loadings values of principal component 1 (p[1]) and principal component 2 (p[2]) of muscle from *Merino* growth and restricted groups (see scores in Figure 4.3B). Highlighted values are the more representatives for the group clustering.

metabolite	p[1]	p[2]
choline/phosphocholine	0,0934444	-0,00916684
lactate	0,187801	-0,0257329
riboflavin	0,108283	-0,0279286
homocysteine	0,13865	-0,0281437
GTP	0,161932	-0,0284619
adenine	0,153523	-0,0293082
adenosine	0,0910446	-0,0313269
isoleucine	0,17947	-0,0335238
methylmalonate	0,187916	-0,0373872
histidine	0,145532	-0,0400434
glucose	0,16066	-0,0483827
leucine	0,176612	-0,0493796
glucose-1-phosphate	0,164189	-0,0517669
beta-alanine	0,140296	-0,0566436
phenylalanine	0,176972	-0,0739192
IMP	0,162193	-0,0880994
tyrosine	0,163835	-0,0916036
inosine	0,13526	-0,0961437
creatine	0,0984559	-0,112648
valine	0,171368	-0,116997
taurine	0,160134	-0,160068
anserine	0,0872286	-0,170245
NAD+/NADP+	0,14135	-0,187977
formate	0,128394	-0,189047
glycine	0,143278	-0,19155
xanthine	-0,015431	-0,20734
glucose-6-phosphate	0,123557	-0,305008
creatine/creatine phosphate	0,18423	0,00272127
nicotinurate	0,174855	0,0223421
aspartate	0,167016	0,0305508
UDP-glucose	0,160648	0,0329277
acetate	0,158195	0,0358494
alanine	0,170251	0,036475
uridine	0,133736	0,0596227
pyruvate	0,106272	0,0650886
carnitine	0,169322	0,0885765
fumarate	0,170695	0,0933712
ADP/AMP/ATP	0,135175	0,106753
proline	0,15098	0,110592
succinate	0,156269	0,119165
betaine	0,048716	0,122313
malonate	0,150058	0,131919
3-methylhistidine	0,0158208	0,136634
carnosine	0,00697562	0,164795
glutamate	0,157805	0,179145
glutamine	0,0984383	0,184886
glutathione	0,0993436	0,244599
acetyl-L-carnitine	0,080261	0,261162
myo-inositol	0,109134	0,261164
citrate	0,118022	0,31952
glycerophosphocholine	0,020397	0,338363

Supplementary Table S4.3

Identified metabolites in liver of *Merino*, *Dorper* and *Damara* sheep breeds (growth and restricted groups). Average concentration (mmol/ g tissue) and standard deviation are shown for each experimental group.

