EFECTO DE LA LÍNEA GENÉTICA HOLSTEIN SOBRE LA CAPACIDAD DE ADAPTACIÓN METABÓLICA DE VACAS MULTÍPARAS EN PASTOREO

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A mi abuela Zenona y mi tía Gonni.

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TABLA DE CONTENIDO

RESUMEN

El objetivo de este trabajo fue estudiar el efecto de la línea genética Hosltein (origen americano, **NAH**, vs. neozelandés, **NZH**) sobre las adaptaciones metabólicas de vacas en pastoreo. El ***trabajo 1*** evaluó el efecto de la línea genética (NAH, n = 20; NZH, n = 20) y dos estrategias de alimentación basadas en un consumo de pasturas en torno al 40 % de la dieta anual (**P30**) o pastoreo maximizado (**PMAX**), sobre el metabolismo energético, proteico y redox. Los animals NZH tuvieron una menor (P = 0,02) producción de leche corregida por grasa y proteína que estuvo asociada a una mayor (P = 0,01) concentración plasmática de glucosa, una tendencia a mayor insulina (P < 0,07) y menores valores del índice de desbalance fisiológico, especialmente en PMAX. Los animales NZH tuvieron una mayor (P < 0,05) concentración plasmática de urea y menor concentración de 3-metilhistidina lo que sugiere una menor movilización de músculo esquelético. También parecen haber tenido un mayor daño oxidativo (concentraciones más altas de TBARS previo al parto y una tendencia a mayores niveles de carbonilos proteicos durante la lactancia, P < 0,10) y mayor respuesta antioxidante [actividad de superóxido dismutasa, **SOD**, que tendió (P = 0,06) a ser alta durante más tiempo, mayor aumento (P < 0,05) de la concentración plasmática de α-tocoferol durante la lactancia]. Finalmente, el análisis de componente principal sugiere que los animales NZH tuvieron un menor *desplazamiento metabólico* entre las distintas etapas de la lactancia. El ***trabajo 2*** tuvo como objetivo determinar el efecto de la línea genética (NAH, n = 8; NZH, n = 8) sobre el perfil metabolómico en plasma a lo largo la lactancia (21 vs. 180 días en leche) en el sistema PMAX. Los resultados indican que el metabolismo de los aminoácidos (**AA**) estuvo afectado (FDR < 0,05) tanto por la etapa de lactancia (19/46 vías afectadas) como por la línea genética durante la lactancia temprana. Esto último dado por menores concentraciones plasmáticas de valina, leucina e isoleucina en los animales NZH vs. NAH (raw-P < 0,05).

Palabras clave: genotipo, Holstein americano, Holstein neozelandés, sistemas pastoriles

EFFECT OF HOLSTEIN GENETIC STRAIN ON METABOLIC ADAPTIVE CAPACITY OF MULTIPAROUS GRAZING COWS

SUMMARY

The aim of this work was to study the effect of Holstein genetic strain (American origin, **NAH**, vs. New Zealand, **NZH**) on metabolic adapations of grazing cows. In the ***article 1***, we assessed the effect of genetic strain (NAH, n = 20; NZH, n = 20) and two feeding strategies (**P30**, fixed pasture around 40 % of annual dry matter intake; **PMAX**, maximized grazing) on energy, protein and redox metabolism. The NZH cows had a lower (P = 0.02) fat and protein corrected milk yield which was associated with greater (P = 0.01) plasma concentrations of glucose, and a tendency (P < 0.07) of greater insulin as well as lower values of physiological imbalance index, especially in PMAX. The NZH cows had greater (P = 0.01) urea and tended to have lower (P = 0.09) 3-methylhistidine concentrations than NAH cows suggesting lower muscle mobilization in the former ones. The NZH cows seemed to have increased oxidative damage (greater thiobarbituric acid-reactive substances prepartum and a tendency for greater protein carbonyls concentrations during lactation, P < 0.10) but also a greater antioxidant response [superoxide dismutase (**SOD**) activity tended (P = 0.06) to remain high for longer during lactation; greater recovery of plasma α-tocopherol during mid lactation]. Finally, principal component analysis indicated that NZH cows had a lower *metabolic displascemnt* across lactation stages. The aim of the ***article 2*** was to determine the effect of genetic strain (NAH, n = 8; NZH, n = 8) and lactation stage (21 vs. 180 days in milk) on metabolomic plasma profile in PMAX. Our results seemed to indicate that several amino acid (**AA**) metabolic pathways were affected (FDR < 0.05) by lactation stage (19/46 pathways), and genetic strain (branched-chain AA metabolism) at early lactation. This latter finding was associated with lower concentrations of valine, leucine and isoleucine in NZH vs. NAH cows (raw-P < 0.05).

Keywords: genotype, North American Holstein, New Zealand Holstein, pasture-based dairy systems

1. INTRODUCCIÓN

En las últimas décadas ha habido un fuerte incremento en la producción lechera uruguaya pasando de 1.505 millones de litros en 2003 a 2.110 litros en 2017 (INALE, 2018). La productividad por vaca es el factor que en forma individual explica la mayor proporción del crecimiento (> 60 %), mientras que el aumento de carga explica un 25-30 % del incremento de la productividad del sector (DIEA, 2018). Parte de este aumento se ha dado por el incremento en el uso de forraje conservado, concentrados y ración mezclada (Fariña y Chilibroste, 2019). Esto ha determinado un proceso de intensificación de la lechería nacional. Sin embargo, la competitividad de los sistemas productivos uruguayos recae en gran medida sobre la producción y utilización de pasturas lo que impacta directamente sobre los costos de producción. En efecto, se ha observado que los sistemas que logran mejores eficiencias productivas son los que logran un alto consumo de pasturas por unidad de superficie (Fariña y Chilibroste, 2019).

A su vez, el aumento de la productividad individual también ha sido consecuencia de la mejora genética del rodeo nacional a lo largo de los años (Ignacio Aguilar, comunicación personal, 25 de mayo de 2019). Sin embargo, si bien los sistemas pastoriles presentan ventajas frente a otros sistemas más intensivos, no son suficientes, por sí solos, para que las vacas de alto mérito genético expresen su máximo potencial productivo (Kolver y Muller, 1998; Horn et al., 2014). Actualmente, el 89% del rodeo lechero uruguayo está constituido por animales de la raza Holstein, de los cuales el 93 % son de origen norteamericano (**NAH**) y un 7% de origen neozelandés (**NZH**) (INALE, 2014). Mientras que las vacas NAH han sido seleccionadas en sistemas estabulados con un fuerte énfasis en la producción de leche individual, las vacas NZH han sido seleccionadas en condiciones de pastoreo considerando no solo la producción de leche individual sino también su composición y la eficiencia reproductiva (Harris y Kolver, 2001). Se ha demostrado que la mayor productividad y menor eficiencia reproductiva de las vacas NAH vs. NZH están asociadas a diferencias en la respuesta endócrino-metabólica (Lucy et al., 2009; Chagas et al., 2009) y la habilidad para el pastoreo (Sheahan et al., 2011). En este sentido, Ingvartsen et al.

(2003) señalaron que la selección de animales de alto mérito genético ha determinado un incremento de la expresión de las vías metabólicas de adaptación homeorhética y del desbalance fisiológico durante la lactancia temprana.

1.1. METABOLISMO ENERGÉTICO, LIPÍDICO Y PROTEICO

El periodo de transición se caracteriza por ser un momento donde el consumo de materia seca es insuficiente para sostener los requerimientos que impone el inicio de la lactancia. Así, los animales sufren un estado catabólico que determina la movilización de reservas corporales para hacer frente al balance energético negativo (BEN) y cubrir los requerimientos de nutrientes (Bauman y Currie, 1980; Meikle et al., 2013). Estas alteraciones homeorréticas están directamente relacionados con el alto metabolismo de la glándula mamaria y a los procesos de adaptación metabólica dados por al eje somatotrópico, el desarrollo de la resistencia a la insulina (Bauman, 2000) y cambios en el perfil metabolómico del plasma sanguíneo (Kénez et al., 2016). Así, los genotipos (razas, líneas genéticas) de mayor productividad suelen tener menores concentraciones de insulina durante la lactancia temprana, particularmente en ambientes nutricionalmente pobres (Pires et al., 2015; O'Hara et al., 2016). Se ha demostrado que las vacas NAH presentan un mayor desacople del eje somatotrópico (Lucy et al., 2009) y una mayor resistencia a la insulina (Chagas et al., 2009) que las NZH cuando son comparadas en condiciones de pastoreo.

Como consecuencia de este estado catabólico durante el BEN, la movilización de tejido adiposo determina un aumento en la concentración de lípidos asociados a un incremento en la actividad de ß-oxidación, que en situaciones de una muy alta movilización reservas corporales conduce a un estado de estrés oxidativo (Pedernera et al., 2010) e inflamación sistémica (Abuelo et al., 2016). Así, los animales con menor mérito genético para la producción de leche suelen tener menores concentraciones plasmática de ácidos grasos no esterificados (**NEFA**) y β-hydroxibutirato (**BHB**) durante el período de transición y la lactancia temprana, como consecuencia de una menor movilización de reservas (Petrera et al., 2015; Pires et al., 2015; Ntallaris et al., 2017). En este sentido, se ha propuesto que el desbalance fisiológico que sufren los animales durante este estado de catabolismo se puede determinar a través del índice

de desbalance fisiológico (Moyes et al., 2013), y se ha reportado que diferencias en este índice están asociadas a diferentes perfiles lipidómicos durante la lactancia temprana (Imashly et al., 2015).

A nivel del metabolismo proteico, la distribución y el uso eficiente de los aminoácidos (**AA**) requeridos durante el inicio de la lactancia implican una coordinación entre el hígado y el músculo esquelético a los efectos de dar respuesta a la alta prioridad de la absorción de AA por parte de la glándula mamaria (Hanigan et al., 2004; Arriola Apelo et al., 2014). Varios trabajos han demostrado un aumento de la concentración plasmática de 3-metilhistidina durante la transición como consecuencia de la movilización del músculo esquelético (Phillips et al., 2003; van der Drift et al., 2012). Overton y Waldron (2004) sugirieron que el rol de los AA durante la transición estaría asociado sobre todo a dar soporte a la gluconeogénesis, para sintetizar la glucosa requerida para la síntesis de leche, siendo los AA responsables de hasta un 30 % de la síntesis de glucosa (Reynolds et al. 2003). Mientras algunos AA (e.g.: metionina, cisteína) pueden aumentar durante la transición, otros como los AA ramificados (**AAR**, valina, leucina e isoleucina) disminuyen en torno al parto probablemente constituyendo un cambio estructural durante este período (Phillips et al., 2003; Luo et al., 2019). Sin embargo, aún no se conocen los mecanismos específicos subyacentes al metabolismo de los AA que participan en el metabolismo energético durante la transición de la vaca lechera, ni el rol específico de los AAR. Se ha reportado mayor concentración plasmática y muscular de AAR en vacas con sobrepeso comparado con vacas con condición corporal (**CC**) moderada, posiblemente indicando una fase de pre-diabetes en estos animales (Ghaffari et al., 2019; Sadri et al., 2020). En humanos (Nie et al., 2018) y en cerdos (Polakof et al., 2016) se ha asociado la resistencia a la insulina con altas concentraciones plasmáticas de los AAR. Si bien se sabe que los AAR tienen un fuerte rol en la señalización de procesos celulares, aún no se conocen los mecanismos ni las relaciones causales específicas entre el desarrollo de la resistencia a la insulina y estos AA específicos en vacas lecheras sometidas a ambientes altamente desafiantes.

1.2. METABOLISMO REDOX Y ESTRÉS OXIDATIVO

Durante el período de transición la vaca lechera puede sufrir un estado de estrés oxidativo, que supone un desbalance entre oxidantes y antioxidantes y puede estar dado tanto por un aumento de los oxidantes, una disminución de la capacidad antioxidante o una combinación de ambas (Sordillo y Aitken, 2009). En todo caso supone el daño de macromoléculas y una disrupción del funcionamiento metabólico y biológico normal (Trevisan et al., 2001). Los agentes oxidantes cumplen funciones naturalmente enmarcadas en los procesos fisiológicos del metabolismo y en el caso de las sustancias reactivas al oxígeno (**ROS**), se trata de radicales libres que son producidos mayoritariamente a nivel mitocondrial en la cadena respiratoria (Sordillo y Aitken, 2009).

Si bien los ROS atacan una gran cantidad de familias de biomoléculas, los lípidos tienen una marcada susceptibilidad (Miller et al., 1993). Dentro de éstos, los ácidos grasos poliinsaturdos son un blanco frecuente de los ROS cuya acción conduce a la producción de oxilípidos que a su vez tienen un rol central, junto con algunos lípidos complejos, en la articulación entre el estrés oxidativo y la respuesta inflamatoria aumentando la inflamación, o estimulando su resolución (Mavangira y Sordillo, 2017). En este sentido, se ha reportado que el sobrepeso al parto en vacas lecheras estuvo asociado a una mayor concentración sanguínea de ceramidas (Rico et al., 2015) lo que podría traducirse en una mayor producción de ROS a nivel mitocondrial (Mavangira y Sordillo, 2017). También las proteínas y el ADN sufren daños por acción de los oxidantes y la medición de carbonilos ha sido usada como marcadores de oxidación proteica. Los carbonilos pueden ser primarios o secundarios. La formación de los primeros ocurre mediante el ataque de ROS hacia los AA prolina, arginina, lisina o treonina en presencia de metales de transición (Fe^{2+}, Cu^{+}, etc.) y consisten en aldehídos o cetonas reactivas. La síntesis de carbonilos secundarios ocurre cuando carbohidratos o lípidos con grupos carbonilos activos actúan sobre los AA de las proteínas (Celi y Gabai, 2015).

Por otra parte, los agentes antioxidantes son aquellas sustancias que detienen, previenen o remueven el daño oxidativo (Halliwell, 2007). La actividad antioxidante a nivel del organismo supone la actuación conjunta y coordinada de los antioxidantes

enzimáticos y no enzimáticos (Mavangira y Sordillo, 2017). Dentro de las enzimas antioxidantes endógenas se destacan la superóxido dismutasa (**SOD**) y glutatión peroxidasas (**GPx**), entre otras, mientras que los antioxidantes provenientes de la dieta son antioxidantes no enzimáticos como los minerales traza (selenio, cobre, zinc), los polifenoles, las vitaminas A, C, D, E y el β-caroteno (Sordillo y Aitken, 2009).

Durante el inicio de la lactancia, como consecuencia del incremento de la demanda metabólica, aumentan los requerimientos de oxígeno y asociado a ello hay un aumento de la síntesis de ROS (Sordillo y Aitken, 2009). En este sentido, Colakoglu et al. (2017) reportaron un aumento de la concentración de sustancias reactivas al ácido tiobarbitúrico (**TBARS**, indicador de oxidación lipídica) hasta el parto y posteriormente una disminución hasta el día 21 post parto. Observaron también mayor concentración de TBARS en animales con buena CC al parto y mayor producción de leche. Asimismo, se ha reportado que la actividad de la GPx disminuyó durante el preparto incrementando durante el inicio de la lactancia (Colakoglu et al., 2017) posiblemente como consecuencia de un estímulo del estrés oxidativo sobre la expresión de los genes codificantes de enzimas antioxidantes (Gessner et al., 2013). Bernabucci et al. (2005) observaron menores actividades de SOD y de GPx en los animales que llegaron al parto con alta CC y tuvieron alta producción de leche durante la lactancia temprana. En este contexto, la literatura no es consistente en encontrar una asociación positiva entre la producción de leche y el estrés oxidativo (Wullepit et al., 2009; Pedernera et al., 2010), sin embargo, hay acuerdo en que el estrés oxidativo estaría asociado fundamentalmente a fuertes movilizaciones de las reservas corporales más que al nivel de producción de leche en sí mismo (Pedernera et al., 2010). De hecho, la producción de ROS es un mecanismo conservado en los procesos adaptativos celulares frente al estrés ambiental. En efecto, Marco-Ramell et al. (2012) reportaron que animales adaptados a la montaña presentaron mayor daño oxidativo y mayor actividad antioxidante que animales de razas no seleccionadas para esas condiciones, sugiriendo que la mayor capacidad de adaptación a un ambiente, esté asociada a una mayor sensibilidad redox. En este sentido, es esperable que el metabolismo redox participe de manera diferencial en las respuestas adaptativas del metabolismo de vacas lecheras con distintos grados de adaptación a las condiciones de pastoreo.

Dados los desafíos nutricionales que implica la lactancia en sí misma y que los sistemas pastoriles suponen desafíos nutricionales y ambientales extra, se ha señalado la necesidad de adecuar el "tipo" de vaca al sistema de producción ya que es posible que el genotipo juegue un rol central en la capacidad de adaptación de los animales en ambientes restrictivos (Delaby et al., 2009; Horn et al., 2014). Que un animal sea capaz de adaptarse al sistema de producción incluye aspectos comportamentales y fisiológicos, dentro de los cuales la capacidad de adaptación metabólica tiene un rol central en la vaca lechera (Blanc et al., 2004). Los trabajos reportados hasta el momento han demostrado que las diferencias entre animales NAH y NZH están dadas, al menos en parte, por diferencias en la movilización de tejido adiposo y en el grado de resistencia a la insulina y desacople del eje somatotrópico. Sin embargo, no se conoce aún el rol que juega el metabolismo aminoacídico y redox, no solo en términos de movilización de reservas o estrés oxidativo, respectivamente, sino en términos de señalización y modulación de la respuesta adaptativa de los animales. En función de los antecedentes, el objetivo central de la tesis es estudiar el efecto del genotipo lechero (NAH vs. NZH) en combinación con la estrategia de alimentación (PMAX: maximización del pastoreo, P30: pastoreo restringido) sobre la capacidad de adaptación metabólica durante la lactancia temprana. En este sentido, la hipótesis que nos plantemos es que las vacas NZH presentaban una mejor capacidad de adaptación en sistemas pastoriles, reflejado en un mejor estatus metabólico, menor movilización de reservas proteicas y menor daño oxidativo en comparación con las vacas NAH.

La estructura central de la tesis consiste en dos artículos científicos, el primer artículo, titulado ***Energy, protein and redox metabolism underlying adaptive responses in New Zealand vs. North American Holstein cows on pasture-based systems*** constituye el capítulo 2 de esta tesis y fue enviado a la revista *Livestock Science* (en revisión). El mismo estudia el efecto del genotipo en interacción con la estrategia de alimentación sobre el metabolismo energético, proteico, estrés oxidativo y estudia de forma integrada la trayectoria metabólica a lo largo de la lactancia a través de un abordaje semi cuantiativo. El segundo artículo se titula **"*Metabolomic analysis reveals branched-chain amino acids play a major role in metabolic adaptation differences between Holstein genetic strains managed under grazing"*** y constituye

el capítulo 3. En este artículo, próximo a ser enviado a *Scientific Reports*, se estudian las vías metabólicas subyacentes a los procesos de adaptación mediante un abordaje metabolómico dirigido con énfasis en el metabolismo primario (AA, aminas biogénicas, ácidos grasos, carbohidratos). En el capítulo IV se presenta una discusión general y conclusiones globales de las preguntas de investigación planteadas en esta tesis.

2. ENERGY, PROTEIN AND REDOX METABOLISM UNDERLYING ADAPTIVE RESPONSES IN NEW ZEALAND VS. NORTH AMERICAN HOLSTEIN COWS ON PASTURE-BASED SYSTEMS

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2.1. RESUMEN

Las diferencias en el metabolismo energético y partición de los nutrientes explican, al menos en parte, las diferencias en productividad y eficiencia reproductiva entre animales Holstein de origen norteamericano (NAH) y neozelandés (NZH). Sin embargo, el metabolismo proteico y redox también podría participar en las respuestas diferenciales entre líneas genéticas. El objetivo de este trabajo fue estudiar el efecto de la interacción entre la línea genética y dos estrategias de alimentación de base pastoril (1/3 de la dieta promedio anual cosechada como pastoreo directo, P30; máximo pastoreo PMAX) sobre el metabolismo proteico y redox y su relación con la respuesta productiva y el metabolismo energético. Se seleccionar vas multíparas don partos de otoño correspondientes a cada uno de los 4 tratamientos: NAH en P30 (NAH-P30, n = 10); NZH en P30 (NZH-P30, n = 10), NAH en PMAX (NAH-PMAX, n = 10) y NZH en PMAX (NZH-PMAX, n = 10). Las vacas NZH produjeron menos (P = 0.02) leche corregida por grasa y proteína, pero tuvieron mayores (P = 0.01) concentraciones plasmáticas de glucosa y tendieron (P < 0.07) a tener más insulina y menores valores del índice de desbalance fisiológico, especialmente cuando eran comparadas en PMAX. Las concentraciones plasmáticas de acidos grasos no esterificados (NEFA) y β-hidroxybutirate (BHB) fueron similares entre líneas genéticas. En cambio, las concentraciones plasmáticas de 3-metilhistidina (3-MH), que indican movilización muscular, fueron mayores en los animales NAH que en NZH previo al parto. A su vez, las vacas NZH tuvieron mayores (P < 0.03) concentraciones de urea en sangre y leche, posiblemente debido a diferencias en la digestibilidad de la materia seca consumida. El daño oxidativo de lípidos y proteínas, indicado a través de las sustancias reactivas al ácido tiobarbitúrico (TBARS) y carbonilos, fue mayor (P = 0.01) previo al parto o tendieron a ser mayor durante la lactancia (P = 0.10) respectivamente, para los animales NZH vs. NAH. Sin embargo, los animales NZH también presentaron una mayor respuesta antioxidante dado que tendieron a mantener una alta actividad de la enzima superóxido dismutasa (SOD) durante más tiempo (P = 0.06) y presentaron una mayor (P < 0.01) concentración plasmática de α -tocoferol más rápidamente durante la lactancia media. La aparentemente menor movilización de músculo esquelético, así

como la mayor sensibilidad redox observada en los animales NZH vs. NAH podría indicar mecanismos subyacentes a la capacidad de adaptación metabólica de las vacas en pastoreo dados por distintas respuestas en términos del metabolismo proteico y redox. En efecto, el análisis cualitativo de las trayectorias metabólicas indicó que los animales NZH tuvieron una trayectoria metabólica con mayor resistencia al cambio, lo que podría ser un indicio de mayor resiliencia.

2.2. SUMMARY

Differences in energy metabolism and nutrients partition are associated to the decreased milk yield and increased reproductive efficiency between New Zealand (**NZH**) and North American (**NAH**) Holstein cows. However, protein and redox metabolism should be involved in the differential adaptive capacity of these genetic strains as muscle mobilization and oxidative stress are major components of metabolic changes during the transition period in dairy cows. Thus, the aim of this work was to study the effect of the interaction between these genetic strains and two pasture-based feeding strategies [1/3 of DMI from grazed pastures (**P30**); maximized grazing (**PMAX**)] on energy, protein and redox metabolism. Forty out of 120 fall-calving multiparous were randomly selected from a larger experiment leading to 4 experimental groups: NAH in P30 (**NAH-P30**, n = 10), NZH in P30 (**NZH-P30**, n = 10), NAH in PMAX (**NAH-PMAX**, n = 10) and NZH in PMAX (**NZH-PMAX**, n = 10). The lower ($P = 0.02$) fat and protein corrected milk (**FPCM**) yield for NZH than NAH cows was related to greater ($P = 0.01$) plasma glucose, and a trend ($P < 0.07$) for greater plasma insulin and lower physiological imbalance index (**PI**) values for the former than the latter cows, especially when cows were fed the strategy that maximized grazing activity. Plasma non-esterified fatty acids (**NEFA**) and β-hidroxybutirate (**BHB**) did not differ between NAH and NZH cows across lactation, suggesting similar tissue mobilization. In contrast, plasma 3-methyl histidine (3-MH) which indicates muscle mobilization, was lower for NZH than NAH cows previous to calving. The NZH cows had greater ($P < 0.03$) plasma and milk urea, probably due to digestive differences compared to NAH cows. Oxidative damage of both, lipids and proteins,

indicated by plasma concentrations of thiobarbituric acid reactive substances (**TBARS**) and protein carbonyls, were greater (P = 0.01) previous to calving or tended to be greater (P = 0.10) during lactation for NZH than NAH cows, respectively. However, NZH seemed to have also a greater antioxidant response as they tended to have greater (P = 0.06) superoxide dismutase (**SOD**) activity and had a greater (P < 0.01) α-tocopherol concentration during mid lactation. Lower muscle mobilization and greater redox reactivity observed for NZH than NAH cows would be indicative of underlying metabolic adaptive responses of dairy cows to challenges imposed by pasture-based systems. Indeed, the qualitative analysis of metabolic trajectories according to multivariate analysis showed greater changes for NAH than NZH cows, particularly in the feeding strategy that maximized grazing activity.

2.3. INTRODUCTION

The major physiological challenges for dairy cows rely upon the need to cope with increased nutrient requirements (Bell and Bauman, 1997) and face the physiological imbalance during negative energy balance (NEB) at early lactation stage (Moyes et al., 2013). The magnitude and duration of this catabolic state, in which dairy cows experience peripheral tissues mobilization, are strongly affected by feeding strategy and cow body reserves at parturition (Meikle et al., 2013).

During the last decades grazing dairy systems – especially those which maximize grazing – are becoming more relevant as they reduce feed cost and offer benefits for animal welfare (Dillon et al., 2006) and environmental care (Basset-Mens et al., 2009). However, it has been indicated that grazing dairy cows are not able to express their potential dry matter intake (DMI) (Kolver and Müller, 1998) and consequently they usually show a deeper and longer NEB of early lactation when compared to their counterparts on total mixed ration (TMR) based-systems (Meikle et al., 2013; Astessiano et al., 2015). Thus, the metabolic challenge of the onset of lactation could be exacerbated in grazing systems.

It has been proposed that cow genotype should be selected according to the production system in order to achieve the milk yield goal for the production system

without compromising animal reproduction, welfare and metabolic status (Delaby et al., 2009). Genetic selection in U.S.A. Holstein (NAH) has led to achieve high individual milk yield and decreased reproductive efficiency (Lucy, 2001). In contrast, New Zealand selection strategy has been focused on milk solid yield and reproductive efficiency of Holstein (NZH) in grazing systems (Lucy et al., 2009). Indeed, when compared to NZH cows, NAH cows presented increased energy and nutrient partitioning towards milk production, sustained by greater loss of body reserves during NEB, a stronger uncoupling of somatotropic axis and an increased insulin resistance (Chagas et al., 2009; Lucy et al., 2009). Body reserve mobilization determine that dairy cows have increased risk of developing production diseases and reduced production or reproduction as a consequence of a physiological imbalance state (PI) (Moyes et al., 2013) which affected lipidomic profile (Imashly et al., 2015) as well as oxidative stress (Pedernera et al., 2010) during early lactation. Oxidative stress could interfere on insulin transduction signal and glucose transport (Turk et al., 2008), being a possible link between insulin resistance development and lipid mobilization (Xu et al., 2014). Indeed, dairy cows fed mixed diets produced more milk and had increased lipid mobilization and oxidative stress than grazing cows (Pedernera et al., 2010). In addition, body reserves mobilization also includes skeletal muscle catabolism as the labile protein can be used to supply amino acids as precursors for liver gluconeogenesis during the peripartum. Protein catabolism has been associated not only with reduced availability of amino acids but also reduced concentrations of insulin (Bell et al., 2000). Plasma concentrations of 3-methylhistidine (3-MH), which reflects skeletal muscle mobilization, increased during the peripartum of dairy cows and were negative correlated to plasma β-hydroxybutyrate (BHB) (van der Drift et al., 2012). Thus, it was suggested that protein mobilization could reduce ketone body synthesis by increasing glucogenic precursors and acting as an anaplerotic role during NEB (van der Drift et al., 2012).

We hypothesize that NAH have a less adaptive capacity to face grazing systems constraints compared to NZH cows which lead to more acute changes in energy and protein metabolism (for instance a deeper catabolic state), leading to a greater oxidative stress in order to sustain greater milk yield in NAH. Thus, the aim of this

work was to assess the effect of Holstein genetic strain (NAH vs. NZH) combined with different feeding strategies varying in the proportion of directly grazed pastures on cow's oxidative metabolism and its association with fat and protein mobilization across lactation.

2.4. MATERIALES Y MÉTODOS

The experiment was carried out as a part of a larger grazing experiment designed to achieve a home-grown forage harvest of 10 ton of dry matter (DM)/ha per year, and an expected milk solids production of 1000 kg/ha per year. The experiment was located at the Experimental Research Station "La Estanzuela" (34°20'S, 57°40' W) belonging to the National Institute of Agronomic Research of Uruguay (INIA). All procedures were approved by the Ethic Committee on Animal Experimentation of INIA (form #INIA_2017.2).

2.4.1. Experimental design and treatments

Forty out of 120 fall-calving multiparous dairy cows of two Holstein genetic strains (NAH, n = 20; NZH, n = 20) fed two different strategies were randomly selected from the larger grazing experiment. Within each genetic strain, cows were paired by parity (3.1 ± 0.9 lactations) and calving date (5/8/2018 ± 18 days), and randomly allocated to one of the two feeding strategies in a 2×2 factorial arrangement. Feeding strategies were designed to achieve the predicted DMI according to (NRC, 2001). The P30 feeding strategy was conducted to reach, in average during lactation, one third of estimated DMI from directly grazed pastures, while in PMAX the objective was to maximize pasture DM intake according to weekly pasture growth rate of the grazing platform, leading to 4 experimental groups: NAH in P30 (NAH-P30, n = 10), NZH in P30 (NZH-P30, n = 10), NAH in PMAX (NAH-PMAX, n = 10) and NZH in PMAX (NZH-PMAX, n = 10).

Previous to calving, NAH cows had a live weight (LW) of 626 ± 15 kg and a body condition score (BCS) of 3.19 ± 0.04, while NZH had a LW of 537 ± 15 kg and a BCS of 3.29 ± 0.04. The 305-days expected milk yield was 7500 and 5500 kg, and

the economic and productive breeding index (Mejoramiento y Control Lechero Uruguayo; https://www.mu.org.uy) was 108 ± 3 and 125 ± 2 (mean ± SE) on average for NAH and NZH cows, respectively. The NAH cows had an expected progeny difference of +34 ± 41 kg, +0.08 ± 0.03 % and +0.02 ± 0.02 % for milk yield, milk fat content, and milk protein content, respectively, compared to the national herd. The NZH cows had an expected progeny difference of -141 ± 40 kg, +0.13 ± 0.03 %, and +0.14 ± 0.01 %, for milk yield, milk fat, and milk protein content, respectively, compared to the national herd.

Cows stayed outdoors all year-round. At calving, cows were assigned to the feeding strategies and grazed pastures of *Dactylis glomerata*, *Medicago sativa* and *Festuca arundinacea*, on a daily rotational grazing system. Daily paddocks with free access to water were allocated to cows in each genetic strain×feeding strategy after milking. Herbage mass (above 5 cm to ground level) was estimated weekly by measuring sward height using a pasture meter (C-Dax pasture meter; C-Dax Ltd, Turitea, New Zealand) and a calibrated equation.

In P30, pasture grazing comprised 1/3 of the diet and was supplemented with a total mixed ration (2/3 of cow DM intake) with a forage to concentrate average ratio of 50:50 ± 9 % (mean ± SD; DM basis; Table 2.1), offered once a day after morning milking. In P30, herbage allowance was adjusted weekly to offer one third of estimated DM intake, and excess growth rate was mechanically harvested as haylage to maintain the same average pasture stock as in PMAX, and to avoid a reduction in pasture quality. In PMAX, herbage allowance (kg DM/cow/day; Table 2.1) was adjusted weekly, based on pasture growth rate in the grazing platform and on stocking rate, to keep a pasture stock of 665 ± 312 (kg DM/ha). In addition, a commercial concentrate (Table 2.1) was offered twice a day at the milking parlor and when pasture allowance was considered restrictive to maintain target DM intake, or in rainy conditions, forage reserves (a mix of corn silage and pasture haylage; 73:27 ± 6 % on a DM basis, respectively) were offered in a feeding parlor immediately before the afternoon milking. Grazing management was conducted as previously described (Talmón et al., 2020) and diets had a similar nutritional composition (Table 2.1).

Table 2.1. Pasture characteristics and average diet composition (mean ± SD) according to feeding strategy

	P30				PMAX			
	-45 DIM	21 DIM	100 DIM	180 DIM	-45 DIM	21 DIM	100 DIM	180 DIM
a. Pasture characteristics								
Herbage mass (kg DM/ha)[1]	-	2964 ± 50	2695 ± 73	1620 ± 611	-	2790 ± 467	2549 ± 64	1563 ± 293
Herbage height (cm)	-	23.6 ± 1.9	19.1 ± 0.9	15.2 ± 4.4	-	24.5 ± 2.3	19.3 ± 0.5	14.9 ± 2.1
Herbage allowance (kg DM/cow·day)	-	11.9 ± 0.3	13.9 ± 3.1	9.7 ± 1.3	-	17.5 ± 1.6	21.8 ± 0.8	21.2 ± 1.0
b. Diet composition								
Pasture (%)[2]	-	35.6 ± 2.5	35.6 ± 2.9	32.5 ± 0.4	-	40.9 ± 0.3	37.8 ± 3.8	55.3 ± 1.6
Concentrate (%)[3]	-	-	-	-	-	36.3 ± 1.8	38.3 ± 0.6	44.7 ± 1.6
Forage reserves (%)[3]	-	-	-	-	-	22.8 ± 3.0	23.9 ± 3.2	0.0 ± 0.0
Total mixed ration (%)[3,4]	100.0 ± 0.0	64.4 ± 2.5	64.4 ± 2.9	67.5 ± 0.8	100.0 ± 0.0	-	-	-
DM (%)[5]	52.9 ± 0.0	47.5 ± 1.8	49.0 ± 0.2	59.2 ± 0.2	52.9 ± 0.0	50.6 ± 3.5	48.7 ± 9.3	52.1 ± 1.0
CP (%)[5,6]	14.0 ± 0.0	17.8 ± 1.1	19.4 ± 0.1	17.2 ± 0.1	14.0 ± 0.0	15.2 ± 2.6	17.1 ± 3.4	20.8 ± 0.1
NDF (%)[5,6]	49.2 ± 0.0	39.2 ± 0.8	38.2 ± 0.3	39.5 ± 0.1	49.2 ± 0.0	37.4 ± 0.5	38.1 ± 1.2	39.3 ± 0.4
ADF (%)[5,6]	33.2 ± 0.0	25.8 ± 0.5	24.4 ± 1.4	25.0 ± 0.1	33.2 ± 0.0	22.5 ± 1.4	24.1 ± 2.1	19.9 ± 0.3
ENL (MJ/kg MS)[5,6,7]	5.8 ± 0.0	6.2 ± 0.1	6.6 ± 0.1	6.4 ± 0.1	5.8 ± 0.0	6.2 ± 0.1	6.8 ± 0.4	7.1 ± 0.1

DIM: days in milk; SD: standard desviation given by variability within feeding strategy during the 5 days of milk recording in each experimental period.

1: Estimated by C-Dax pasture meter (C-Dax Ltd, Turitea, New Zealand); 2: Estimated by the difference between offered and refused herbage mass at ground level; 3: Estimated by the difference between the feed offered and refused; 4: Diet composition at -45 DIM correspond to the offered diet in a unique herd; 5: Estimated as average composition based on diet composition and nutritional quality of each aliment; 6: Expressed on DM basis; 7: According to NRC (2001).

2.4.2. Animal measurements and sampling

Cows were milked twice a day at 0400 and 1400 h. Milk yield was measured daily using an automated recording system (Dairy Plan; GEA Farm Technologies, Düsseldorf, Germany) and milk samples preserverd with potassium dichromate (Lactopol®, Grupo Benzo, Uruguay) were collected every 14 days to determine fat, protein and milk urea (MUN) concentration by a milk analyzer (Combi FOSS FT+, Foss Electric, HillerhØd, Denmark). Cow LW and BCS (scale from 1 to 5) (Edmonson et al., 1989) were measured every 14 days from -45 to 180 ± 18 DIM.

At -45, 21, 100 and 180 ± 18 DIM blood samples were taken by coccygeal venipuncture using 10 mL heparinized Vacutest ® tubes (Vacutest Kima, Vacutest Kima, Arzergrande, Italia). Plasma samples were immediately harvested by centrifuging at 4000×g during 12 min, aliquoted and stored at -80 °C until analysis.

2.4.3. Metabolites, hormones and enzymatic activities determinations

Plasma concentrations of glucose, non-esterified fatty acids (NEFA), BHB, cholesterol, urea, total protein and albumin were quantified by spectrophotometry using a 96-well microplate reader (Multiskan FC, Thermo Fisher Scientific Inc., MA, USA) and commercial kits (Biosystems SA, Barcelona, Spain for glucose, cholesterol, urea, total protein, and albumin; and Randox Laboratories Ltd., Crumlin, UK for NEFA and BHB). For all assays, the intra-assay and inter-assay CV for low and high controls were < 16.5 and 12.0 % respectively. Plasma concentrations of 3-MH were quantified after derivatization with fluorescamine according to (Houweling et al., 2012) using HPLC tandem mass spectrometry (HPLC-MS/MS). Insulin concentrations were determined by radioinmunometry in a single assay (DIAsource Immuno Assays, Brussels, Belgium). Intra-assay coefficients of variation were 3.7 and 9.4 % for the low and high controls, respectively.

Lipid and protein oxidation biomarkers were assessed determining plasma concentrations of thiobarbituric acid reactive species (TBARS) and protein carbonyls, respectively. Concentrations of TBARS were measured using a colorimetric method at 532 nm according to (Wernicki et al., 2006) using a 96-well microplate reader (Multiskan FC, Thermo Fisher Scientific Inc., MA, USA). The concentration of

malondialdehyde (MDA) was calculated using its extinction coefficient (156 000 mol/cm; adjusted for the path length of the solution in the well). Protein carbonyls concentration was determined through absorbance of 2,4-dinitrophenylhydrazine (DNPH) derived carbonyls at 380 nm according to (Ceci et al., 2015) using a 96-well microplate reader (Multiskan FC, Thermo Fisher Scientific Inc). Concentrations were calculated using the DNPH molar extinction coefficient (22 000 mol/cm; adjusted for the path length of the solution in the well). Results were expressed as nmol of DNPH/mg of total protein.

Antioxidant system was assessed by enzyme activity of superoxide dismutase (SOD) and glutathione peroxidase (GPx) in plasma as well as plasma concentration of αtocopherol (vitamin E). The activity of SOD and GPx were measured spectrophotometrically by kinetic assays using commercial kits (Randox Laboratories Ltda, Crumlin, UK) in a Varioskan Flash microplate reader (Thermo Fisher Scientific Inc.,) according to manufacturer's protocol. For GPx activity determination plasma samples were not diluted while SOD protocol had a minor modification: prior to apply manufacturer's protocol, 30 µL of chloroform and 50 µL of ethanol were added to 100 µL plasma samples, mixed and vortexed during 1 minute. Subsequently, samples were centrifuged at 17 000×g during 1 min and 4 °C. Supernatant was recovered and 9 µL diluted with 0.01 M phosphate solution (pH = 7.0) in a 25:75 relationship (vol/vol, sample: diluent). Results were expressed as mU of activity per mg of total protein measured in in plasma. Plasma α-tocopherol (vitamin E) concentration was determined using a reversed-phase HPLC method using a 1100 Agilent quaternary pump HPLC (Agilent Technologies, Waldbronn, Germany) with a fluorescent detector according to (Schweigert et al., 2003).

2.4.4. Calculation and statistical analyses

Fat and protein corrected milk (FPCM) yield was calculated according to Østergaard et al. (2003) with the following equation (1):

FPCM = [0.383(% fat) + 0.242(% protein) + 0.7832] [3.14] [MY]

Where, MY is milk yield (kg/day).

Insulin sensitivity was estimated by the RQUICKI index (Holtenius and Holtenius, 2007), while the risk of developing production metabolic diseases was estimated by the PI index (Moyes et al., 2013), calculated according to equations 2 and 3, respectively:

RQUICKI = 1/ (log [glucose] + log [insulin] +log [NEFA])

PI = ln [NEFA] + ln [BHB] – [glucose]

Where, glucose concentration is in mg/dL, insulin is in μU/mL and NEFA is in mmol/L in eq. 2, while in eq. 3 [NEFA] is expressed in mEq/L, [BHB] in mmol/L and [glucose] in mmol/L.