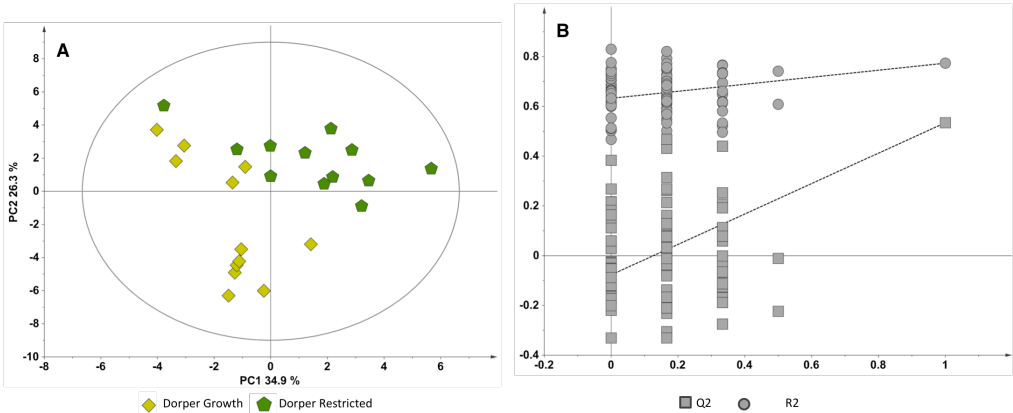
	Merino		Dorper		Damara	
	growth	restricted	growth	restricted	growth	restricted
3-Hydroxybutyrate ^a	3.30E-04 ± 1.00E-04	4.67E-04* ± 1.40E-04	2.35E-04 ± 9.97E-05	3.18E-04 ± 1.40E-04	2.91E-04 ± 7.93E-05	2.67E-04 ± 1.38E-04
Methylhistidine	1.30E-04 ± 1.39E-04	1.05E-04 ± 1.01E-04	1.85E-04 ± 1.88E-04	1.01E-04 ± 1.03E-04	5.79E-05 ± 6.53E-05	6.70E-05 ± 7.89E-05
ADP AMP ATP	8.00E-04 ± 3.30E-04	9.49E-04 ± 3.27E-04	7.20E-04 ± 3.51E-04	7.56E-04 ± 2.87E-04	9.19E-04 ± 1.74E-04	6.90E-04 ± 3.80E-04
Acetate ^a	5.89E-04 ± 1.26E-04	8.29E-04** ± 1.72E-04	4.94E-04 ± 2.32E-04	5.77E-04 ± 2.47E-04	6.08E-04 ± 1.31E-04	4.32E-04* ± 2.31E-04
Adenine ^a	3.30E-04 ± 1.46E-04	3.03E-04 ± 1.63E-04	1.64E-04 ± 9.73E-05	1.87E-04 ± 1.02E-04	3.03E-04 ± 4.54E-05	1.63E-04** ± 1.19E-04
Adenosylhomocysteine	4.41E-05 ± 2.52E-05	5.45E-05 ± 2.36E-05	2.67E-05 ± 2.02E-05	4.36E-05 ± 2.24E-05	4.75E-05 ± 1.94E-05	4.17E-05 ± 2.20E-05
Alanine ^a	1.97E-03 ± 4.86E-04	1.38E-03** ± 4.18E-04	1.78E-03 ± 8.76E-04	1.37E-03 ± 4.57E-04	2.38E-03 ± 2.77E-04	1.53E-03** ± 7.19E-04
Ascorbate ^a	1.24E-03 ± 3.70E-04	7.67E-04** ± 3.53E-04	9.23E-04 ± 4.95E-04	7.21E-04 ± 3.23E-04	1.15E-03 ± 2.52E-04	8.22E-04* ± 4.12E-04
Aspartate	8.23E-04 ± 1.34E-04	9.19E-04 ± 3.37E-04	7.53E-04 ± 3.23E-04	9.52E-04 ± 3.94E-04	1.17E-03 ± 2.95E-04	9.09E-04 ± 4.43E-04
Benzoate	2.37E-05 ± 1.65E-05	2.30E-05 ± 1.53E-05	1.35E-05 ± 1.59E-05	1.21E-05 ± 9.44E-06	2.67E-05 ± 1.46E-05	1.93E-05 ± 3.36E-06