Data were analysed as repeated measures with a mixed model using the MIXED procedure (SAS® University Edition, SAS Institute Inc., Cary, NC, USA). The model included genetic strain, feeding strategy, DIM and its interactions as fixed effects, and cow as a random effect. Calving date and days in pregnancy were tested as covariables and were removed because of the lack of significance ($P > 0.1$). The spatial power (SP(POW)) was chosen for covariance structure due to unequally spaced sampling periods (Littell et al., 2006). For all results, means considered to differ when $P \leq 0.05$, and trends were identified when $0.05 < P \leq 0.10$. Least-squares means were compared using Tukey's test. Correlation analysis was done in R (www.r-project.org) using the *hlmisc* package. A metabolic trajectory analysis was performed considering the metabolic parameters and based on principal component analysis (PCA) as previously reported (Zhang et al., 2013)

Table 2.2. Productive performance and metabolic variables according to genetic strain and feeding strategy interaction

	Average mean					*P*-value					
	P30		PMAX		SEM	GS	FS	DIM	GS×FS	GS×DIM	FS×DIM
	NAH	NZH	NAH	NZH							
a. Productive perfomance											
FPCM (kg/day)	31.6	27.4	33	30.4	2.0	0.02	0.12	< 0.01	0.59	0.95	0.10
LW (kg)	598.1	523.9	588.6	523.4	14.5	< 0.01	0.63	0.06	0.66	< 0.01	0.16
BCS	2.67	2.74	2.62	2.68	0.06	0.09	0.19	< 0.01	0.83	0.90	0.16
b. Energy metabolism											
NEFA (mmol/L)	0.198	0.226	0.253	0.239	0.050	0.84	0.29	< 0.01	0.52	0.44	0.02
BHB (mmol/L)	0.300	0.277	0.300	0.291	0.035	0.52	0.78	< 0.01	0.76	0.37	0.37
Cholesterol (mmol/L)	2.8	3.42	3.26	4.37	0.28	< 0.01	< 0.01	0.04	0.21	0.48	< 0.01
Glucose (mmol/L)	3.26	3.38	2.99	3.83	0.27	0.01	0.64	0.02	0.07	0.23	0.02
Insulin (mU/mL)	8.05	8.64	7.01	7.96	0.60	0.07	< 0.01	< 0.01	0.69	0.19	< 0.01
c. Protein metabolism											
MUN[3] (g/kg)	18.9	20.5	17.2	20.2	0.6	< 0.01	0.03	< 0.01	0.13	0.04	0.69
Urea (g/L)	4.21	5.33	4.45	4.65	0.33	0.01	0.33	0.29	0.06	0.15	< 0.01
3-MH (mmol/L)	3.92	3.60	4.68	4.04	0.40	0.09	0.03	< 0.01	0.56	0.04	< 0.01
Total protein (g/L)	63.3	63.4	64.4	63.6	3.6	0.9	0.80	0.18	0.88	0.64	0.58
Albumin (g/L)	26.6	25.2	27.2	26.2	1.2	0.16	0.38	0.42	0.84	0.04	0.49
d. Metabolic indexes											
RQUICKI	0.56	0.53	0.54	0.52	0.04	0.20	0.44	<0.01	0.87	0.26	0.03
PI index	-0.17[ab]	0.17[b]	0.85[c]	-0.27[a]	0.29	0.06	0.16	0.02	<0.01	0.20	<0.01

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GS: genetic strain; FS: feeding strategy; DIM: days in milk; NAH: North American Holstein; NZH: New Zealand Holstein; FPCM: fat and protein corrected milk; LW: live weight; BCS: body condition score; NEFA: non-esterified acids; BHB: β-hidroxybutirate; MUN: milk urea nitrogen; 3-MH: 3-methylhistidine. Different letters mean significant differences ($P \leq 0.05$) according to Tuckey test. Variables were not affected by the triple interaction between GS, FS and DIM except FPCM ($P < 0.01$), LW ($P < 0.01$), glucose ($P = 0.06$) and insulin ($P = 0.06$).

Table 2.3. Oxidative metabolism variables according to the interaction between genetic strain and feeding strategy

	Average mean					*P*-value					
	P30		PMAX		SEM	GS	FS	DIM	GS×FS	GS×DIM	FS×DIM
	NAH	NZH	NAH	NZH							
TBARS (mmol MDA/L)	1.02	1.07	1.02	1.08	0.06	0.17	0.87	0.86	0.87	0.06	0.16
Carbonyls (nmol DNPH/mg TP)	0.39	0.42	0.32	0.41	0.15	0.10	0.33	0.20	0.38	0.64	0.09
SOD (mU/mg TP)	48.2[a]	49.9[a]	75.9[b]	71.8[b]	1.2	0.60	0.01	0.04	0.16	0.10	0.10
GPx (mU/mg TP)	2.66	2.36	2.55	2.39	0.28	0.83	0.76	0.27	0.42	0.22	0.83
α-Tocopherol (μg/mL)	2.24[a]	1.99[a]	2.80[b]	2.88[b]	0.46	0.79	0.03	0.01	0.62	0.10	0.05

GS: genetic strain; FS: feeding strategy; NAH: North American Holstein; NZH: New Zealand Holstein; DIM: days in milk; TBARS: thiobarbituric acid reactive species; MDA: malondialdehyde; DNPH: Dinitrophenylhydrazine; SOD: superoxide dismutase; GPx: glutathione peroxidase; α-Toc: α-Tocopherol; TP: total protein. Different letters mean significant differences ($P \leq 0.05$) according to Tuckey test. Variables were not affected by the triple interaction between GS, FS and DIM ($P > 0.10$ in all cases).

2.5. RESULTS

2.5.1. Milk yield, milk composition, live weight and body condition score

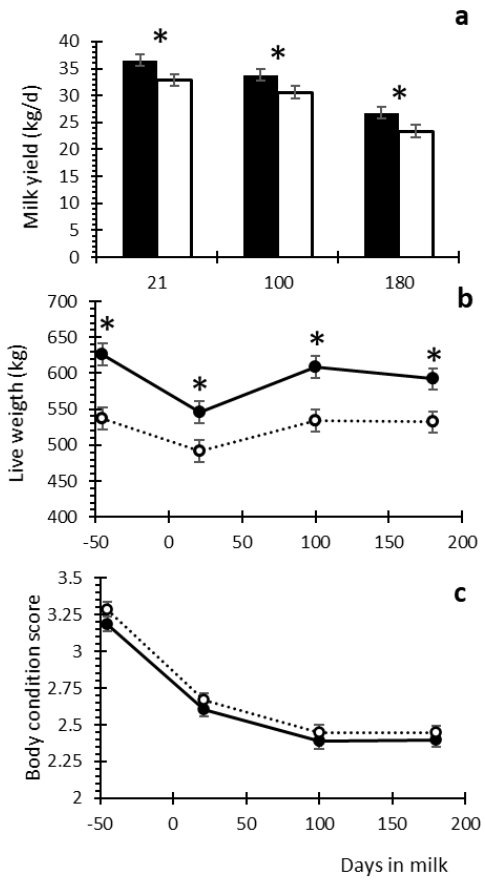

Figure 2.1. Fat and protein corrected milk yield (a), live weight (b) and bodycondition for North American and New Zealand cows (full black: NAH; empty: NZH) across lactation (days in milk). Significative differences ($P \leq 0.05$) between genetic strains at any given time are denoted by *.

Fat and protein corrected milk yield was greater ($P = 0.02$) for NAH than in NZH cows (32.3 vs. 28.9 ± 1.0 kg/day, respectively, Table 2.2) and decreased ($P < 0.04$) from 21 to 180 DIM for all cows (Figure 2.1a). However, FPCM yield tended to be affected ($P = 0.10$) by the interaction between feeding strategy and DIM as PMAX cows had a greater ($P = 0.02$) FPCM yield than P30 only at 21 DIM. Cow LW was greater ($P < 0.01$) for NAH than NZH cows (Table 2.2, Figure 2.1b) and was affected by DIM, and by the interaction between genetic strain, feeding strategy and DIM ($P <$

0.01) as LW was lost in all cows from -45 to 21 DIM except for NZH-PMAX which showed no significative changes at this time (data not shown). Then, all cows increased ($P < 0.01$) by 10 % their LW from early to mid-lactation and remained unchanged until 180 DIM, except for NAH-PMAX which decreased their LW, reaching a similar values than NZH-P30 and NZH-PMAX at 180 DIM. In contrast, cow BCS tended to be greater ($P = 0.09$) for NZH than NAH cows and was affected by DIM ($P = 0.01$), as it decreased from -45 to 100 DIM, remaining then unchanged until 180 DIM in all cows (Figure 2.1c).

2.5.2. Energy and protein metabolism

Plasma NEFA concentrations were affected ($P < 0.01$) by DIM and by the interaction between feeding strategy and DIM (Table 2.2; Figure 2.2a) as plasma NEFA increased ($P < 0.01$) in both feeding strategies from -45 to 21 DIM, but decreased faster in P30 than PMAX cows, reaching lower concentrations at 100 DIM in the former than the latter ones. Plasma BHB was only affected by DIM ($P < 0.01$), increasing from -45 to 21 DIM and decreasing then at 100 DIM (Table 2.2; Figure 2.2b). Cholesterol concentrations were greater ($P < 0.01$) for NZH than NAH cows and PMAX than P30 cows (Table 2.2). However, plasma cholesterol was affected by the interaction between feeding strategy and DIM ($P < 0.01$) as it increased from 21 to 180 DIM only for PMAX cows, determining that differences between feeding strategies were evident only in mid-late lactation (Figure 2.2c).

Concentrations of glucose were greater ($P = 0.01$) for NZH than NAH cows and tended to be affected ($P = 0.07$) by the interaction between genetic strain and feeding strategy as differences between NZH and NAH cows were only significant in PMAX (Table 2.2). Plasma glucose was also affected ($P = 0.02$) by DIM and by the interaction between feeding strategy and DIM as it was greater for PMAX than P30 during the prepartum (Figure 2.3b). Also, the interaction between genetic strain, feeding strategy, and DIM tended to be significant ($P = 0.06$) as glucose concentrations did not differ along with lactation for NAH-P30 and NZH- P30 cows but were always greater for NZH-PMAX than NAH-PMAX except at 21 DIM (data not shown). In NAH-PMAX

glucose concentrations decreased constantly from -45 to 180 DIM ($P < 0.01$). Insulin concentrations tended to be greater ($P = 0.07$) for NZH than NAH cows and was greater ($P < 0.01$) for P30 than PMAX cows (Table 2.2). However, plasma insulin was affected by the interaction between feeding strategy and DIM ($P < 0.01$), as it decreased in both feeding strategies from -45 until 21 DIM but, although it increased at 100 DIM for all cows, it increased more ($P < 0.01$) for P30 than PMAX cows (Table 2.2; Figure 2.3d).

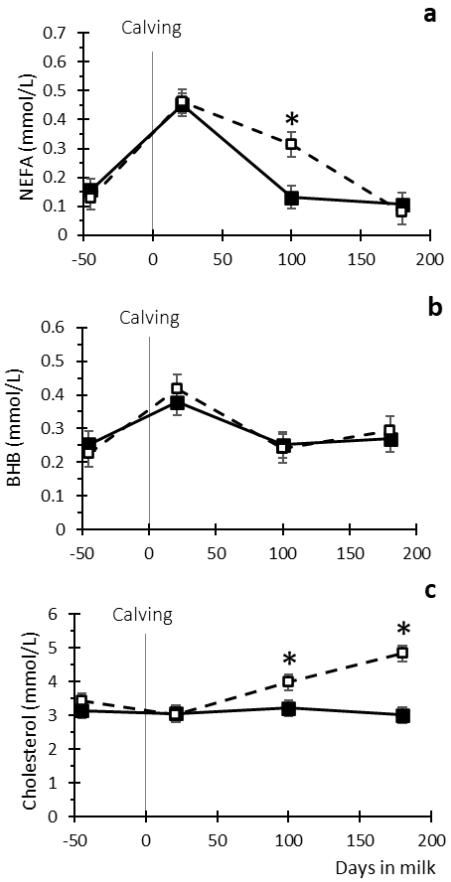

Figure 2.2. Plasma concentrations of non-esterified fatty acids (a), b-hidroxybutirate (b) and cholesterol (c) for cows fed different feeding strategies (fullblack: P30; empty: Pmax) across lactation. Significant differences ($P \leq v0.05$) between feeding strategies at any given time are denoted by *.

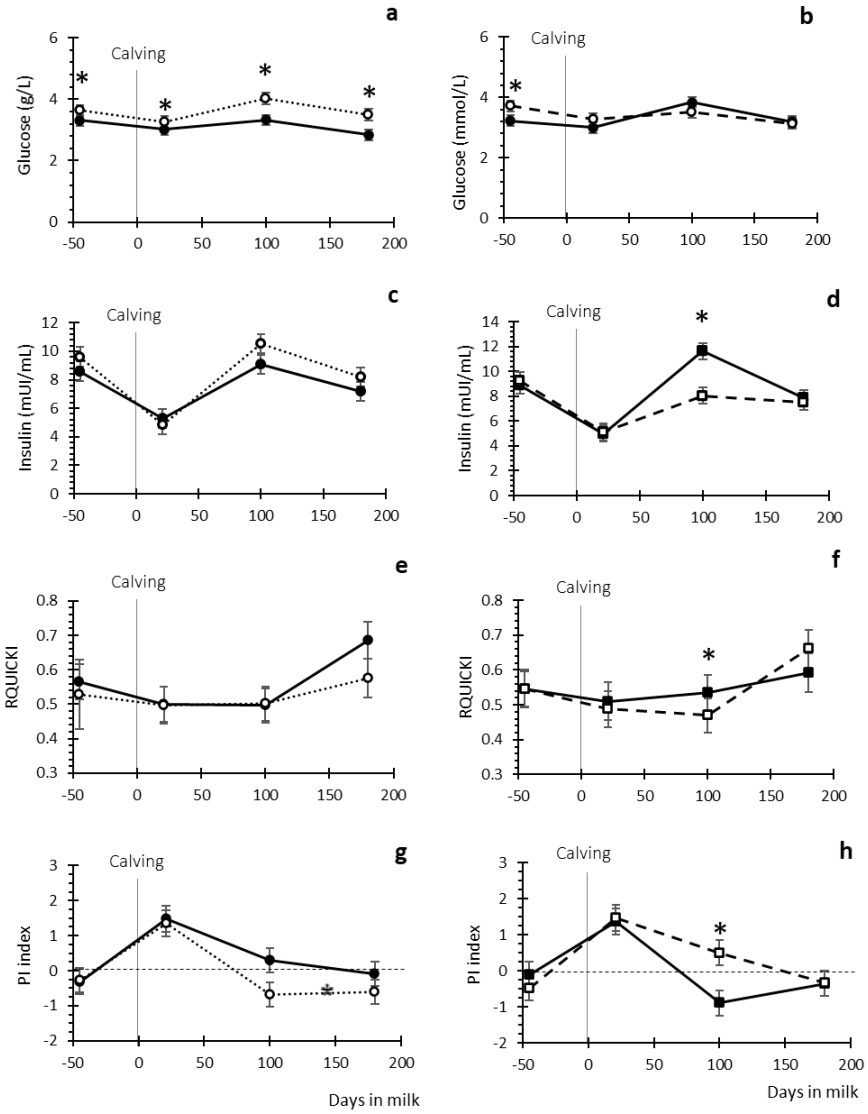

Figure 2.3. Plasma concentrations of glucose (a, b) and insulin (c, d) and metabolic indexes RQUICKI (e, f) and PI (g, h) for North-American and New Zealand cows (a, c, e, g) and different feeding strategies (b, d, f, h) across lactation. Genetic strains are depicted by circles (full black: NAH; empty: NZH), and feeding strategies by squares (full black: P30; empty: Pmax). Significative differences ($P \leq 0.05$) between genetic strains or feeding strategies at any given time are denoted by *.

The RQUICKI was affected ($P < 0.03$) by DIM and by the interaction between feeding strategy and DIM as in P30 it was low from -45 to 21 DIM and then increased until 180 DIM while for PMAX cows it decreased ($P < 0.01$) from -45 to 100 DIM and increased at 180 DIM (Table 2.3; Figure 2.3f). This determined that RQUICKI was greater ($P < 0.01$) at 100 DIM for P30 than PMAX cows. The PI index tended to be lower ($P = 0.06$) for NZH than NAH cows (Table 2.2) and was affected by the interaction between genetic strain and feeding strategy ($P < 0.01$) as it was the highest for NAH-PMAX cows. It was also affected by the interaction between feeding strategy and DIM ($P < 0.01$) as it increased after calving at 21 DIM in both feeding strategies but remained high until 100 DIM only for PMAX cows (Table 2.2; Figure 2.3g, h).

Concentrations of MUN were greater ($P < 0.03$) on average for NZH than NAH cows and P30 than PMAX, and it also was affected ($P = 0.04$) by the interaction between genetic strain and DIM as in NAH cows it decreased ($P < 0.05$) from 21 to 100 DIM while it remained unchanged in NZH cows at this time being lower in the former than in the latter ones at 100 and 180 DIM (Figure 2.4a). Finally, MUN increased ($P < 0.01$) in both genetic strains at 180 DIM. Plasma urea concentrations were greater on average ($P < 0.01$) for NZH than NAH cows and tended to be affected ($P = 0.06$) by the interaction between genetic strain and feeding strategy it was the greatest ($P < 0.05$) for NZH-P30 cows (Table 2.2). Besides, plasma urea was also affected by the interaction between feeding strategy and DIM as it increased ($P < 0.01$) from -45 to 100 DIM and decreased ($P < 0.01$) at 180 DIM for P30 cows, while it remained low from -45 to 100 DIM and increased ($P < 0.01$) at 180 DIM for PMAX cows (Figure 2.4d). This determined that plasma urea was greater ($P < 0.01$) at 100 DIM but lower ($P < 0.01$) at 180 DIM for P30 than PMAX cows (Figure 2.4d). Concentrations of 3-MH tended to be greater ($P = 0.09$) for NZH than NAH cows and were greater ($P = 0.03$) for PMAX than P30 cows. Plasma 3-MH decreased ($P < 0.01$) from -45 to 21 DIM for all cows (Table 2.2) and was affected ($P < 0.04$) by the interactions between genetic strain and DIM, and between feeding strategy and DIM as it was greater for NAH than NZH cows during the prepartum (-45 DIM; Figure 2.4e) and for PMAX than P30 cows at 100 DIM (Figure 2.4f). Plasma total protein concentrations were not affected by genetic strain, feeding strategy, DIM nor their

interactions (Table 2.2). Albumin concentration was only affected by the interaction between genetic strain and DIM (Table 2.2) as it remained unchanged during the whole experiment in NZH cows while in NAH cows it decreased (P = 0.02) between -45 and 21 DIM increasing (P = 0.02) then at 100 DIM and decreasing again at 180 DIM. This determined that NAH cows tended (P = 0.06) to have and had (P = 0.04) greater albumin concentration than NZH cows at -45 and 100 DIM, respectively.

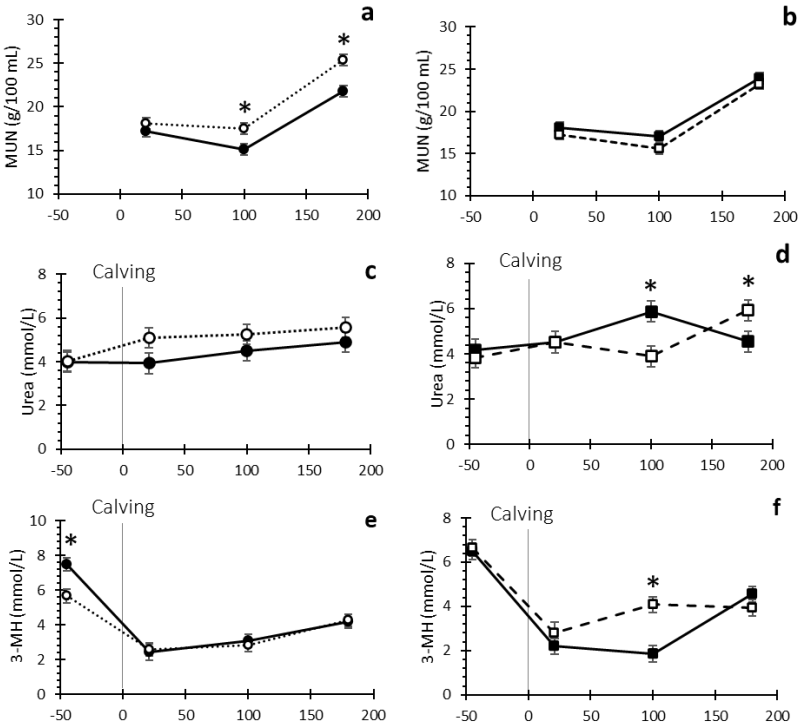

Figure 2.4. Concentrations of milk urea nitrogen, urea and 3-methyilhistidinefor North-American and New Zeland (a, c and e, respectively) and different feeding strategies (b, d and f, respectively) across lactation. Genetic strains are depicted by circles (full black: NAH; empty: NZH), and feeding strategies by squares (full black: P30; empty: Pmax). Significant differences ($P \leq 0.05$) between genetic strains or feeding strategies at any given time are denoted by *.