Betaine	4.22E-04 ± 8.76E-05	4.73E-04 ± 8.99E-05	3.07E-04 ± 2.03E-04	4.22E-04 ± 1.97E-04	3.55E-04 ± 1.54E-04	3.18E-04 ± 1.84E-04
Carnitine ^a	5.68E-04 ± 3.16E-04	5.77E-04 ± 3.47E-04	6.40E-04 ± 3.45E-04	9.74E-04* ± 4.27E-04	7.03E-04 ± 2.61E-04	7.08E-04 ± 3.79E-04
Carnosine	3.24E-05 ± 2.48E-05	4.80E-05 ± 1.72E-05	4.11E-05 ± 2.34E-05	3.57E-05 ± 3.56E-05	5.20E-05 ± 6.45E-05	2.74E-05 ± 1.92E-05
Choline/ Acetylcholine/ Phosphocholine ^a	5.97E-04 ± 3.04E-04	8.77E-04 ± 6.55E-04	4.55E-04 ± 3.61E-04	5.39E-04 ± 2.70E-04	6.21E-04 ± 2.58E-04	3.43E-04* ± 2.27E-04
Citrate ^a	3.64E-04 ± 1.22E-04	2.57E-04 ± 1.34E-04	3.23E-04 ± 1.36E-04	2.49E-04 ± 1.08E-04	4.87E-04 ± 1.21E-04	2.92E-04** ± 1.83E-04
Creatine/ Creatine phosphate ^a	1.66E-03 ± 5.20E-04	2.60E-03** ± 7.24E-04	1.47E-03 ± 6.77E-04	1.76E-03 ± 8.34E-04	1.59E-03 ± 3.28E-04	1.68E-03 ± 1.04E-03
Creatinine	2.62E-04 ± 2.11E-04	2.59E-04 ± 5.52E-05	2.33E-04 ± 1.61E-04	2.33E-04 ± 1.35E-04	2.24E-04 ± 6.99E-05	2.12E-04 ± 1.23E-04
Formate ^a	1.21E-04 ± 7.59E-05	1.34E-04 ± 5.55E-05	3.89E-05 ± 3.40E-05	4.99E-05 ± 5.04E-05	1.21E-04 ± 5.60E-05	4.80E-05** ± 2.52E-05
Fumarate	1.07E-04 ± 4.85E-05	1.26E-04 ± 6.44E-05	8.43E-05 ± 3.99E-05	1.20E-04 ± 5.73E-05	1.06E-04 ± 3.06E-05	1.11E-04 ± 7.94E-05
Glucose	8.68E-02 ± 1.81E-02	7.76E-02 ± 1.53E-02	8.03E-02 ± 3.70E-02	7.12E-02 ± 2.63E-02	9.10E-02 ± 1.51E-02	7.71E-02 ± 3.11E-02
Glutamate	3.98E-03 ± 9.35E-04	3.38E-03 ± 1.20E-03	4.16E-03 ± 1.94E-03	3.90E-03 ± 1.73E-03	3.87E-03 ± 8.97E-04	3.74E-03 ± 1.77E-03

Glutathione ^a	2.53E-03 ± 7.38E-04	1.20E-03** ± 4.27E-04	2.24E-03 ± 1.36E-03	1.51E-03 ± 6.41E-04	2.54E-03 ± 4.75E-04	1.93E-03 ± 1.26E-03
Glycerophosphocholine	1.15E-02 ± 1.48E-03	1.15E-02 ± 2.13E-03	7.36E-03 ± 2.92E-03	8.79E-03 ± 3.12E-03	9.40E-03 ± 1.00E-03	8.40E-03 ± 2.85E-03
Glycine	2.98E-03 ± 2.28E-03	5.04E-03 ± 3.07E-03	2.95E-03 ± 2.62E-03	3.52E-03 ± 2.60E-03	2.51E-03 ± 2.20E-03	3.23E-03 ± 2.13E-03
Histamine	7.22E-05 ± 4.98E-05	7.17E-05 ± 5.11E-05	8.66E-05 ± 1.28E-04	5.94E-05 ± 6.40E-05	7.67E-05 1.05E-04	4.48E-05 ± 6.62E-05
Histidine	1.76E-05 ± 1.77E-05	3.90E-05 ± 6.67E-05	4.60E-05 ± 6.54E-05	6.57E-05 ± 8.10E-05	4.27E-05 ± 4.42E-05	2.96E-05 ± 2.65E-05
Inosine	1.38E-03 ± 3.39E-04	1.15E-03 ± 5.63E-04	1.04E-03 ± 5.13E-04	1.16E-03 ± 4.13E-04	1.15E-03 ± 1.61E-04	1.00E-03 ± 4.30E-04
Isoleucine	2.16E-04 ± 3.05E-05	2.35E-04 ± 4.58E-05	1.97E-04 ± 9.67E-05	2.05E-04 ± 7.34E-05	2.17E-04 ± 3.94E-05	2.01E-04 ± 9.25E-05
Lactate ^a	2.33E-02 ± 4.36E-03	1.97E-02* ± 2.99E-03	1.65E-02 ± 7.78E-03	1.50E-02 ± 5.30E-03	2.10E-02 ± 2.70E-03	1.48E-02** ± 5.34E-03
Leucine	5.15E-04 ± 1.33E-04	5.56E-04 ± 1.40E-04	3.98E-04 ± 1.92E-04	4.63E-04 ± 1.47E-04	5.15E-04 ± 7.97E-05	4.53E-04 ± 1.86E-04
Lysine	1.84E-04 ± 8.02E-05	2.01E-04 ± 8.48E-05	2.27E-04 ± 9.18E-05	2.33E-04 ± 1.03E-04	2.77E-04 ± 7.91E-05	2.30E-04 ± 1.05E-04
myo-Inositol	1.79E-03 ± 8.03E-04	2.39E-03 ± 6.31E-04	1.50E-03 ± 6.14E-04	1.57E-03 ± 5.63E-04	1.61E-03 ± 6.65E-04	1.34E-03 ± 6.57E-04
NAD ⁺ /NADP ⁺ /NADPH	3.39E-04 ± 7.57E-05	3.27E-04 ± 8.69E-05	2.22E-04 ± 1.10E-04	2.43E-04 ± 9.46E-05	3.08E-04 ± 3.12E-05	2.58E-04 ± 1.28E-04
Nicotinurate	4.05E-04 ± 1.20E-04	3.85E-04 ± 2.04E-04	3.64E-04 ± 2.19E-04	3.14E-04 ± 1.42E-04	3.37E-04 ± 6.96E-05	2.70E-04 ± 1.15E-04
Phenylalanine	9.77E-05 ± 2.70E-05	9.26E-05 ± 1.97E-05	7.96E-05 ± 3.84E-05	8.75E-05 ± 3.51E-05	8.81E-05 ± 2.65E-05	8.19E-05 ± 3.41E-05
Pyruvate	1.01E-04 ± 5.32E-05	1.22E-04 ± 6.77E-05	8.44E-05 ± 7.04E-05	1.09E-04 ± 7.39E-05	1.02E-04 ± 7.93E-05	6.27E-05 ± 5.67E-05
Sarcosine ^a	2.50E-05 ± 1.06E-05	6.57E-05* ± 4.68E-05	1.91E-05 ± 9.89E-06	4.99E-05** ± 3.33E-05	2.54E-05 ± 9.92E-06	2.84E-05 ± 1.94E-05
Succinate ^a	1.94E-03 ± 7.03E-04	1.10E-03* ± 7.40E-04	1.40E-03 ± 9.42E-04	1.01E-03 ± 5.22E-04	1.58E-03 ± 6.35E-04	1.35E-03 ± 6.22E-04
Taurine	3.14E-03 ± 1.43E-03	2.90E-04 ± 5.48E-04	2.11E-03 ± 6.67E-04	2.05E-03 ± 1.33E-03	2.25E-03 ± 1.36E-03	2.46E-03 ± 1.13E-03
Tyrosine	1.06E-04 ± 2.14E-05	1.13E-04 ± 1.97E-05	9.53E-05 ± 4.17E-05	1.07E-04 ± 3.94E-05	1.18E-04 ± 1.97E-05	9.84E-05 ± 4.55E-05
UDP-glucose/ UDP-glucuronate ^a	2.37E-04 ± 6.03E-05	1.69E-04* ± 6.06E-05	1.79E-04 ± 8.35E-05	1.99E-04 ± 8.58E-05	1.82E-04 ± 4.43E-05	1.35E-04* ± 5.16E-05
UMP	2.16E-04 ± 6.39E-05	1.99E-04 ± 9.61E-05	1.73E-04 ± 1.20E-04	2.06E-04 ± 1.06E-04	2.37E-04 ± 6.70E-05	1.91E-04 ± 1.19E-04
Uridine	2.00E-04 ± 8.53E-05	1.89E-04 ± 7.52E-05	1.73E-04 ± 9.29E-05	2.16E-04 ± 9.06E-05	2.52E-04 ± 4.53E-05	1.79E-04 ± 1.47E-04
Valine	2.85E-04 ± 4.72E-05	2.68E-04 ± 5.60E-05	2.89E-04 ± 1.49E-04	3.24E-04 ± 1.10E-04	2.84E-04 ± 7.18E-05	2.92E-04 ± 1.45E-04
Xanthine	9.03E-05 ± 1.28E-04	5.88E-05 ± 6.43E-05	1.07E-04 ± 2.08E-04	1.94E-04 ± 2.51E-04	1.51E-04 ± 1.73E-04	1.10E-04 ± 2.10E-04
Xanthosine	5.53E-05 ± 1.95E-05	6.54E-05 ± 1.56E-05	3.13E-05 ± 1.46E-05	4.47E-05 ± 1.85E-05	3.99E-05 ± 9.11E-06	3.73E-05 ± 1.57E-05