2.5.3. Oxidative metabolism compounds and enzymes

Plasmatic TBARS levels tended (P = 0.06) to be affected by the interaction between genetic strain and DIM as it was greater (P = 0.01) for NZH than NAH cows during the prepartum (Figure 2.5a). The concentration of plasma protein carbonyls tended to be greater (P = 0.10) for NZH than NAH cows and it also tended (P = 0.09) to be affected by the interaction between feeding strategy and DIM as protein carbonyls remained unchanged from -45 to 180 DIM for P30 cows while it decreased (P < 0.01) from 21 to 180 DIM for PMAX cows. This determined that plasma protein carbonyls at 180 DIM were greater (P = 0.04) for P30 than PMAX cows (Figure 2.5d).

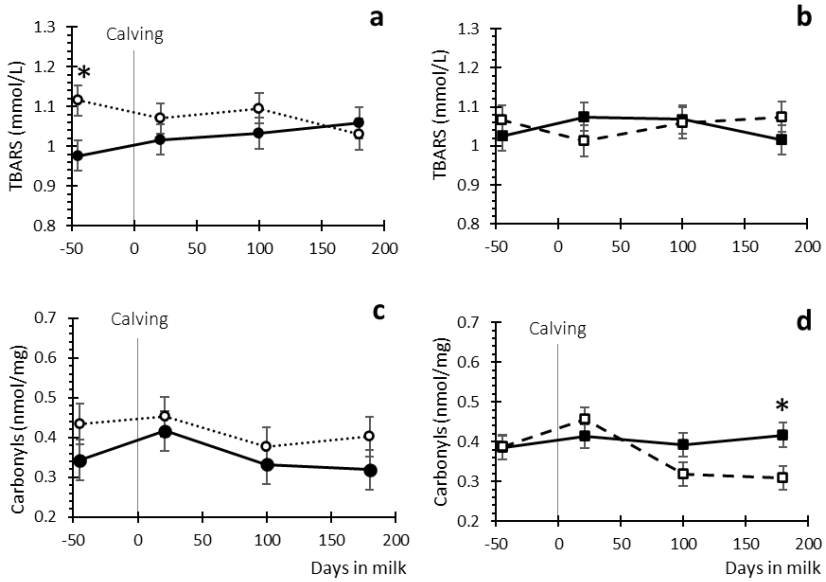

Figure 2.5. Plasma concentrations of thiobarbituric acid reactive species and protein carbonyls for North-American and New Zealand cows (a, c) and different feeding strategies (b, d, respectively) across lactation. Genetic strains are depicted by circles (full black: NAH; empty: NZH), and feeding strategies by squares (full black: P30; empty: PMAX). Significant differences (P ≤ 0.05) between genetic strains at any given time are denoted by *.

Plasma GPx activity was not affected by genetic strain, feeding strategy, DIM nor their interactions (Table 2.3; Figure 2.6a, b). In contrast, SOD activity was greater

(P < 0.01) for PMAX than P30 cows and decreased (P = 0.04) from -45 to 180 DIM. However, the decrease was earlier in lactation for P30 than PMAX cows, determining that at 21 DIM, plasma SOD activity was less (P = 0.02) for the former than the latter ones (Figure 2.6d). Also, plasma SOD activity at 21 DIM tended to be greater (P = 0.06) for NZH than NAH cows (Figure 2.6c). Plasma α-tocopherol was affected DIM as it increased from -45 to 100 DIM to decrease again at 180 DIM (P < 0.05; Table 2.3), and tended to be affected by the interaction between genetic strain and DIM as it increment until 100 DIM was significant (P < 0.05) only for NZH cows (Figure 2.7e). Plasma α-tocopherol was also affected by the interaction between feeding strategy and DIM as it was greater P < 0.01) for PMAX than P30 cows later in lactation (at 100 and 180 DIM; Figure 2.6f).

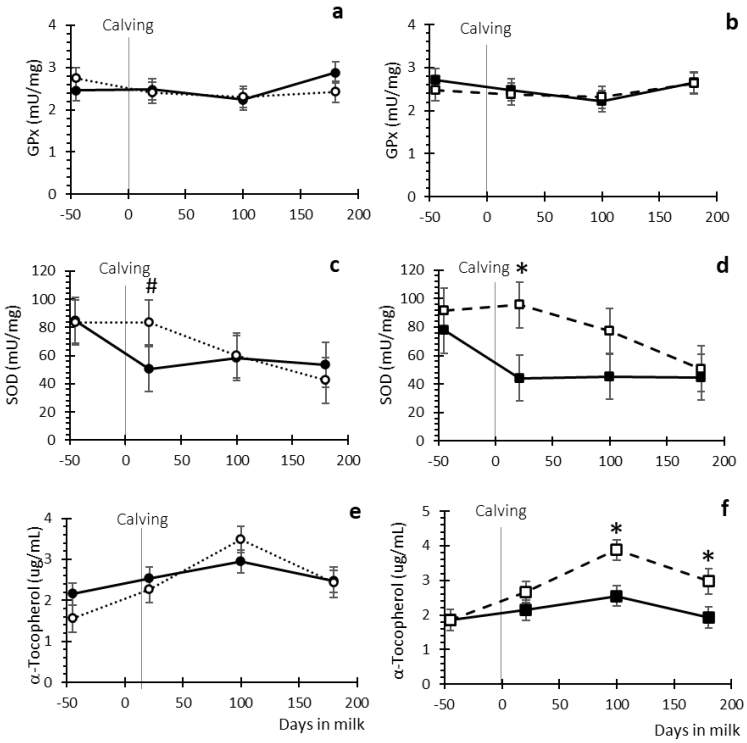

Figure 2.6. Plasma activity of glutathione peroxidase, superoxide dismutase and plasma concentration of α-tocopherol for North-American and NewZealand cows (a, c, e) and different feeding strategies (b, d, f) across lactation. Genetic strains are depicted by circles (full black: NAH; empty: NZH), and feeding strategies by squares (full black: P30; empty: PMAX). Significant differences (P ≤ 0.05) between genetic strains at any given time are denoted by *.

2.5.4. Correlation analysis

The FPCM yield had a positive correlation with plasma NEFA and BHB ($r \leq 0.20$, $P < 0.03$) and PI index ($r = 0.31$, $P < 0.01$). The plasma concentration 3-MH had a positive correlation with BCS ($r = 0.41$, $P < 0.01$), and negative with plasma NEFA, BHB ($r = -0.20$, $P = 0.02$ in both cases) and albumin ($r = -0.34$, $P < 0.01$). Plasma cholesterol was negatively correlated with BCS, and positively with αtocopherol ($r = 0.36$, $P < 0.01$). Plasma carbonyls, SOD and GPx were positively correlated between them ($r > 0.38$, $P < 0.01$ in all cases).

Differences in plasma concentrations between -45 and 21 DIM for 3-MH and glucose were positively correlated ($r = 0.50$, $P < 0.01$), while those for 3-MH with NEFA and BHB were both negatively correlated ($r = -0.22$, $P < 0.03$ in both cases). The plasma differences between -45 and 21 for carbonyls and BHB were positively correlated ($r = 0.19$, $P = 0.04$).

2.5.5. Metabolic trajectories analysis

Metabolic trajectory analysis by PCA showed greater metabolic trajectories for PMAX than P30 cows, happening the largest displacements between -45 and 21 DIM for all cows (Figure 2.7); then at 100 and 180 DIM, cows reached intermediate states located between -45 and 21 DIM positions. In addition, clear differences between genetic strains were observed at PMAX, as the NZH-PMAX cows had a greater progression towards the initial state with the progression of lactation compared to NAH-PMAX cows (Figure 2.7b, d).

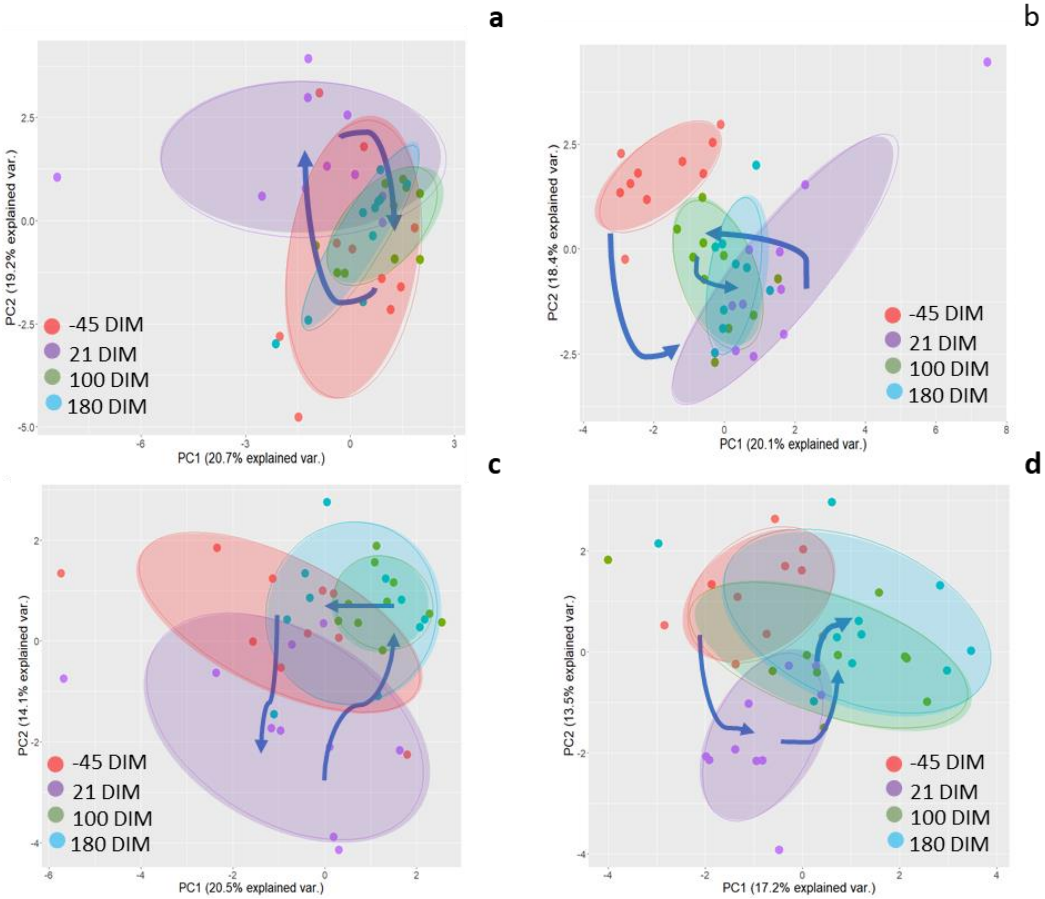

Figure 2.7. Metabolic trajectory for North-American cows in P30 (a) and PMAX (b), and New Zeland cows in P30 (c) and PMAX (d) according to score plots of principal component analysis. Days in milk are denoted by different colours (-45: pink, 21: violet, 100: green, 180: sky blue). The ellipses indicate 95 % confidence.

2.6. DISCUSION

Several studies reported until now have clearly demonstrated a significant effect of the interaction genotype×environment on productive, reproductive and metabolic parameters when comparing NAH vs. NZH genetic strains under different feeding strategies (Roche et al., 2006, Lucy et al., 2009; Chagas et al., 2009). Physiological studies have addressed differences between these genetic strains at the level of glucose metabolism and adipose tissue mobilization, sustained by differences in somatotropic uncoupling and insulin resistance during transition. In the current

work, we demonstrate that also protein and redox metabolism underlie the adaptive responses differences, as NZH cows mobilized lower muscle protein and displayed more reactive redox responses.

2.6.1. Genetic strain

The greater FPCM yield together with the trend to have a lower BCS observed for NAH cows throughout the experiment are in agreement with the genetic selection focused on individual milk yields and applied during several decades on this genetic strain (Harris and Kolver, 2001; Horan et al., 2005). The greater milk production in NAH cows may be sustained by a greater DMI as previously reported in relationship with its greater LW when compared to NZH cows (Sheahan et al., 2011). In fact, adipose tissue mobilization rate did not seem to differ between NAH and NZH cows as BCS, and changes in plasma NEFA and BHB across time were similar between genetic strains. However, the greater FPCM yield in NAH cows led to a worse metabolic status compared to NZH cows as denoted by the lower glucose and insulin (Bjerre-Harpøth et al., 2012). These differences were exacerbated in the PMAX feeding strategy, indicating more challenging conditions for the NAH cows as grazing activity was increased (Kolver et al., 2000). Furthermore, the NAH cows displayed a greater physiological imbalance state according to the PI index, suggesting these cows had a greater risk of metabolic diseases (Moyes et al., 2013), especially when grazing activity was maximized (PMAX). Taken together, these results would suggest these genetic strains displayed different homeorhetic and homeostatic responses according to the feeding strategy to sustain differences in energy and nutrients partition priorities.

Interestingly, the elevated concentrations of 3-MH previous to calving indicated that all cows were mobilizing labile protein at the time (Houweling et al., 2012), possibly due to a state of amino acids deficiency previous to the massive mobilization of adipose tissue in early lactation (van der Drift et al., 2012). In fact, the negative association between 3-MH and BHB observed in our work would suggest that the preferential use of gluconeogenic substrates (amino acids vs. fatty acids) changed during lactation (van der Drift et al., 2012). Moreover, in our best knowledge, this is the first work studying the effect of the Holstein genetic strain on protein

reserves mobilization. Our results demonstrated that skeletal muscle mobilization was more acute for NAH than NZH cows, possible due to a more negative protein balance and a lower proteolysis inhibition caused by lower concentrations of plasma insulin (van der Drift et al., 2012). The greater plasma and milk urea observed in the NZH cows suggest a decreased nitrogen use efficiency, probably due to greater nitrogen absorption as a consequence of digestive differences when compared to NAH cows. These results may be related to frame differences (Aikman et al., 2008) as lower nitrogen use efficiency in lower-sized cows (Kauffman and St-Pierre, 2001) have been related to a better distribution of the ingestion across the day, and differences in ruminal daily kinetics as a consequence of relative lower mouth and bite sizes (Aikman et al., 2008). These results indicate that not only glucose and lipid metabolism can be affected by the Holstein genetic strain but the protein metabolism could also be displaying a central role within the metabolic adaptations, at least when comparing NAH vs. NZH cows in pasture-based systems.

During the transition period, dairy cows can undergo an oxidative stress state (Castillo et al., 2005) as a consequence of the imbalance between pro-oxidants (e.g.: reactive oxygen species; ROS) and antioxidants given by the increase of the former ones, a decrease of the latter ones, or a combination of both processes (Abuelo et al., 2013). It implies oxidative damage of macromolecules such as lipid and proteins (Miller et al., 1993; Ceci et al., 2015). In the present work, greater TBARS concentrations previous to calving as well as the trend for greater plasma carbonyls observed in the NZH cows, suggested a greater ROS production in these animals and thus a stronger oxidative load, bringing out probable differences between genetic strains in terms of immunologic responses against the metabolic challenge of the transition period. Reactive oxidative species are secreted by neutrophils and phagocytes during the immune response leading to an oxidative stress that can be properly managed by the animal (Celi and Gabai, 2015). Moreover, the negative association observed between carbonyls and PI changes between -45 and 21 DIM, indicated increased protein oxidation when cows had a better physiological balance. Natural functions of ROS include its participation within structural mechanisms of cellular defence as well as an evolutionary mechanism of cellular signalling against

stress conditions, probably playing an essential role within the evolutive process and species survival (Sordillo and Atiken 2009; Tian et al., 2015; Taverne et al., 2018). The apparently greater oxidative damage observed in the NZH cows could denote a greater adaptive response as they did not only show greater oxidative load but also a higher SOD activity for longer during lactation, and a greater recovery of α-tocopherol during mid lactation when compared with NZH cows. These results were in agreement with (Marco-Ramell et al., 2012), who found an increased state of GPx and SOD activity in a local-breed of cows adapted to mountain grazing compared to non-adapted breeds, and suggested that higher responses of redox metabolism could constitute structural mechanism of adaptive strategies in cattle subjected to challenging conditions.

2.6.2. Feeding strategy

The greater FPCM yield observed in PMAX cows during early lactation, would have been probably given by a greater DMI than for P30 cows. In fact, we did not observe metabolic differences neither at energy nor protein metabolism that denoted differences between feeding strategies in terms of body reserves mobilization. In00terestingly, PMAX cows decreased their milk yield to similar production levels than P30 cows at mid lactation, and this was associated with greater plasma NEFA, and lower insulin and urea concentrations, suggesting a decrease in DMI (Cavestany et al., 2005). Furthermore, the increase of 3-MH observed in PMAX cows at this time would indicate a new protein catabolic stage (van der Drift et al., 2012). It has been postulated that the mobilization of labile protein is circumscribed to the onset of lactation. However, in grazing conditions DMI is very constrained at winter due to grass shortfalls (Fariña and Chilibroste, 2019), and it could imply muscle mobilization in mid lactation-cows triggered by a nutrient deficit.

Differences observed in plasma carbonyls and antioxidant activity (SOD, α-tocopherol) denote the PMAX cows suffered a greater oxidative load, probably related to a greater metabolic stress. Despite there were not differences between feeding strategies in plasma TBARS, the changes on plasma carbonyls would indicate that protein oxidation decreased with the advance of lactation for PMAX cows (Tsiplakou

et al., 2017), probably due to a greater activity of SOD after calving. In fact, plasma carbonyls were positively correlated with GPx and SOD activities, and negatively with the α-tocopherol concentrations, which highlighted that the greater oxidative load stimulated an increased response from the antioxidant system in PMAX cows when compared to P30 cows (Pedernera et al., 2010). It has been widely suggested that GPx activity increase after calving would be the result of an activated state of gene expression given by the increased oxidative load (Gessner et al., 2013). Moreover, the greater recovery of αtocopherol in PMAX than P30 cows at mid lactation could be consequence of a lower oxidative damage at this time and thus a reduced waste of exogenous antioxidants in the former due to greater antioxidant response during early lactation (greater SOD activity). However, it could also be related to a higher intake of αtocopherol as pasture intake was greater for PMAX than P30 cows (Kay et al., 2005).

2.6.3. Integrated metabolic trajectories

Adaptive strategies include both behavioural, physiological and metabolic responses (Friggens et al., 2017). Historically, metabolic changes across lactation have been studied based on single metabolic parameters in a reductionist approach based on repeated measures analysis and association analyses between variables through regression and correlations. However, multivariate approaches have been successfully used in order to describe metabolic trajectories based on metabolomic data (Polakof et al., 2018). In our work, we attempted to apply a multivariate approach despite not metabolomic data but several metabolic parameters were available. Despite metabolomic database are generally based on hundreds of features, it has been previously reported successfully analysis of metabolic trajectories by PCA based on a small number of metabolites likewise in the work reported by (Zhang et al., 2013) in which 20 metabolties were used. In this sense, our results demonstrate that PCA can be used with succes in order to assess metabolic trajectories in dairy cows in the medium-term scale of lactation. Specifically, the obtained PCA synthesize the original variables by combining them in latent variables containing most variation (Wehrens, 2011). In brief, the PCA score plots highlighted that PMAX cows had a greater

metabolic displacement¸ this is, more acute changes in metabolic parameters across lactation. In addition, within the PMAX feeding strategy, the NZH cows were able to return earlier than NAH cows to metabolic states close to the initial state (previous to calving). In a whole, our results suggest the metabolic load was greater according to the increase of grazing activity in the feeding strategy and it implied greater challenges, especially for NAH cows, which seemed to have had a lower adaptive capacity to face it.

2.7. CONCLUSIONS

When compared to NAH, the NZH cows seem to have developed a better adaptive capacity to cope with the grazing conditions as suggested by their better metabolic status, lower mobilization of labile protein and the greater redox sensibility. Furthermore, metabolic adaptive mechanism would probably include not only energy, but protein metabolism changes and redox sensibility.

2.8. DECLARATION OF COMPETING INTEREST

None of the authors have any conflict of interest to declare.