Key: (a) metabolites with significant differences between growth and restricted groups in at least one breed; (*) $p < 0.05$ when compared with growth groups of the same breed; (**) $p < 0.01$ when compared with growth groups of the same breed.

Supplementary Figure S4.2

Multivariate analysis of gastrocnemius liver (aqueous fraction) metabolites concentration. (A) PLS scores plot of *Dorper* growth and restricted groups (NC = 3, $R^2 = 0.697$, $Q^2 = 0.535$, $p_{CV-ANOVA} = 0.022$); (B) Permutation test of the PLS obtained from the *Dorper* growth and restricted groups (100 permutations; Intercepts: $R^2 = (0.0, 0.661)$; $Q^2 = (0.0, -0.0199)$).



Supplementary Table S4.4

PCA loadings values of principal component 1 (p[1]) and principal component 2 (p[2]) of liver from all experimental groups (see scores in Figure 4.5A). Highlighted values are the more representatives for the group clustering.

metabolite	p[1]	p[2]
lysine	0,127616	-0,00836563
tyrosine	0,217153	-0,0101978
leucine	0,213215	-0,0182982
fumarate	0,16816	-0,0194202
isoleucine	0,218857	-0,0270153
valine	0,193653	-0,0282933
myo-inositol	0,126898	-0,0344818
aspartate	0,175622	-0,0405529
betaine	0,182334	-0,0429109
carnitine	0,0864109	-0,0503634
glycerophosphocholine	0,199769	-0,0594424
3-hydroxybutyrate	0,17255	-0,0637507
phenylalanine	0,195149	-0,0656461
creatine/creatine phosphate	0,150869	-0,0656852
sarcosine	0,0993925	-0,117427
NAD+/NADP+/NADPH	0,15416	-0,123242
acetate	0,181122	-0,124148
histamine	0,0689646	-0,162512
taurine	0,0889907	-0,165381
xanthosine	0,13541	-0,182926
carnosine	0,0751018	-0,186254
formate	0,11196	-0,254873
pyruvate	0,10112	-0,257031
adenosylhomocysteine	0,0905311	-0,265901
benzoate	0,0699395	-0,303525
glycine	0,0717862	-0,344065
choline/acetylcholine/phosphocholine	0,126998	0,00421237
histidine	0,0423974	0,0065039
ADP/AMP/ATP	0,160462	0,0274156
methylhistidine	0,0542767	0,0421994
glutamate	0,185099	0,0518193
adenine	0,13848	0,0601666
UDP-glucose/UDP-glucuronate	0,153788	0,0665798
uridine	0,154128	0,0822441
UMP	0,142359	0,0970162
creatine	0,109538	0,105426
ascorbate	0,136358	0,118421
xanthine	0,0749608	0,134583
nicotinurate	0,163784	0,140839
lactate	0,196794	0,147192
inosine	0,177502	0,154198
citrate	0,133787	0,158362
glucose	0,18902	0,164965
alanine	0,171358	0,208244
glutathione	0,143614	0,219198
succinate	0,102549	0,337444

Supplementary Table S4.5

PCA loadings values of principal component 1 (p[1]) and principal component 2 (p[2]) of liver from the three restricted groups (see scores in Figure 4.5B). Highlighted values are the more representatives for the group clustering.

metabolite	p[1]	p[2]
isoleucine	0,208316	-0,000775746
phenylalanine	0,188558	-0,00329607
nicotinurate	0,143321	-0,00548825
lactate	0,190172	-0,00862534
creatine/creatine phosphate	0,170103	-0,0123637
UDP-glucose/UDP-glucuronate	0,14332	-0,0217326
tyrosine	0,206108	-0,0388418
lysine	0,13228	-0,0388863
creatine	0,138003	-0,0522449
histidine	0,0606518	-0,0612253
aspartate	0,17443	-0,071565
glutamate	0,17281	-0,0871191
ascorbate	0,15292	-0,0905637
citrate	0,158871	-0,103218
fumarate	0,161439	-0,107697
valine	0,185765	-0,115555
UMP	0,148757	-0,121438
carnitine	0,116598	-0,131282
glutathione	0,142582	-0,154829
glucose	0,18041	-0,167011
uridine	0,145556	-0,174022
inosine	0,146805	-0,18577
alanine	0,168406	-0,205698
xanthine	0,0942055	-0,238436
succinate	0,0769011	-0,279004
ADP/AMP/ATP	0,180266	0,00599214
leucine	0,198651	0,0248222
NAD ⁺ /NADP ⁺ /NADPH	0,158748	0,028534
glycerophosphocholine	0,18985	0,060954
betaine	0,191049	0,0765777
adenine	0,121361	0,0798769
myo-inositol	0,136747	0,0845144
methylhistidine	0,046819	0,0851829
sarcosine	0,120288	0,12591
choline/acetylcholine/phosphocholine	0,117606	0,126589
3-hydroxybutyrate	0,18354	0,131976
histamine	0,0530351	0,145616
acetate	0,172174	0,152669
carnosine	0,12277	0,184341
xanthosine	0,15219	0,207178
taurine	0,0854165	0,228516
pyruvate	0,0859815	0,234773
benzoate	0,0707271	0,255549
glycine	0,0886229	0,263101
adenosylhomocysteine	0,0886352	0,274663
formate	0,121623	0,296229

Supplementary Table S4.6

PCA loadings values of principal component 1 (p[1]) and principal component 2 (p[2]) of liver from Merino control and restricted groups (see scores in Figure 4.5C). Highlighted values are the more representatives for the group clustering.

metabolite	p[1]	p[2]
xanthine	-0,130986	-0,0215009
phenylalanine	0,227905	-0,0283117
UMP	0,127628	-0,0521813
uridine	0,0977984	-0,0590317
adenine	0,0628757	-0,0592536
methylhistidine	0,141309	-0,0787875
glutamate	0,153095	-0,097344
nicotinurate	0,251954	-0,0973478
ascorbate	-0,0910994	-0,0998218
UDP-glucose/UDP-glucuronate	0,038097	-0,100275
citrate	-0,00360237	-0,144997
creatine	0,125737	-0,155774
glutathione	-0,00830276	-0,225313
inosine	0,051935	-0,228885
alanine	0,119323	-0,257885
glucose	0,0754439	-0,273554
lactate	0,166109	-0,287578
succinate	0,075375	-0,320218
fumarate	0,168831	0,00107662
lysine	-0,107692	0,00221375
glycerophosphocholine	0,193145	0,0142183
valine	0,233315	0,0247463
NAD ⁺ /NADP ⁺ /NADPH	-0,065732	0,0299473
leucine	0,256368	0,0316425
tyrosine	0,284198	0,039383
choline/acetylcholine/phosphocholine	0,195512	0,0450083
taurine	0,0405945	0,0504528
betaine	0,288543	0,0543983
histidine	0,0189755	0,0555584
aspartate	0,200786	0,0787987
isoleucine	0,308628	0,0831473
3-hydroxybutyrate	0,224642	0,0971955
ADP/AMP/ATP	0,0458819	0,115534
xanthosine	0,118581	0,119332
pyruvate	0,0877144	0,125332
carnitine	-0,164151	0,131304
sarcosine	0,179643	0,135232
histamine	0,0375752	0,138799
creatine/creatine phosphate	-0,0620337	0,16219
carnosine	0,0048895	0,169906
adenosylhomocysteine	0,0725635	0,171096
myo-inositol	0,0228209	0,180632
acetate	0,140317	0,201524
benzoate	-0,00294643	0,211504
formate	0,0243885	0,227965
glycine	0,077779	0,280858