2.9. ACKNOWLEDGMENTS

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3. METABOLOMIC ANALYSIS REVEALS BRANCHED-CHAIN AMINO ACIDS PLAY A MAJOR ROLE IN METABOLIC ADAPTATION DIFFERENCES BETWEEN HOLSTEIN GENETIC STRAINS MANAGED UNDER GRAZING

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3.1. RESUMEN

Durante el periodo de transición, la vaca lechera sufre un incremento dramático en los requerimientos de nutrientes mientras que el consumo de materia seca (CMS) está disminuido. Esto determina que el animal experimente cambios homeorréticos con el fin de sostener el inicio de la lactancia. En los sistemas de base pastoril, los desafíos ambientales y nutricional están exacerbados y por lo tanto la vaca necesita tener una alta capacidad de adaptación. El objetivo de este trabajo fue evaluar el efecto de la línea genética Holstein (origen norteamericano, NAH, n = 8; neocelandéz, NZH, n = 8)) en un sistema de base pastoril sobre el perfil metabolómico a lo largo de la lactancia (21 vs. 180 días en leche). Sesenta y siete de 172 metabolitos presentaron una concentración diferencial (FDR < 0,05) de acuerdo a los días en leche, mientras que la alanina fue el único metabolito afectado (FDR = 0.02) por la interacción entre la línea genética y los días en leche. De acuerdo con el análisis ASCA y el ranking MEBA, varios metabolitos tales como la alanina y los amino ácidos ramificados (AAR) difirieron entre las línea genéticas en forma dependiente a los días en leche. Nuestros resultados indicaron que el metabolismo de los AA estaría estructuralmente vinculado a los cambios homeorréticos que ocurren entre los 21 y 180 días en leche. Más aún, la menor (FDR < 0,05) concnetración de los AAR observada en los animales NZH vs. NAH durante la lactancia temprana sugiere que estos compuestos posiblemente participen en las diferencias adaptativas entre estas líneas genéticas.

3.2. ABSTRACT

During the transition period, dairy cows experience a dramatic increase in nutrient requirements while dry matter intake (DMI) is decreased leading to homeorhetic changes in order to sustain the onset of lactation. In pasture-based systems, the nutritional and environmental challenges are exacerbated and thus, dairy cows require an enhanced adaptive capacity. The aim of this work was to evaluate the effect of Holstein genetic strain (North American Holstein, NAH, n = 8; New Zealand Holstein, NZH, n = 8) in a pasture-based system on plasma metabolomic profile changes across lactation (21 vs. 180 days in milk). Sixty seven out of 172 metabolites

were differentially expressed (FDR < 0.05) according to DIM, while only alanine was affected (FDR = 0.02) by the interaction between genetic strain and DIM. According to ASCA and MEBA ranking, several metabolites such as alanine and the branched-chain amino acids (BCAA) differed between genetic strains in a DIM-dependent manner. Our results indicated that AA metabolism may be structurally involved in homeorhetic changes between 21 and 180 DIM. Furthermore, the lower (FDR < 0.05) BCAA concentrations observed for NZH than NAH at early lactation, suggest these compounds might underly adaptive differences between genetic strains.

3.3. INTRODUCTION

Adaptive strategies of animals when facing challenging conditions are given by behavioural and physiological responses[1]. In the dairy cow, at the beginning of lactation dry matter intake is not enough to sustain the high rate of milk synthesis, thus a negative energy balance is stablished, and adipose and skeletal muscle are mobilized[2,3]. Indeed, early lactation cows are characterized by a catabolic state associated with the uncoupling of somatotropic axis and a state of insulin resistance[4].

Metabolomic approach has been successfully used in the last decade to better understand metabolic changes in dairy cows during transition[5]. Indeed, changes in plasma metabolome occur in early lactation suggesting that metabolic adaptive strategies imply several metabolic pathways[6]. Negative associations between plasma short chain acylcarnitines and non-esterified fatty acids (NEFA) as well as between large-chain acylcarnitines and insulin suggest that the phospholipidome is involved in the development of insulin resistance[7]. Furthermore, Luo et al.[8] reported during the transition of dairy cows that some amino acids (AA) (e.g.: lysine, valine, isoleucine, arginine) undergo significative changes in plasma and suggested that urea cycle might be affected at parturition, possibly related to the need of gluconeogenic precursors during the negative energy balance, and linkages between AA metabolism, urea and Krebs cycles were also suggested. Recently, Ghaffari *et al.* [9] reported several metabolic pathways involved in AA and protein metabolism such as branched-chain AA (BCAA) degradation and methyl-histidine metabolism were affected during transition as well as by cow body condition score and suggested that these metabolites

could be associated to insulin resistance. Indeed, dairy cows with greater lipid mobilization rates have been reported to have unique plasma AA profile (i.e.: increased plasma glycine, decreased threonine) at early lactation[7].

Productive and reproductive differences between dairy breeds or genetic strains are a result, at least in part, of metabolic differences, particularly during the transition and early lactation periods. The greater milk yield and lower reproductive efficiency reported for **NAH** than **NZH** cows in grazing systems have been associated with a stronger uncoupling of somatotropic axis and an increased insulin resistance[10, 11,12] which result in a more pronounced loss of body reserves in the former ones. In addition, differences in homeorhetic strategies between these Holstein strains would also include AA and protein metabolic pathways. Greater milk and plasma urea concentrations, as well as lower skeletal muscle mobilization indicated by decreased plasma 3-methyl histidine concentrations, and greater protein oxidative damage were reported for NZH than NAH cows, especially when fed with a strategy that maximized pasture grazing[*]. Therefore, the aim of this study was to characterize the metabolic adaptive responses of early and mid-lactation NAH and NZH cows managed under grazing conditions by evaluating the metabolic profile.

3.4. MATERIALS AND METHODS

3.4.1. Location, experimental design, feeding strategy and herd management

The experiment was carried out the Experimental Research Station "La Estanzuela" (34°20' S, 57°40' W) belonging to the Instituto Nacional de Investigación Agropecuaria (INIA) of Uruguay. All procedures were approved by the Ethic Committee on Animal Experimentation of INIA (form INIA_2017.2).

Cows were randomly selected from a larger experiment designed to study the interaction between genetic strains and grazing-based feeding strategies[13]. Multiparous dairy cows (3.1 ± 0.9 lactations; fall calving 5/15/2018 ± 12 days) of NAH (n = 8) and NZH (n = 8) genetic strains (593 ± 17 and 560 ± 17 kg of body weight and 2.94 ± 0.06

[*] Corresponde al Cap. 1 de esta tesis.

and 3.28 ± 0.06 of body condition score at calving for NAH and NZH cows, respectively), fed a strategy that maximized pasture grazing were used. Cows had at least 87.5% of American (USA or Canada) or New Zealand ancestries for NAH and NZH cows, respectively (Mejoramiento y Control Lechero Uruguayo; https://www.mu.org.uy). The 305-days expected milk yield was 7500 and 5500 kg and the economic and productive selection index was 104 and 130 on average for NAH and NZH cows, respectively. The NAH cows had an expected progeny difference of +70.4 kg, +0.01 % fat and -0.01 % protein for milk yield, milk fat and milk protein content, respectively when compared to the national herd. The NZH cows had an expected progeny difference of -185.7 kg, +0.23 % fat and +6.19 % protein for milk yield, milk fat and milk protein content, respectively when compared to the national herd.

The diet was designed to maximize pasture grazing according to weekly pasture growing rate and concentrate (at a rate of 33% of the predicted daily dry matter intake[14]) was offered twice a day individually at the milking parlour. Cows were outdoors all year-round and throughout the experiment cows grazed *Dactylis glomerata* + *Medicago sativa* (75% of the time) or *Festuca arundinacea* (25% of the time) on a rotational grazing system with strips assigned after milking and free access to freshwater. Details on pasture and grazing management were previously described[13]. Forage reserves were offered in a feeding parlour immediately before the afternoon milking. On average, diet was composed of 64% of grazed pastures, 5% of concentrate and 31 % of reserved forage (corn silage and pasture haylage mix; 73:27 ± 8% on dry matter basis, respectively).

3.4.2. Animal measurements and sampling

Cows were milked twice a day at 0400 and 1400 h, milk yield was recorded daily and weekly milk samples were collected for composition analysis (Combi FOSS FT+, Foss Electric, HillerhØd, Denmark). Cow body weight and body condition score (scale from 1 to 5, Edmonson's scale[15]) were recorded biweekly. Fat and protein corrected milk (FPCM) yield was calculated according to Østergaard et al.[16]. Blood samples were collected at early and mid-lactation (21 and 180 ± 3 days in milk; DIM)

(mean ± SE) by caudal venipuncture using 10 mL heparinized Vacutest ® tubes (Vacutest Kima, Vacutest Kima, Arzergrande, Italia). Plasma samples were harvested by centrifugation (4000 *x*g, 12 min) and immediately stored at -80 °C until analysis. Plasma glucose, NEFA, β-hydroxybutyrate (BHB), urea and insulin concentrations were determined as previously reported[17].

3.4.3. Targeted metabolomic analysis and annotation

Metabolomic analysis and ion annotation procedures were performed by gas chromatography time-of-flight mass spectrometry (GCToF/MS) according to Fiehn et al.[18] at the core lab *West Metabolomic Center* (UC Davis Genome Center, Davis, CA, USA) and 200 prominent metabolites were analysed. A column of 30 m length x 0.25 mm internal diameter with 0.25 µm film made of 95% dimethyl/5%diphenylpolysiloxane (Rtx-5Sil MS, Restek® Corporation,) was used for chromatography analyses. A Leco® Pegasus IV mass spectrometer (S^t Jospeh, MI, USA) was used with unit mass resolution at 17 spectra/s from 80 to 500 Da and ionization energy set in -70 eV and equipped with a 1800 V detector voltage, 230 °C transfer line and a 250°C ion source. Chromatographic data were pre-processed without smoothing and peak width was set at 3 s, the baseline subtraction was done just above the noise level, and automatic mass spectral deconvolution and peak detection at signal/noise levels of 5:1 were automatically performed throughout the chromatogram. Annotation of derivatized ions were performed using the BinBase algorithm based on deconvoluted spectra and peak metadata (retention index, unique ion, spectral similarity, signal/noise ratio, peak purity) from the LecoChromaTOF software using a multi-tiered filtering system with stringent thresholds, specifically developed for GCToF/MS data annotation. Data is reported as peak height expressed as relative-to the samples' average sum of all peak heights of annotated ions.

3.4.4. Statistical analysis

Productive performance and metabolite and endocrine concentrations were analysed as repeated measure using the MIXED procedure (SAS University Edition, SAS Institute Inc., Cary, NC, USA). The model included genetic strain, lactation stage

(21 vs. 180 DIM) and their interaction as fixed effect, and cow within genetic strain as a random effect.

Metabolomic data pre-processing and statistical analysis were performed using MetaboAnalyst©[19]. Prior to statistical analysis, data were normalized by the median, cube root transformed and auto-scaled. Data quality was assessed through multivariate analysis comparing individual samples' data against the pooled samples. Outliers were assessed based on principal component analysis (**PCA**) score plot and heatmap confirmation. Data were subjected to multivariate analysis; clustering was assessed by PCA and classification models were studied by partial-least square discriminant analysis (**PLS-DA**)[20]. Data was also subjected to ANOVA analysis considering a time-series model in which genetic strain (NAH vs. NZH), lactation stage (21 vs 180 DIM) and its interaction were considered as fixed effects, while the cow was considered the experimental unit. Raw-P values were adjusted for multiple hypothesis testing[21] at a false discovery rate of 5 % (FDR $\leq$ 0.05). The time-trend of metabolites was complementary assessed both by ANOVA simultaneous component analysis (ASCA)[22] in which metabolite were considered to be well-modelled by the fixed effect when leverage > 0.040 and SPE < 1.5×10^{-30}), and by multivariate empirical Bayes statistics analysis (MEBA) based on Hotelling's T^2 ranking[23].

Metabolic pathways analysis was based on the *Boss Taurus* KEGG database, combining the *Globaltest*, which calculates the association between the metabolite sets and the phenotype without referring to a background[24], and a topologic analysis based in the *betweenness centrality*, which is a measure of the importance of a compound within a given metabolic pathway[19]. Significative enrichment of metabolic pathway was set at FDR $\leq$ 0.05. The metabolic pathway analyses were performed based on three comparisons: a) 21 vs. 180 DIM, b) NAH vs. NZH at 21 DIM, and c) NAH vs. NZH at 180 DIM.

3.5. RESULTS

3.5.1. Productive performance and metabolite and endocrine profile

The fat and protein-corrected milk yield tended to be greater for NAH than NZH cows (P = 0.076, Table 3.1). The LW tended (P = 0.052) to be lower for NZH than NAH, and BCS did not differ between genetic strains, however, BCS was greater (P < 0.001) at 21 than 180 DIM. Plasma NEFA and BHB were greater at 21 than 180 DIM (P < 0.020) while no differences were detected between Holstein strains. While urea concentrations increased (P < 0.001) for both genetic strains at 180 vs. 21 DIM, both glucose and insulin were affected by the interaction between genetic and DIM. Glucose concentration increased (P < 0.001) at 180 DIM only in NZH cows, while insulin increased (P < 0.001) for both strains but NZH reached greater concentrations than NAH cows at this time (P < 0.050).

Table 3.1. Phenotypic and metabolic classic variables in NAH and NZH cows at early and mid lactation

	Average mean			***P-value***		
	NAH	NZH	SEM	GS[1]	DIM[2]	GS × DIM
FPCM[3] (kg/day)	34.1	31.1	1.6	0.08	< 0.01	0.21
LW (kg)	556	519	20	0.05	0.18	0.60
BCS	2.44	2.53	0.09	0.32	< 0.01	0.06
NEFA (mmol/L)	0.266	0.308	0.063	0.52	< 0.01	0.43
BHB (mmol/L)	0.371	0.372	0.048	0.99	0.02	0.32
Urea (g/L)	18.0	21.5	0.7	< 0.01	< 0.01	0.07
Glucose (g/L)	47.5	66.1	5.5	< 0.01	0.374	0.01
Insulin (mUI/mL)	5.9	6.4	0.7	0.52	< 0.01	0.04

1: genetic strain; 2: days in milk; 3: average mean of 5 days around sampling date
FPCM: fat and protein corrected milk yield, LW: live weight; BCS: body condition score; NEFA: non-esterified fatty acid; BHB: β-hydroxybutirate

3.5.2. Metabolomic profiling and cluster analysis

The final metabolomic dataset accounted for 172 out of 200 assayed metabolites by the GCToF/MS method which were effectively quantified (i.e.

metabolite identifier ion with peak intensity greater than the limit of detection in at least 80 % of the plasma samples) (Supl. Table 1). To characterize the differences in metabolic profiles between the NAH and NZH cows, PCA and PLS-DA were conducted. Principal components analysis showed that metabolomics profiles can be separated by stage of lactation (early vs. mid-lactation; 21 vs. 180 DIM) for NZH but not for NAH cows; DIM had a stronger effect than genetic strain on PCA clustering (Figure 3.1a). Principal components 1 and 2 accounted in total for 33.7 % of the variation. The PLS-DA confirmed that data could be better discriminated by stage of lactation for NZH than for NAH cows (Figure 3.1b) and the best classification model was obtained based on the first 3 components and had good validation values (R^2 = 0.94, Q^2 = 0.54).

Fifty-nine (out of the 172) differential metabolites were identified with a value in importance projection (VIP) > 1.0, among which seven metabolites (oxoproline, 5-hydroxynorvaline, isoleucine, p-tolyl glucuronide, leucine, α-aminoadipic acid and L-pipecolate) showing a VIP > 2.0; for all of them the lowest concentration was observed for NZH at 21 DIM (Figure 3.1c).

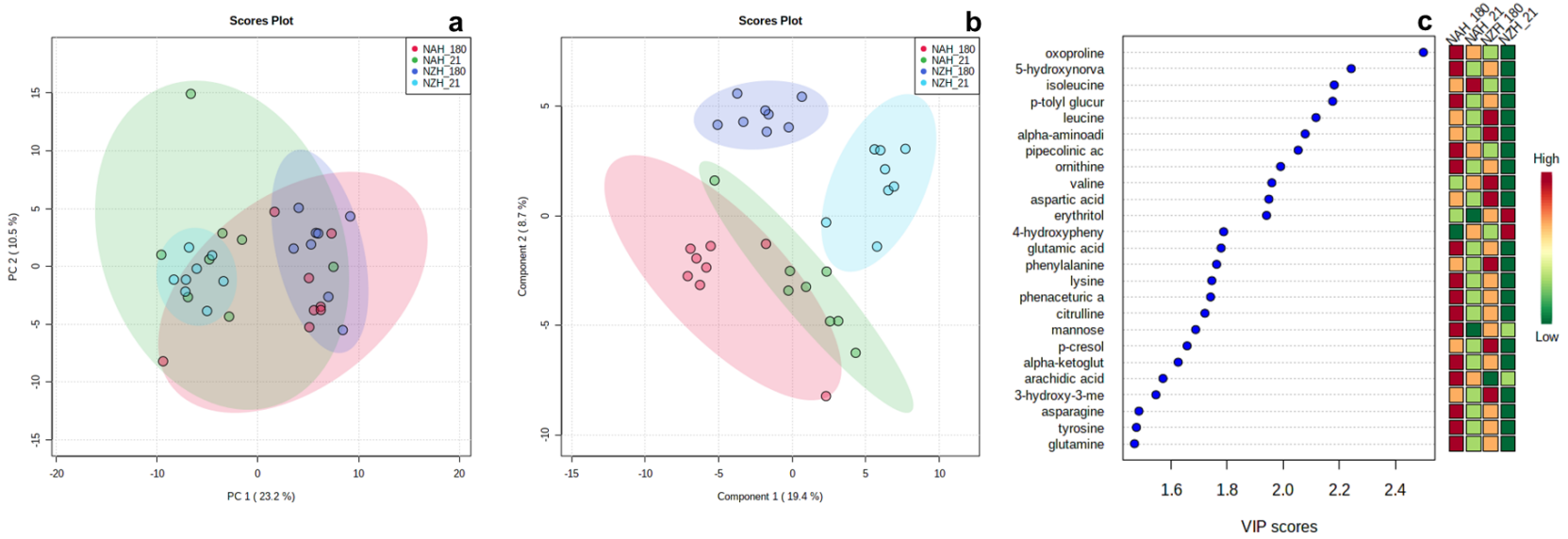

Figure 3.1. Scores plot for PCA (a), PLS-DA (b) of NAH and NZH cows at 21 (green and sky-blue dots, respectively) and 180 (red and blue dots, respectively) days in milk. The VIP scores plot (c) is based on the top 25 metabolites with the highest VIP values for the 1st component of PLS-DA.

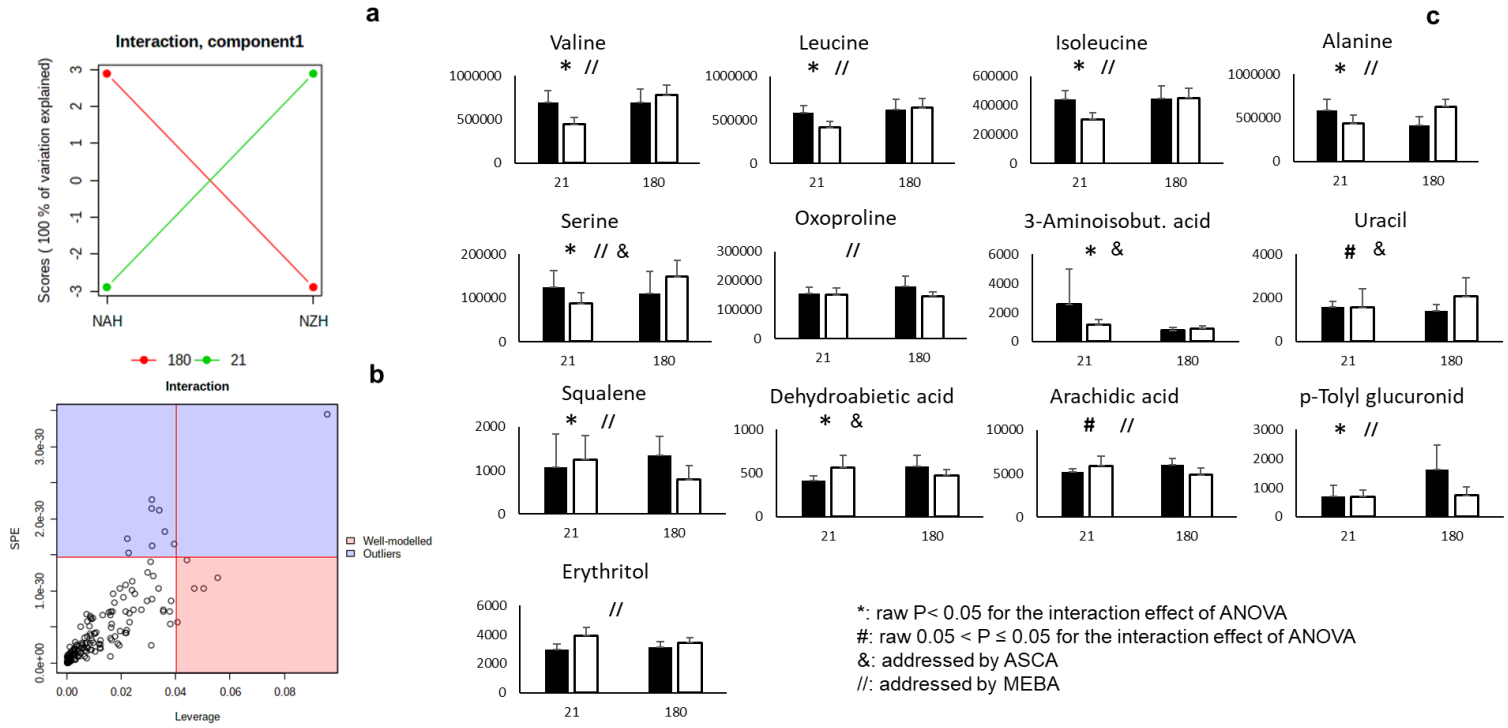

Figure 3.2. Interaction effect (genetic strain and days in milk) plot based on 1st component of ASCA (a), metabolites well modeled for ASCA interaction model (b) and bar plot of metabolites addresed to have different temporal paterns between NAH and NZH cows according to ASCA and/or top 10 MEBA's ranking. Raw P-values < 0.05 for the interaction effect of ANOVA are depicted by *. The x-axis denote days in milk, the y-axis denote ion intensisty peak height and rrror bars indicate standard deviation.