Supplementary Table S4.7

PCA loadings values of principal component 1 (p[1]) and principal component 2 (p[2]) of liver from *Damara* growth and restricted groups (see scores in Figure 4.5D). Highlighted values are the more representatives for the group clustering.

metabolite	p[1]	p[2]
glycine	0,0244041	0,330112
methylhistidine	0,0395212	0,142924
adenosylhomocysteine	0,049878	0,225319
carnosine	0,0571769	0,139833
histamine	0,0649823	0,352769
pyruvate	0,069471	0,29267
taurine	0,0777508	0,187248
histidine	0,0854182	-0,0733942
sarcosine	0,089201	0,0101228
benzoate	0,0895776	0,308548
succinate	0,114866	-0,277505
choline/acetylcholine/phosphocholine	0,117652	-0,172304
myo-inositol	0,117797	-0,073074
formate	0,120196	0,0919685
betaine	0,121406	-0,0799325
creatine	0,125324	-0,0839733
nicotinurate	0,126424	-0,147877
xanthine	0,127075	-0,07777
carnitine	0,138737	-0,0065273
lysine	0,146226	0,151516
xanthosine	0,146916	0,0853477
UDP-glucose/UDP-glucuronate	0,147759	-0,160506
UMP	0,154387	-0,0736292
3-hydroxybutyrate	0,15474	-0,105817
adenine	0,161653	-0,167643
phenylalanine	0,162582	0,154978
fumarate	0,170056	0,0778041
aspartate	0,170086	0,10321
uridine	0,171311	-0,0805676
ADP/AMP/ATP	0,172519	-0,202394
acetate	0,174108	-0,072834
creatine/creatine phosphate	0,174122	-0,0683038
citrate	0,174285	-0,0359612
glutathione	0,175249	-0,0786109
valine	0,175946	0,123836
glutamate	0,177562	0,15096
lactate	0,178027	-0,11048
alanine	0,178856	-0,0699502
ascorbate	0,179167	-0,071083
NAD+/NADP+/NADPH	0,182011	0,106688
glycerophosphocholine	0,186098	0,0281151
inosine	0,190939	-0,00196768
leucine	0,191633	0,00893044
tyrosine	0,192082	0,0391107
isoleucine	0,192687	0,0726498
glucose	0,198681	0,082695

Supplementary Table S4.8

Summary of previous results of the same animals.

Live weight variation (%) between day 0 and day 42: (-) decrease, (+) increase, significant differences ($p < 0.05$) were found for all experimental groups in a comparison of day 42 and day 0, and between the two groups of the same breed at day 42.

Carcass weights (kg) and yields (%): Data shown as mean $\pm$ standard deviation. Different letters (a,b) in each row for each nutritional treatment indicate significant differences ($p < 0.05$) between breeds. Different letters (A, B, C) indicate significant differences ($p < 0.05$) between nutritional treatments for the same breed: A – *Merino*, B – *Damara*, C – *Dorper*.