Table 3.2. Metabolites affected by genetic strain, days in milk or its interaction

Compound family	Metabolite	Peak relative-height average mean						GS[3]		DIM[4]		GS × DIM	
		NAH	NZH	SEM	21 DIM	180 DIM	SEM	*raw-P*[5]	*FDR*[6]	*raw-P*	*FDR*	*raw-P*	*FDR*
α-keto acids	pyruvic acid	7.6E+03	1.0E+04	1.4E+03	7.5E+03	1.1E+04	1.5E+03	0.50	0.79	0.05	0.10	0.58	0.79
AA	Isoleucine	4.4E+05	3.8E+05	2.1E+04	3.7E+05	4.5E+05	2.0E+04	0.01	0.27	0.01	0.04	0.01	0.29
AA	Leucine	6.0E+05	5.3E+05	3.1E+04	4.9E+05	6.3E+05	2.8E+04	0.01	0.27	0.01	0.03	0.03	0.30
AA	Valine	6.9E+05	6.1E+05	4.2E+04	5.7E+05	7.4E+05	3.8E+04	0.02	0.33	0.01	0.02	0.00	0.21
AA	glutamic acid	5.8E+04	4.7E+04	6.0E+03	4.7E+04	5.7E+04	6.0E+03	0.07	0.58	0.03	0.08	0.09	0.34
AA	aspartic acid	1.2E+04	1.2E+04	8.9E+02	9.9E+03	1.4E+04	7.0E+02	0.09	0.58	< 0.01	0.00	0.16	0.46
AA	Asparagine	9.5E+03	9.0E+03	5.1E+02	8.5E+03	1.0E+04	4.7E+02	0.22	0.62	0.04	0.08	0.58	0.79
AA	Phenylalanine	9.7E+04	9.8E+04	4.7E+03	8.5E+04	1.1E+05	3.3E+03	0.24	0.62	< 0.01	0.00	0.37	0.66
AA	Glutamine	2.4E+05	2.0E+05	1.6E+04	1.9E+05	2.4E+05	1.6E+04	0.24	0.62	0.02	0.06	0.24	0.58
AA	Lysine	1.3E+05	1.2E+05	9.9E+03	9.9E+04	1.4E+05	8.3E+03	0.24	0.62	< 0.01	0.01	0.96	0.98
AA	Threonine	6.7E+04	6.6E+04	4.9E+03	5.6E+04	7.6E+04	4.2E+03	0.29	0.62	0.01	0.03	0.06	0.33
AA	Tyrosine	2.3E+05	2.3E+05	1.8E+04	1.9E+05	2.7E+05	1.5E+04	0.40	0.74	0.00	0.02	0.69	0.87
AA	Cysteine	4.7E+03	4.8E+03	4.3E+02	3.7E+03	5.9E+03	3.3E+02	0.58	0.85	< 0.01	0.00	0.19	0.51
AA	Methionine	3.3E+04	3.4E+04	2.1E+03	2.9E+04	3.8E+04	1.8E+03	0.60	0.85	0.01	0.03	0.38	0.66
AA	histidine	5.2E+04	5.5E+04	3.4E+03	4.5E+04	6.2E+04	2.6E+03	0.73	0.92	< 0.01	0.00	0.81	0.92
AA	glycine	4.1E+05	4.4E+05	3.4E+04	4.9E+05	3.6E+05	3.0E+04	0.87	0.98	0.04	0.09	0.18	0.50
AA	alanine	5.0E+05	5.3E+05	3.4E+04	5.1E+05	5.2E+05	3.5E+04	0.91	0.98	0.50	0.66	< 0.01	0.02
AA	tryptophan	1.9E+05	2.1E+05	1.4E+04	1.7E+05	2.4E+05	1.0E+04	0.93	0.98	< 0.01	0.00	0.78	0.91
AA related	oxoproline	1.7E+05	1.5E+05	6.3E+03	1.5E+05	1.6E+05	6.7E+03	0.00	0.22	0.04	0.09	0.43	0.70
AA related	pipecolinic acid	6.3E+03	4.0E+03	7.5E+02	4.2E+03	6.0E+03	7.8E+02	0.01	0.27	0.04	0.09	0.11	0.38
AA related	homocystine	4.4E+02	4.2E+02	2.3E+01	4.4E+02	4.3E+02	2.3E+01	0.03	0.49	0.97	0.98	0.46	0.72
AA related	5-hydroxynorvaline	7.6E+02	6.9E+02	3.7E+01	6.5E+02	8.0E+02	3.3E+01	0.04	0.51	0.00	0.01	0.75	0.90
AA related	α-aminoadipic acid	1.8E+03	1.6E+03	1.4E+02	1.4E+03	2.0E+03	1.2E+02	0.05	0.51	< 0.01	0.00	0.03	0.30
AA related	N-acetylglycine	4.4E+03	3.6E+03	5.2E+02	5.4E+03	2.5E+03	3.4E+02	0.06	0.58	< 0.01	0.00	0.36	0.66
AA related	2-ketoisocaproic acid	1.1E+04	9.0E+03	7.2E+02	1.0E+04	9.5E+03	7.4E+02	0.08	0.58	0.92	0.97	0.08	0.33
AA related	creatinine	4.0E+05	4.0E+05	1.4E+04	4.0E+05	4.0E+05	1.4E+04	0.09	0.58	0.38	0.54	0.03	0.30
AA related	3-hydroxy-3-methylglutaric acid	3.4E+02	2.9E+02	3.2E+01	2.8E+02	3.6E+02	3.0E+01	0.10	0.58	0.07	0.14	0.12	0.38
AA related	ornithine	1.5E+05	1.4E+05	1.2E+04	1.0E+05	1.8E+05	7.2E+03	0.14	0.58	< 0.01	0.00	0.54	0.78
AA related	citrulline	2.7E+04	2.6E+04	1.3E+03	2.4E+04	2.9E+04	1.2E+03	0.14	0.58	0.01	0.03	0.55	0.78
AA related	3-aminoisobutyric acid	1.7E+03	1.0E+03	2.7E+02	1.9E+03	8.3E+02	2.5E+02	0.15	0.58	0.00	0.01	0.01	0.24
AA related	phenaceturic acid	1.9E+04	1.8E+04	1.2E+03	1.6E+04	2.1E+04	1.0E+03	0.15	0.58	0.00	0.02	0.50	0.76
AA related	kynurenine	4.6E+03	6.0E+03	6.2E+02	3.8E+03	6.8E+03	4.8E+02	0.19	0.58	< 0.01	0.00	0.50	0.76

AA related	guanidinosuccinate	2.5E+02	3.0E+02	2.1E+01	2.3E+02	3.2E+02	1.9E+01	0.27	0.62	0.00	0.01	0.35	0.66
AA related	allantoic acid	5.5E+04	6.4E+04	3.6E+03	6.6E+04	5.3E+04	3.3E+03	0.31	0.64	0.02	0.04	0.11	0.36
AA related	cystine	7.8E+03	9.3E+03	7.6E+02	6.7E+03	1.0E+04	6.1E+02	0.37	0.71	< 0.01	0.00	0.45	0.72
AA related	5-aminovaleric acid	2.5E+03	2.5E+03	2.7E+02	2.9E+03	2.1E+03	2.5E+02	0.40	0.74	0.04	0.10	0.04	0.30
AA related	aminomalonate	1.8E+04	1.9E+04	1.4E+03	2.1E+04	1.6E+04	1.2E+03	0.57	0.85	0.04	0.09	0.21	0.55
AA related	3-(4-hydroxyphenyl)propionic acid	3.0E+02	3.3E+02	2.0E+01	2.8E+02	3.5E+02	1.9E+01	0.73	0.92	0.00	0.02	0.35	0.66
AA related	trans-4-hydroxyproline	1.9E+04	2.1E+04	1.5E+03	2.4E+04	1.6E+04	1.1E+03	0.91	0.98	0.00	0.01	0.35	0.66
AA related	phenylacetic acid	2.5E+03	2.7E+03	1.7E+02	2.2E+03	2.9E+03	1.5E+02	0.94	0.98	0.00	0.01	0.28	0.60
AA related	cystathionine	1.4E+03	1.5E+03	7.6E+01	1.3E+03	1.5E+03	7.5E+01	0.96	0.98	0.01	0.02	0.10	0.35
Bile acids	cholic acid	2.4E+04	2.9E+04	8.1E+03	4.0E+04	1.3E+04	7.6E+03	0.31	0.64	0.00	0.01	0.04	0.31
Bile acids	deoxycholic acid	2.9E+03	3.4E+03	1.1E+03	4.6E+03	1.7E+03	1.0E+03	0.34	0.67	0.01	0.02	0.07	0.33
Biogenic amines	phenylethylamine	1.7E+03	2.9E+03	6.2E+02	8.6E+02	3.7E+03	5.2E+02	0.25	0.62	< 0.01	0.00	0.01	0.24
Carbohydrates	xylose	6.3E+03	5.8E+03	4.1E+02	6.6E+03	5.5E+03	3.9E+02	0.00	0.22	0.16	0.28	0.94	0.97
Carbohydrates	erythritol	3.1E+03	3.7E+03	1.1E+02	3.5E+03	3.3E+03	1.3E+02	0.01	0.22	0.90	0.97	0.30	0.62
Carbohydrates	1,5-anhydroglucitol	6.0E+03	5.3E+03	6.9E+02	6.9E+03	4.4E+03	6.1E+02	0.06	0.58	0.02	0.04	0.32	0.65
Carbohydrates	xylulose	1.8E+03	1.3E+03	2.2E+02	1.8E+03	1.3E+03	2.2E+02	0.08	0.58	0.17	0.30	0.42	0.70
Carbohydrates	mannose	8.8E+04	9.0E+04	3.7E+03	8.2E+04	9.6E+04	3.4E+03	0.23	0.62	< 0.01	0.01	0.26	0.59
Carbohydrates	glycerol-α-phosphate	2.9E+03	4.1E+03	4.9E+02	2.8E+03	4.2E+03	4.7E+02	0.28	0.62	0.01	0.04	0.04	0.31
Carbohydrates	α-ketoglutarate	3.2E+03	2.9E+03	4.5E+02	2.2E+03	3.9E+03	3.8E+02	0.30	0.63	0.00	0.01	1.00	1.00
Carbohydrates	gluconic acid	9.3E+02	8.5E+02	1.3E+02	6.9E+02	1.1E+03	1.2E+02	0.63	0.87	0.01	0.03	0.72	0.88
Carbohydrates	glucose	1.2E+05	1.3E+05	6.7E+03	1.2E+05	1.3E+05	6.8E+03	0.73	0.92	0.01	0.02	0.22	0.55
Carbohydrates	galactonic acid	8.6E+02	8.4E+02	1.4E+02	6.4E+02	1.1E+03	1.3E+02	0.75	0.92	0.01	0.03	0.54	0.78
Carboxylic acids	isocitric acid	2.8E+03	2.6E+03	2.0E+02	3.0E+03	2.4E+03	1.9E+02	0.11	0.58	0.06	0.12	0.16	0.48
Carboxylic acids	aconitic acid	6.3E+02	6.0E+02	4.7E+01	7.1E+02	5.3E+02	4.0E+01	0.15	0.58	0.01	0.04	0.49	0.76
Carboxylic acids	citric acid	1.7E+05	1.6E+05	1.3E+04	1.9E+05	1.5E+05	1.2E+04	0.16	0.58	0.02	0.04	0.05	0.33
Carboxylic acids	salicylic acid	4.3E+02	4.7E+02	3.6E+01	3.8E+02	5.2E+02	3.1E+01	0.96	0.98	0.00	0.01	0.63	0.84
Cresols	p-tolyl glucuronide	1.2E+03	7.1E+02	1.3E+02	7.0E+02	1.2E+03	1.3E+02	0.05	0.51	< 0.01	0.00	0.00	0.21
Cresols	p-cresol	1.9E+04	1.7E+04	2.1E+03	1.3E+04	2.3E+04	1.6E+03	0.19	0.58	0.00	0.01	0.33	0.65
Fatty acids	arachidic acid	5.6E+03	5.4E+03	2.2E+02	5.5E+03	5.4E+03	2.3E+02	0.00	0.22	0.71	0.82	0.08	0.33
Fatty acids	stearic acid	1.4E+06	1.4E+06	9.6E+04	1.6E+06	1.2E+06	8.4E+04	0.04	0.51	0.03	0.07	0.05	0.33
Fatty acids	nonadecanoic acid	2.0E+03	2.0E+03	1.8E+02	2.4E+03	1.6E+03	1.5E+02	0.14	0.58	0.02	0.04	0.08	0.33
Fatty acids	linoleic acid	3.4E+03	3.3E+03	2.9E+02	4.0E+03	2.8E+03	2.4E+02	0.14	0.58	0.01	0.03	0.12	0.38
Fatty acids	myristic acid	8.0E+03	8.4E+03	1.1E+03	1.1E+04	5.5E+03	7.9E+02	0.41	0.74	< 0.01	0.01	0.06	0.33
Fatty acids	heptadecanoic acid	2.2E+04	2.4E+04	2.8E+03	3.1E+04	1.6E+04	2.1E+03	0.44	0.75	0.00	0.01	0.07	0.33
Fatty acids	palmitic acid	2.0E+05	2.1E+05	1.5E+04	2.3E+05	1.7E+05	1.2E+04	0.47	0.76	0.00	0.02	0.07	0.33
Fatty acids	arachidonic acid	1.4E+03	1.5E+03	1.6E+02	1.9E+03	1.1E+03	1.2E+02	0.65	0.87	0.00	0.02	0.22	0.55

Fatty acids	isolinoleic acid	6.5E+02	7.4E+02	6.6E+01	6.0E+02	7.8E+02	6.1E+01	0.78	0.93	0.04	0.09	0.98	0.99
Fatty acids	palmitoleic acid	2.6E+03	3.2E+03	6.1E+02	4.7E+03	1.2E+03	4.2E+02	0.85	0.97	< 0.01	0.00	0.06	0.33
Fatty acids	9-myristoleate	6.0E+03	6.4E+03	8.0E+02	7.6E+03	4.7E+03	7.0E+02	0.87	0.98	0.01	0.03	0.03	0.30
Fatty acids	cerotinic acid	3.5E+02	3.6E+02	3.1E+01	2.9E+02	4.2E+02	2.6E+01	0.98	1.00	0.01	0.03	0.29	0.61
Glycerides	1-monopalmitin	7.7E+03	9.1E+03	5.3E+02	7.2E+03	9.5E+03	4.6E+02	0.25	0.62	< 0.01	0.00	0.26	0.58
Glycerides	1-monostearin	4.6E+03	5.0E+03	3.1E+02	4.1E+03	5.6E+03	2.4E+02	0.96	0.98	< 0.01	0.00	0.37	0.66
Hydroxy acids	2-hydroxyglutaric acid	9.3E+02	8.7E+02	8.6E+01	1.1E+03	7.4E+02	7.2E+01	0.32	0.66	0.00	0.01	0.54	0.78
Hydroxy acids	2-hydroxybutanoic acid	1.6E+04	1.5E+04	2.1E+03	2.1E+04	1.1E+04	1.6E+03	0.45	0.75	0.00	0.02	0.28	0.60
Hydroxy acids	4-hydroxybutyric acid	2.3E+03	2.4E+03	1.9E+02	2.7E+03	1.9E+03	1.6E+02	0.70	0.92	0.00	0.02	0.85	0.96
Imides	maleimide	7.0E+03	8.9E+03	6.0E+02	7.1E+03	8.8E+03	6.1E+02	0.17	0.58	0.04	0.09	0.09	0.34
Indoles	indole-3-propionic acid	2.8E+03	4.4E+03	3.8E+02	3.4E+03	3.9E+03	4.4E+02	0.09	0.58	0.02	0.06	0.74	0.90
Indoles	indoxyl sulfate	6.2E+02	6.5E+02	7.0E+01	4.7E+02	8.0E+02	5.7E+01	0.88	0.98	< 0.01	0.00	0.93	0.97
Inorganic	phosphate	1.2E+05	1.3E+05	6.9E+03	1.1E+05	1.4E+05	6.9E+03	0.43	0.75	0.01	0.03	0.01	0.29
Keto-acids	2-ketobutyric acid	3.8E+03	3.8E+03	3.6E+02	3.1E+03	4.5E+03	2.9E+02	0.59	0.85	0.02	0.04	0.21	0.55
Nitrogenous bases	uric acid	9.3E+03	7.2E+03	9.8E+02	1.0E+04	6.4E+03	8.4E+02	0.01	0.28	0.01	0.02	0.38	0.66
Nucleoside and nucleotides	pseudo uridine	8.8E+03	9.6E+03	9.2E+02	1.2E+04	6.8E+03	6.8E+02	0.94	0.98	0.00	0.01	0.44	0.70
Phenol esters	4-hydroxyphenylacetic acid	9.8E+02	1.3E+03	1.4E+02	1.5E+03	8.2E+02	1.2E+02	0.17	0.58	0.00	0.01	0.53	0.78
Quinolones and derivatives	2,8-dihydroxyquinoline	2.3E+02	2.2E+02	1.9E+01	2.6E+02	1.8E+02	1.6E+01	0.26	0.62	0.00	0.01	0.57	0.79
Sterols	cholesterol	1.6E+05	2.2E+05	1.1E+04	1.8E+05	2.0E+05	1.3E+04	0.05	0.51	0.09	0.17	0.88	0.97
Sugar alcohol	xylitol	7.7E+03	7.4E+03	5.7E+02	8.6E+03	6.5E+03	5.0E+02	0.07	0.58	0.02	0.06	0.14	0.42
Sugar alcohol	isothreitol	1.1E+03	1.1E+03	5.5E+01	1.2E+03	9.4E+02	4.0E+01	0.49	0.79	< 0.01	0.00	0.64	0.85
Sugar alcohol	glycerol	1.3E+05	1.3E+05	1.1E+04	1.6E+05	1.0E+05	8.7E+03	0.60	0.85	0.00	0.02	0.39	0.66
Terpenoids and derivatives	phytanic acid	8.3E+03	9.4E+03	7.2E+02	7.3E+03	1.0E+04	5.9E+02	0.44	0.75	0.00	0.02	0.59	0.80
Vitamins and cofactors	α-tocopherol	6.1E+03	8.3E+03	5.6E+02	6.2E+03	8.2E+03	5.7E+02	0.03	0.49	0.01	0.04	0.24	0.58

1: Target retention index in the BinBase database system (Fiehn Lab), where RI is obtained as the conversion of absolute retention times to relative retention times based on a set of pre-defined internal standards, using Fiehn retention indices; 2: m/z value used to quantify peack height for each BinBase database entry; 3: genetic strain; 4: days in milk; 5: raw P-value according to ANOVA; 6: P-value adjusted by FDR.