Liver weight (g): Data shown as mean $\pm$ standard deviation. No significant differences ($p < 0.05$) were observed between breeds. Star (*) indicates significant differences between nutritional treatments ($p < 0.05$).

Meat characteristics (colour, pH, eye muscle/longissimus dorsi muscle dimensions): Data shown as mean $\pm$ standard deviation. Different letters (a, b, c) in each row indicate significant differences ($p < 0.05$) between breeds, for each parameter (colour and dimensions). Star (*) indicates significant differences between nutritional treatments ($p < 0.05$). Colour (redness, yellowness and lightness), higher values mean redder/yellower/lighter.

Plasma concentrations of leptin and insulin ($\mu\text{U/ml}$): Data shown as means $\pm$ standard error. No interaction effect of breed and feed restriction was observed. Different letters (a, b) in each row indicate significant differences. Asterisks: (*) indicates significant differences ($p < 0.05$), (**) indicates significant differences ($p < 0.001$). Leptin: significant differences ($p < 0.001$) were observed between feed restriction and growth groups between day 0 and day 42 in all groups.

Adapted from: Almeida et al. (2013) Trop Anim Health Prod. 45:1305-1311 (Reference No. 7) and Almeida et al. (2016) PlosOne. DOI:10.1371 (Reference No. 13). Shown here for contextual reasons only.

		Merino		Dorper		Damara	
		Growth	Restricted	Growth	Restricted	Growth	Restricted
Live weight variation (%)		+10	-14	+13	-12	+7	-12
Carcass hot weight (kg)		14.81±2.18 a	11.11±1.10 aA	20.17±1.55 b	14.90±1.93 bC	20.27±3.61 b	16.70±3.06 bB
Carcass yield (%)		39.11±1.55 a	38.02±2.62 a	44.53±2.66 b	43.30±2.66 b	43.89±3.17 b	44.64±3.17 b
Liver weight (g)		612.85±76.54 *	375.40±40.98	696.34±94.00 *	419.33±95.85	637.69±101.80 *	470.45±61.57
Colour	redness	14.39±0.78 a*	16.28±1.03 a	14.50±1.54 a	15.31±1.05 ab	13.88±0.93 a	14.11±1.08 b
	yellowness	6.15±0.88 a	6.06±1.02 a	5.97±0.90 a	5.84±0.75 a	5.64±1.37 a	5.57±0.60 a
	lightness	42.35±2.47 a	41.26±1.12 a	40.00±1.41 b	40.78±1.89 a	38.07±1.76 b	37.82±1.74 b
pH		5.79±0.06 a*	5.92±0.61 a	5.89±0.06 b	5.96±0.08 a	5.78±0.05 a	5.83±0.04 b
Eye muscle	area (cm ²)	1065.33±211.90 a	800.87±159.89 a	1569.31±286.45 b*	1177.07±186.60 b	1485.96±241.86 b	1373.33±246.73 b
	diameter (mm)	23.08±3.37 a*	18.71±3.25 a	30.18±4.24 b*	24.75±2.67 b	29.91±3.97 b	27.83±2.89 b
	width (mm)	57.33±4.23 a	53.27±3.28 a	64.64±3.63 b*	59.17±3.81 b	62.00±3.74 ab	61.33±5.07 b
Leptin	Day 0	0.501±0.015	0.541±0.018 a**	0.547±0.032	0.533±0.023 a**	0.773±0.084	0.810±0.088 b**
(µU/ml)	Day 42	0.883±0.057	0.638±0.049 a*	0.951±0.055	0.627±0.048 a*	1.084±0.126	0.873±0.104 b*
Insulin	Day 0	3.671±0.469	2.703±0.271 a**	3.463±0.580	2.815±0.278 a**	7.632±1.259	5.828±0.981 b**
(µU/ml)	Day 42	4.223±0.470	2.322±0.232 a**	4.318±0.348	2.067±0.11 a**	6.938±0.821	3.958±0.468 b**