3.5.3. Univariate analysis

According to ANOVA analysis, 67 of the quantified metabolites varied with DIM (FDR < 0.05; Table 3.2) whereas there was no metabolite affected by genetic strain (FDR > 0.10). Amino acids (n = 12) or AA-related compound (n =15) represented 40% of the metabolites differing between stage of lactation, being most of them (23/27) associated with lower concentrations at 21 than 180 DIM (relative peak height -25 % on average, FDR < 0.05). Few AA-related compounds (e.g.: N-acetylglycine, 3-aminoisobutyric acid, trans-4-hydroxyproline, and allantoic acid) were greater (FDR ≤ 0.05) at 21 than 180 DIM (Table 3.2). In addition, almost all the fatty acids (8/9) affected by DIM as well as all the hydroxy acids and derivatives (3/3), sugar alcohols (2/2) and bile acids (1/2) were greater (FDR ≤ 0.05) at 21 than 180 DIM, while glycerides (2/2) and carbohydrates and related compounds (6/7) were less at 21 when compared with 180 DIM (Table 3.2). Despite none of the quantified metabolites had an FDR ≤ 0.05 when comparing NAH vs. NZH cows, 16 metabolites had a raw-P ≤ 0.05 for the genetic strain effect, being most of them AA or AA-related compounds (n = 12) and fatty acids (n = 2) with lower concentrations for NZH than NAH cows (Table 3.2).

Additionally, only the alanine was affected (FDR = 0.02) by the interaction between genetic strain and DIM as it was lower at 21 DIM but greater at 180 DIM for NZH vs. NAH dairy cows (Table 3.2; Figure 3.2). However, changing patterns of metabolites between genetic strains and stage of lactation (DIM) were also assessed by both, ASCA and the ranking top 10 MEBA. Valid models for DIM (P < 0.01, 1/100 permutations) and for the interaction between genetic strain and DIM (P = 0.04, 4/100 permutations) (Suppl. Figure 1). Four metabolites were well modelled (leverage > 0.040, SPE < 1.5×10^{-30}) by the interaction, among which dehydroabietic acid was increased while 3-aminoisobutyric acid was decreased for NZH than NAH cows only at 21 DIM. Serine increased at mid lactation only for NZH cows. and uracil were greater for NZH than NAH cows at 180 DIM (Figure 3.2). Alanine, BCAA (valine, isoleucine, leucine), arachidic acid, oxoproline, squalene, p-tolyl glucuronide, erythritol and serine were in the top-10 ranked metabolites according to Hotteling's T^2 (Hotelling's T^2 > 10.0, Suppl. Table 3). Plasma BCAA were less while erythritol

concentrations were greater for NZH than NAH cows at 21 DIM. In addition, p-tolyl glucuronide increased at 180 DIM for NAH cows while it remained unchanged for the NZH ones (Figure 3.2c). Except for uracil (raw-P = 0.054), arachidic acid (raw-P = 0.08), erythritol and oxoproline (raw-P > 0.10, Figure 3.2), all of the metabolites affected by the interaction —identified by both by ASCA and/or the ranking top 10 MEBA— had a raw P ≤ 0.05 for the genetic strain by DIM interaction assessed by ANOVA.

3.5.4. Metabolic pathway analysis

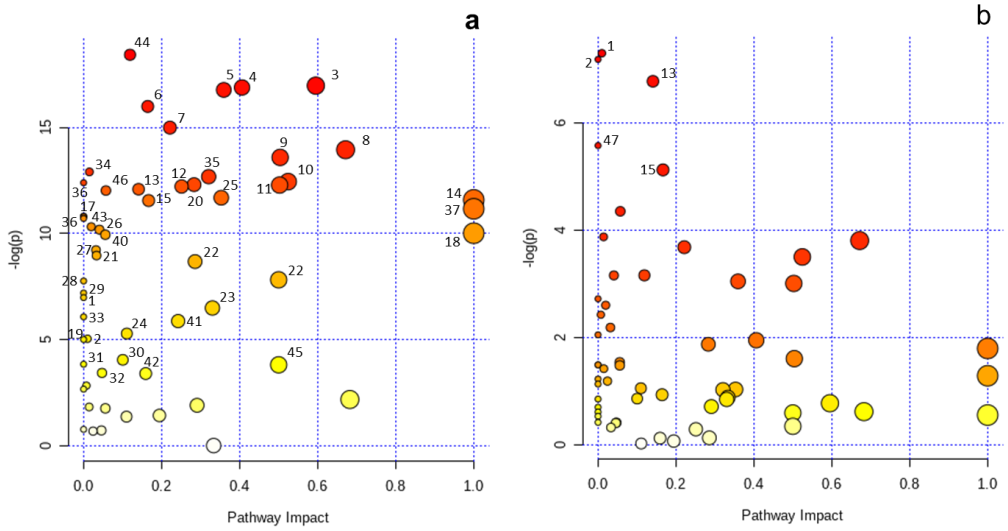

Figure 3.3. Metabolic pathway análisis based on metabolic enrichment anaysis and topoligc analysis of metabolomic profiles comparing metabolic pathways shifts when comparing 21 and 180 days in milk (a) or NZH vs. NAH cows at 21 days in milk. Increasingly red colors indicate lower P-values (more significative shift in the pathway), greater circle size indicates greater pathway impact. Numerous depict pathways with signicatively regulation shift: 1: Branched-chain aminoacids biosynthesis, 2: branched-chain aminoacids degradation, 3: phenylalanine metabolism, 4: arginine biosynthesis, 5: arginine and proline metabolism, 6: tyrosine metabolism, 7: histidine metabolism, 8: alanine, aspartate and glutamate metabolism,

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9: β-Alanine metabolism, 10: glycine, serine and threonine metabolism, 11: cysteine and methionine metabolism, 12:tryptophan metabolism, 13: lysine degradation, 14: phenylalanine, tyrosine and tryptophan biosynthesis, 15: aminoacyl-tRNA biosynthesis, 16: taurine and hypotaurine metabolism, 17: thiamine metabolism, 18: glutamine and glutamate metabolism, 19: nitrogen metabolism, 20: glyoxylate and dicarboxylate metabolism, 21: fructose and mannose metabolism, 22: galactose metabolism, 23: pentose and glucuronate interconversions, 24: amino sugar and nucleotide sugar metabolism, 25: citrate cycle, 26: propanoate metabolism, 27: butanoate metabolism, 28: ubiquinone and other terpenoid-quinone biosynthesis, 29: biotin metabolism, 30: glycolysis / gluconeogénesis, 31: synthesis and degradation of ketone bodies, 32: pentose phosphate pathway, 33: porphyrin and chlorophyll metabolism, 34: fatty acid biosynthesis, 35: arachidonic acid metabolism, 36: fatty acid elongation, 37: linoleic acid metabolism, 38: biosynthesis of unsaturated fatty acids, 39: fatty acid degradation, 40: primary bile acid biosynthesis, 41: glycerolipid metabolism, 42: inositol phosphate metabolism, 42: purine metabolism, 43: glutathione metabolism, 44: ascorbate and aldarate metabolism, 45: pantothenate and CoA biosynthesis.

This analysis revealed that 46 metabolic pathways differed (FDR $\leq$ 0.05) in their regulated state when comparing early and mid-lactation. Most of them (19/46) corresponded to AA and protein metabolism including, BCAA metabolism (both biosynthesis and degradation), essential AA metabolism (all of them), and glutamine and glutamate metabolism, among others (Table 3.3). With the exception of few metabolites (e.g.: glycine, glycerate, 4-hydroxiphenylacetate, indole-3-acetate, β-alanine and 3-ureidopropionate), these pathways were associated with decreased concentrations at 21 than 180 DIM (Figure 3.4; Supl. Figure 2). In addition, some pathways involved in carbohydrates metabolism likewise glyoxylate and dicarboxylate metabolism, and pentose and glucuronate interconversions were also affected (FDR < 0.05) by the lactation stage being half of the metabolites involved in

these pathways increased (e.g.: glycine, citrate) and half of them decreased (e.g.: pyruvate, glutamine) at 21 vs. 180 DIM. Half of the metabolites involved in glyoxylate and dicarboxylate metabolism pathway were associated with positive foldchanges (e.g.: glycine, citrate; raw-P < 0.05) and half of them with negative foldchanges (e.g.: pyruvate, glutamine; raw-P < 0.05) concentrations at 21 vs. 180 DIM, however, non-significant differences were detected after raw-P adjustment (FDR > 0.05; Suppl. Table 3.2; Suppl. Figure 3A). Within the pentose and glucuronate interconversions pathway, most of its metabolites (5/6) had positive foldchange for 21 vs. 180 DIM, but significant differences were not detected (FDR > 0.05) for none of them while a raw-P < 0.05 was only observed for xylitol (Supl. Figure 3A, Suppl. Table 2). Central energy pathways included citrate 62 cycle, butanoate metabolism, ketone bodies synthesis and degradation among others (Table 3.3) and were related to some metabolites such as citrate and cis-aconitate increased (FDR < 0.05) at 21 vs. 180 DIM, while α-ketoglutarate and α-ketobutyric acid were decreased (FDR ≤ 0.04) at this time (Suppl. Figure 3B).

Others metabolic pathways affected (FDR < 0.05) by DIM included primary bile acids biosynthesis which was related with positive foldchange of the involved metabolites for 21 vs. 180 DIM (cholic acid, FDR = 0.01; glycine, FDR = 0.09; Supl. Figure 3C). In addition, 9 metabolic pathways that participate in lipid metabolism were affected (FDR < 0.05) by DIM, depicting metabolic changes as lactation advanced such as in the biosynthesis of fatty acids and unsaturated fatty acids, fatty acid degradation, arachidonic and linoleic acids, and myo-inositol phosphate metabolism. The shifts in these metabolic pathways were mostly related with increased concentrations of fatty acids and metabolic intermediates from 21 to 180 DIM. These included myristic, linoleic, palmitic, stearic, glycerol, glycerate and glucuronate (Figure 3.4; Supl. Figure 3D). Finally, metabolic pathways belonging to nitrogen metabolism as well as redox, and vitamins and coenzymes metabolism were also observed to be affected by DIM. Nitrogen metabolism changes included purine metabolism as uric and allantoic cid had a positive fold change for 21 vs. 180 DIM (FDR < 0.05). Redox metabolism included ascorbate and aldarate metabolism, and glutathione metabolism but no clear trend was observed within the measured

metabolites participating in these pathways (Supl. Figure 3E, 3F and 3G). Finally, most of metabolites included in the pantothenate and CoA biosynthesis pathway such as valine and aspartate were decreased (FDR < 0.05) at 21vs. 180 DIM.

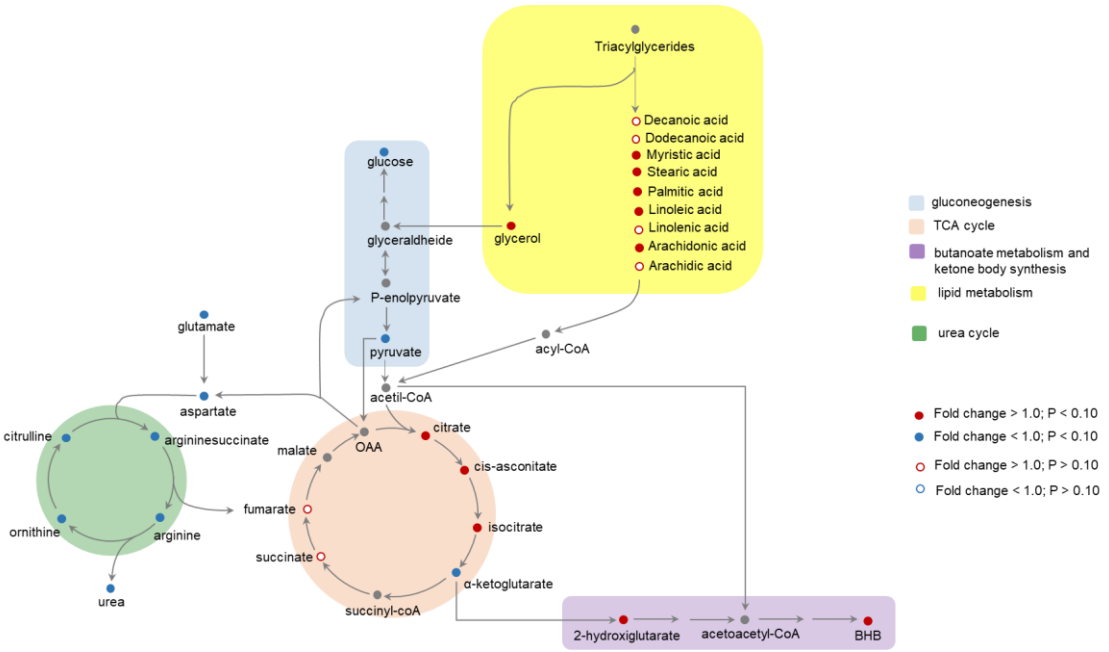

Figure 3.4. Main metabolic pathways shift regulated at early vs. mid lactation stage. Coloured cirlces depict efectively measured compound while gray circles correspond to not measured or detected compounds.

Table 3.3. Metabolic pathways differentially regulated accross lactation stages or genetic strains

Main metabolism	**Metabolic pathways**	**Total comp**[1]	**Hits**[2]	***raw-P***[3]	***FDR***[3]	**Impact**[4]
1. 21 vs. 180 DIM						
AA and protein	Phenylalanine metabolism	12	6	4.3E-08	7.6E-07	0.60
AA and protein	Arginine biosynthesis	14	8	4.6E-08	7.6E-07	0.41
AA and protein	Arginine and proline metabolism	38	6	5.2E-08	7.6E-07	0.36
AA and protein	Tyrosine metabolism	42	4	1.1E-07	1.3E-06	0.16
AA and protein	Histidine metabolism	16	3	3.1E-07	3.0E-06	0.22
AA and protein	Alanine, aspartate and glutamate metabolism	28	11	8.7E-07	7.2E-06	0.67
AA and protein	β-Alanine metabolism	21	5	1.3E-06	9.1E-06	0.50
AA and protein	Glycine, serine and threonine metabolism	34	8	3.9E-06	1.9E-05	0.52
AA and protein	Cysteine and methionine metabolism	33	8	4.6E-06	1.9E-05	0.50
AA and protein	Tryptophan metabolism	41	4	4.9E-06	1.9E-05	0.25
AA and protein	Lysine degradation	25	3	5.6E-06	2.0E-05	0.14
AA and protein	Phenylalanine, tyrosine and tryptophan biosynthesis	4	2	9.3E-06	2.8E-05	1.00
AA and protein	Aminoacyl-tRNA biosynthesis	48	19	9.5E-06	2.8E-05	0.17
AA and protein	Taurine and hypotaurine metabolism	8	1	2.0E-05	5.0E-05	0.00
AA and protein	Thiamine metabolism	7	1	2.0E-05	5.0E-05	0.00
AA and protein	D-Glutamine and D-glutamate metabolism	5	3	4.4E-05	9.2E-05	1.00
AA and protein	Valine, leucine and isoleucine biosynthesis	8	6	9.2E-04	1.5E-03	0.00
AA and protein	Valine, leucine and isoleucine degradation	40	4	6.4E-03	9.3E-03	0.01
AA and protein	Nitrogen metabolism	6	2	6.6E-03	9.4E-03	0.00
Carbohydrates	Glyoxylate and dicarboxylate metabolism	32	9	4.5E-06	1.9E-05	0.28

Carbohydrates	Fructose and mannose metabolism	20	2	1.3E-04	2.4E-04	0.03
Carbohydrates	Galactose metabolism	27	10	1.7E-04	3.1E-04	0.29
Carbohydrates	Pentose and glucuronate interconversions	18	6	4.0E-04	7.0E-04	0.50
Carbohydrates	Amino sugar and nucleotide sugar metabolism	37	5	5.0E-03	7.5E-03	0.11
Energy central	Citrate cycle (TCA cycle)	20	7	8.4E-06	2.7E-05	0.35
Energy central	Propanoate metabolism	23	4	3.8E-05	8.1E-05	0.04
Energy central	Butanoate metabolism	15	6	9.8E-05	1.9E-04	0.03
Energy central	Ubiquinone and other terpenoid-quinone biosynthesis	9	1	4.2E-04	7.2E-04	0.00
Energy central	Biotin metabolism	10	1	7.4E-04	1.2E-03	0.00
Energy central	Glycolysis / Gluconeogenesis	26	1	1.7E-02	2.4E-02	0.10
Energy central	Synthesis and degradation of ketone bodies	5	1	2.1E-02	2.9E-02	0.00
Energy central	Pentose phosphate pathway	22	3	3.2E-02	4.1E-02	0.05
Heme biosynthesis	Porphyrin and chlorophyll metabolism	30	2	2.3E-03	3.5E-03	0.00
Lipid	Fatty acid biosynthesis	47	4	2.5E-06	1.6E-05	0.01
Lipid	Arachidonic acid metabolism	37	1	3.1E-06	1.8E-05	0.32
Lipid	Fatty acid elongation	39	1	4.1E-06	1.9E-05	0.00
Lipid	Linoleic acid metabolism	5	1	1.4E-05	3.9E-05	1.00
Lipid	Biosynthesis of unsaturated fatty acids	36	6	2.1E-05	5.0E-05	0.00
Lipid	Fatty acid degradation	39	2	2.2E-05	5.2E-05	0.00
Lipid	Primary bile acid biosynthesis	46	3	4.8E-05	9.5E-05	0.06
Lipid	Glycerolipid metabolism	16	2	1.5E-03	2.4E-03	0.33
Lipid	Inositol phosphate metabolism	30	4	3.3E-02	4.2E-02	0.16
Nitrogenous bases	Purine metabolism	66	4	3.3E-05	7.3E-05	0.02

Redox	Glutathione metabolism	28	5	1.0E-08	5.8E-07	0.12
Redox	Ascorbate and aldarate metabolism	10	4	2.2E-02	2.9E-02	0.50
Vitamins and coenzymes	Pantothenate and CoA biosynthesis	19	7	6.0E-06	2.0E-05	0.06
Lipid	Steroid hormone biosynthesis	75	1	5.8E-02	7.2E-02	0.01
Anitbiotic biosynthesis	Neomycin, kanamycin and gentamicin biosynthesis	2	2	6.9E-02	8.3E-02	0.00
2. NAH vs. NZH cows at early lactation (21 DIM)						
AA and protein	Valine, leucine and isoleucine degradation	40	4	6.8E-04	2.2E-02	0.01
AA and protein	Valine, leucine and isoleucine biosynthesis	8	6	7.7E-04	2.2E-02	0.00
AA and protein	Lysine degradation	25	3	1.2E-03	2.2E-02	0.14
Redox	Selenocompound metabolism	20	1	3.8E-03	5.5E-02	0.00
AA and protein	Aminoacyl-tRNA biosynthesis	48	19	6.0E-03	6.9E-02	0.17

1: Total metabolites theoretically considered by the KEGG database for the current metabolic pathways

2: Metabolites efectively quantified in the current study and belonging to the identified pathway

3: Raw P and FDR-adjusted P values obtained with the Global Test

4: Topological analysis of impact of the current metabolic pathway

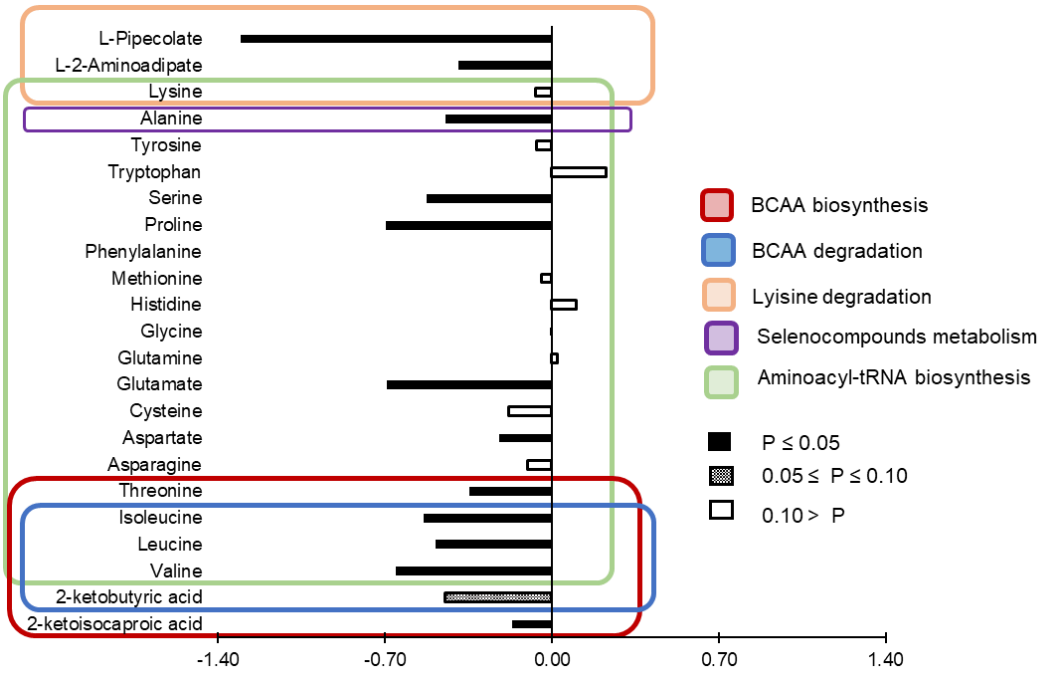

Figure 3.5. Log 2 fold change of quantified metabolites belonging to metabolic pathways with differentially regulated (FDR < 0.10) between NAH and NZH cows at 21 days inmilk. Negative values mean lower ion intensity in NZH compared to NAH cows. Black bars indicate $P \leq 0.05$, while gray are metabolites with $0.05 < P \leq 0.10$ and white not signifcant difference between genetic strains according to anova analysis at this time. Background colours indicate metabolic pathways.

With a deeper insight at early lactation (21 DIM), metabolic pathways such as BCAA biosynthesis and degradation (FDR = 0.022), and lysine degradation (FDR = 0.022) were differentially-regulated when comparing NZH vs. NAH cows (Figure 3.3, Figure 3.5). Changes in BCAA metabolism pathways were related to decreased concentrations of valine, leucine, isoleucine, 2-ketobutyric and 2-ketoisocaproic acid for NZH than NAH cows. In addition, selenocompounds metabolism as well as aminoacyl-tRNA biosynthesis tended to be affected (FDR < 0.070) when comparing genetic strains as most of its involved metabolites (16/19) had a negative fold change when NZH were compared to NAH cows. No metabolic pathways were detected to be differentially expressed when comparing genetic strains at 180 DIM.

3.6. DISCUSION

Metabolomics has successfully been used to describe metabolic changes occurring during the transition period, not only related to lipid metabolism[6,7,25] but also to AA and protein metabolism[26,27]. Our results indicated that homeorhetic changes in grazing dairy cows involved general AA metabolism rather than specific or isolated AA and AA-related compounds. Furthermore, differences in the metabolic adaptive responses between NAH and NZH cows included several changes in AA metabolism which could be associated to glucose homeostasis and insulin sensitivity[11] and a chronic activation of the mTOR pathway[28].

The general trend for decreased plasma concentrations of AA at early lactation was in agreement with previous reports[29] and indicated that AA balance was reduced at this time when compared with mid-lactation stage. Plasma AA reflect the balance between protein synthesis and degradation and thus, decreased AA concentrations reflected the negative protein balance. Negative protein balance is associated to increased AA requirements for milk protein synthesis and gluconeogenesis together with a decreased dietary supply due to the restricted dry matter intake after calving[30,31]. In contrast to the general trend for decreased plasma AA, the greater plasma glycine and trans-4-hydroxyproline in early than mid-lactation (21 relative to 180 DIM) was in agreement with previous reports[32]. Muscle protein catabolism coupled with increased AA *de novo* synthesis and limited utilization by the mammary gland led to plasma enrichment and muscle concentration nadir of these two AA after calving[32,29]. Furthermore, trans-4-hydroxyproline is released during collagen breakdown, therefore greater plasma concentrations could be indicative of increased muscle protein breakdown[33]. Indeed, increased skeletal muscle catabolism during the transition period for the cows of the current experiment was previously indicated by increased 3-methylhistidine previous to calving (data non shown).

In addition to increased muscle catabolism, liver uptake of AA was reported to be enhanced at parturition (reviewed by Larsen and Kristensen[34]) and decreased AA plasma concentrations during the transition period has also been related to a downregulation state of the urea cycle[32,8]. In the present study, all measured intermediates of the urea cycle were decreased at early lactation which was consistent

with the decreased urea concentrations observed at 21 DIM. Previous studies have also observed a decreased activity of urea cycle enzymes around parturition[35,36] associated with increased hepatic triglyceride accumulation which may account for decreased ureagenesis[37].

Several AA well known to be metabolic shuttles between AA metabolism and energy pathways, differed between lactation stages. Specifically, glutamine which is a major gluconeogenic AA was decreased at early lactation which is in agreement with the increased requirement of glucose for milk synthesis. Furthermore, both, not only glutamine but glutamate, which can act as a shuttle between urea cycle and TCA, were decreased at early lactation. Recently, Luo et al.[8] found that plasma concentrations of glutamate decreased from prepartum to calving and suggested this should be related with urea cycle changes affecting TCA activity through the formation of fumarate which in turn was increased at early lactation in the present study.

The increase on energy and nutrient requirements for milk synthesis after calving[38] highlight the central role that TCA plays in cell's energy supply as indicated by the increased in most of the TCA cycle intermediates in this work. Indeed, metabolic pathway analysis indicated that TCA cycle was enhanced at early lactation[39]. In fact, increased plasma concentrations of citrate and isocitrate observed in our work agreed with previous research which have demonstrated that citrate synthase and isocitrate dehydrogenase activities were increased during negative energy balance of early lactation[40]. Moreover, increased TCA activity during early lactation is concordant with increased lipid mobilisation and fatty acid oxidation for energy production and glucose demand[41].

Adipose tissue mobilization after calving, increases fatty acids availability for energy production through β-oxidation[36] which was reflected by the increased plasma NEFA and particularly, by the increase of saturated fatty acids concentrations[42]. In this regard, the increased plasma concentrations of palmitic and stearic acids, in early than late lactation would indicate enhanced lipolysis as they are main components of adipose tissue. Additionally, unsaturated fatty acids such as linoleic, linolenic and arachidonic acids, which have been suggested to be associated with oxidative stress and proinflammatory states, were also increased at early lactation[7]. Increased plasma

concentrations of polyunsaturated fatty acids would provide a nexus between lipid mobilization and oxidative stress and inflammation during the transition period[43]. Polyunsaturated fatty acids are also known precursors for oxylipid synthesis[44] and can be metabolized through the cyclooxygenase/lipoxygenase pathway leading to eicosanoid synthesis. Eicosanoids can act as signalling molecules in the regulation of inflammation process as they participate in its initiation, progression and resolution[43]. Our data suggested increased oxidative load at early lactation which could be associated with increased antioxidant activity[†]. In fact, metabolic pathways analysis revealed that ascorbic acid and glutathione metabolism pathways were shift-regulated at early when compared to mid lactation. Ascorbic acid metabolism was linked to greater plasma concentrations of D-glucuronic acid, saccharic acid and myo-inositol suggesting increased synthesis of vitamin C. Glucose is the sole precursor for vitamin C in cattle via D-glucuronic[45]. It has been suggested that vitamin C synthesis might be highly prioritized during early lactation as similar plasma concentrations were observed in ketotic vs. healthy cows despite their different energy status[46].

When hepatic fatty acid oxidation exceeds TCA oxidative capacity, ketone body synthesis is an alternative pathway to provide energy fuels to peripheral tissues as glucose availability decreased due to milk synthesis[47]. In the current experiment, decreased plasma concentrations of ketogenic AA such as lysine, tyrosine and phenylalanine at early lactation may account for increased ketone body synthesis as it has been previously observed in dairy cows experiencing ketosis[48]. This is further supported by the metabolic pathway analysis that revealed the butanoate metabolism was differentially expressed between stages of lactation. The decreased concentrations of α-ketoglutarate in early lactation in could be due, at least in part, to an increased activity of 2-hydroxyglutarate dehydrogenase leading to the observed greater concentrations of 2-hydroxyglutarate. Increased 2-hydroxyglutarate could be related to increased ketone body synthesis as it is used in the synthesis of acetoacetyl-CoA which is metabolized via acetoacetyl-CoA and β-hydroxy-β-methylglutaryl-CoA (HMG-CoA) in the liver mitochondria[49]. Taken together, our metabolomic results

[†] Corresponde al artículo 1, en evaluación en *Livestock Science*, capítulo II de esta tesis.

confirmed increased ketogenesis during early lactation[50] in agreement with the greater concentrations of BHB.

Metabolic pathway analysis found differences between NAH and NZH cows only at early lactation, suggesting that the effect of genetic strain on metabolic adaptive responses is exacerbated in highly challenging conditions such as the onset of lactation. Differences were mostly related to plasma concentrations of AA or AA-related compounds which most of these metabolites were increased for NAH than NZH cows at early lactation. Indeed, greater BCAA plasma concentrations for NAH than NZH cows could be related to their higher insulin resistance in early lactation[11]. (Fig. 3.6) Circulating BCAA, which are poorly catabolised in the liver, can act as signalling molecules sensing the nutritional state for peripheral tissues and bringing out cellular signalling cascades[51]. Notwithstanding that the relationship between insulin resistance and BCAA has been recently suggested in ruminants[27], it is well stablished other species[52], the exact mechanisms remain to be known. In this sense, insulin and BCAA upregulate the mTOR signalling pathway[48], which participate in several cell process including protein and lipid synthesis[53]. Despite upstream activation pathways of mTOR differ between insulin and BCAA, insulin fails to stimulate mTOR downstream cascade in the absence of AA[54]. Moreover, in humans, it has been proposed that high BCAA concentrations would indirectly impair insulin sensitivity through the chronic activation of the mTOR pathway leading to over-phosphorylation of the insulin receptor substrate 1[28,55] which decreased expression and activity of glucose transporter 4[56]. However, the linking mechanism between insulin resistance and BCAA is not completely understood and whether high concentrations of BCAA are cause or consequence of insulin resistance development still remains to be known.

On the other hand, greater BCAA concentrations for NAH than NZH cows could also be consequence of increased muscle protein mobilisation at early lactation, which was in agreement with their higher plasma concentrations of 3-methylhistidine (data not shown). Hypothetically increased activity of mTOR for NAH cows would enhance protein synthesis[54]. Thus, it is possible that NAH cows had not only greater muscle mass catabolism but also protein synthesis, leading to increased protein and

AA turnover, when compared with NZH cows. Indeed, Ghaffari et al.[9] suggested that lower insulin sensitivity in obese dairy cows would be associated with increased protein turnover when compared to lean cows.

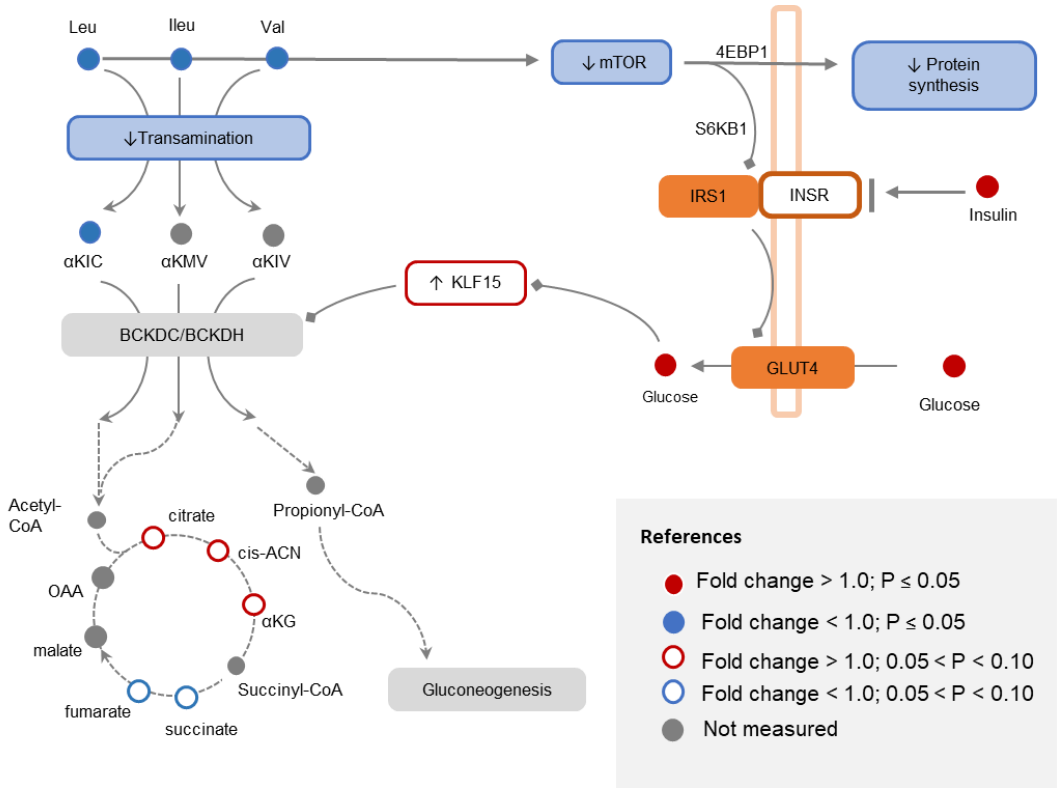

Figure 3.6. Integrative metabolic interpreation map of BCAA and insulin sensitivity in NZH vs. NAH cows at early lactation stage. Lower BCAA concentrations in NZH cows are expected to down-regualte mTOR chronic activity leading to decreased downstream cascades and so that lower phosphorilation of the insulin substrate receptor I, which in turn could determine a greater insulin sensitivity associated with increased gene expresión of GLUT 4 transporter. In addition, greater intracelular concentrations of glucose would be linked with an up-regulated state of the negative feedback on BCAA decarboxylation through the KLF 15 gene factor. Finnally, BCAA degradation products interact with energy production by entering into the Krebs cycle or gluconeogenesis pathways.

Plasma concentrations of alanine and α-ketoisocaproic were greater for NAH than NZH cows suggesting an increased BCAA catabolism[57]. It has been observed that low intracellular glucose concentrations, due to reduced glucose uptake by peripheral tissue associated with the diminished insulin sensitivity would enhance BCAA degradation through the glucose- Krüppel-like factor 15 (KLF15)-BCAA axis[58]. These authors demonstrated that low glucose down-regulate the negative feedback of the KLF15 transcriptional regulator factor on BCAA decarboxylation step which is the limiting rate step of BCAA catabolism. In addition, both alanine as well as degradation products of BCAA replenish the oxalacetate pool or enter TCA cycle in order to produced glucose or energy, reflecting the interconnection between protein and energy metabolism[56]. Actually, a greater oxalacetate redirection from the TCA cycle towards gluconeogenesis would determine a down-regulation of this cycle (cataplerosis) leading to reduced citrate, aconitate and a-ketoglutarate concentrations for NAH than NZH cows (Fig. 3.6) in early lactation. On the other hand, increased valine catabolism in addition to greater DMI leading to greater propionyl-CoA in the NAH cows could explain their plasma concentrations of increased succinate and fumarate. In a whole, a greater BCAA degradation rate for NAH vs. NZH cows would be in agreement with the reduced plasma glucose concentrations and decreased TCA cycle activity.

Moreover, other metabolites such as α-aminoadipic, L-pipecoalate and 3-aminoisobutiryc that were increased for NAH than NZH cows, are probably also related to greater insulin resistance and protein and AA turnover in the former ones. The α-aminoadipic acid is a product of lysine degradation and has been previously reported as pre-diabetic biomarker in rodents and humans[59] as well as in dairy cows[9]. Both, α-aminoadipic acid and L-pipecolate, another metabolite of the lysine degradation pathway, have been reported to be elevated in blood of patients with liver injury and peroxisomal disorders in humans[60]. Although no differences in plasma NEFA were observed between genetic strains, the greater concentrations of 3-aminoisobutiryc, which is known to activate the expression of thermogenic genes through the peroxisome proliferator-activated receptor α receptors, could indicate increased lipolysis for NAH than NZH cows in agreement with reduced BCS and body retained energy[13].

3.7. CONCLUSIONS

Although muscle mass mobilization is well known to structurally participate in homeorhetic changes during the transition period, our metabolomic study demonstrated that AA and AA-related compounds participated in metabolic adaptations during the onset of lactation. Decreased plasma AA were observed concomitant with increased fatty acid concentrations in early lactation and a downregulated state of the urea cycle together with an upregulation of the TCA cycle. In addition, AA metabolism appeared to be also implicated on genetic strain adaptive capacity to cope with transition challenges, probably through insulin resistance modulation by BCAA signaling roles. Moreover, BCAA and other specific compounds related to lipid mobilization were greater for NAH than NZH cows suggesting these metabolites may be involved in metabolic responses due to the negative energy balance of the onset of lactation and particularly to challenges imposed by grazing systems.

3.8. AKNOWLEDGEMENTS

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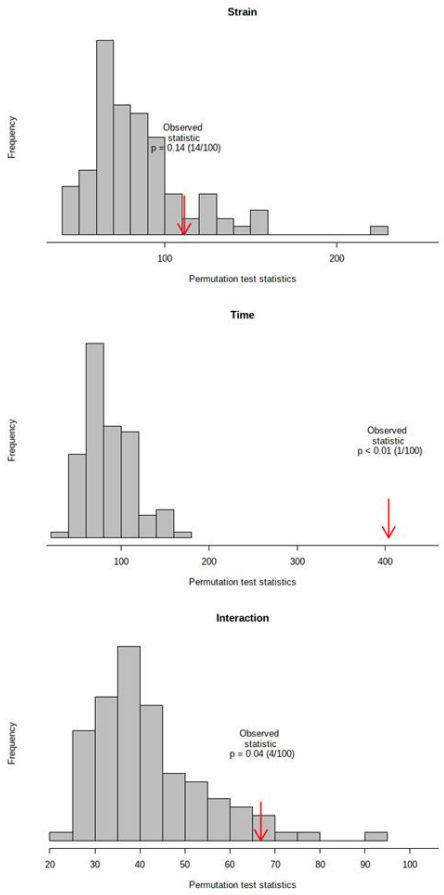
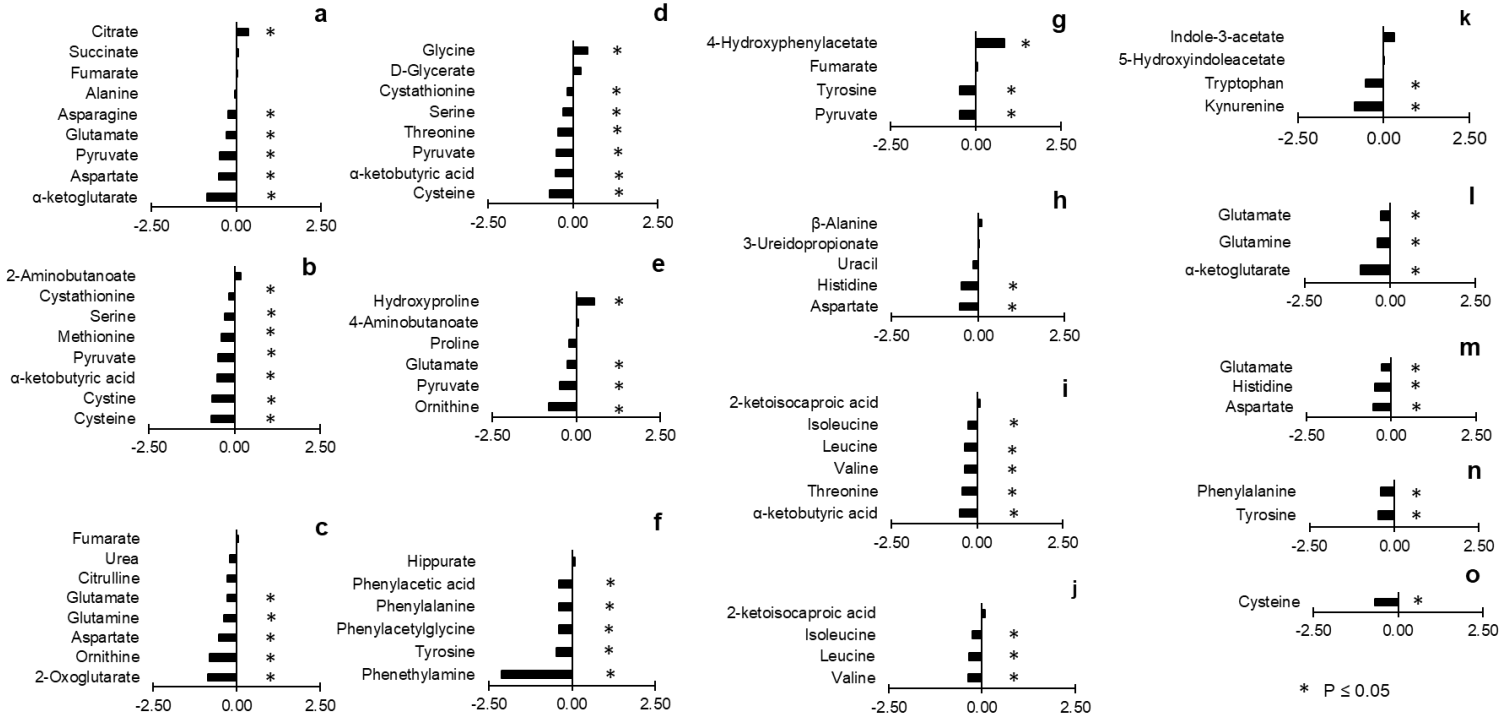
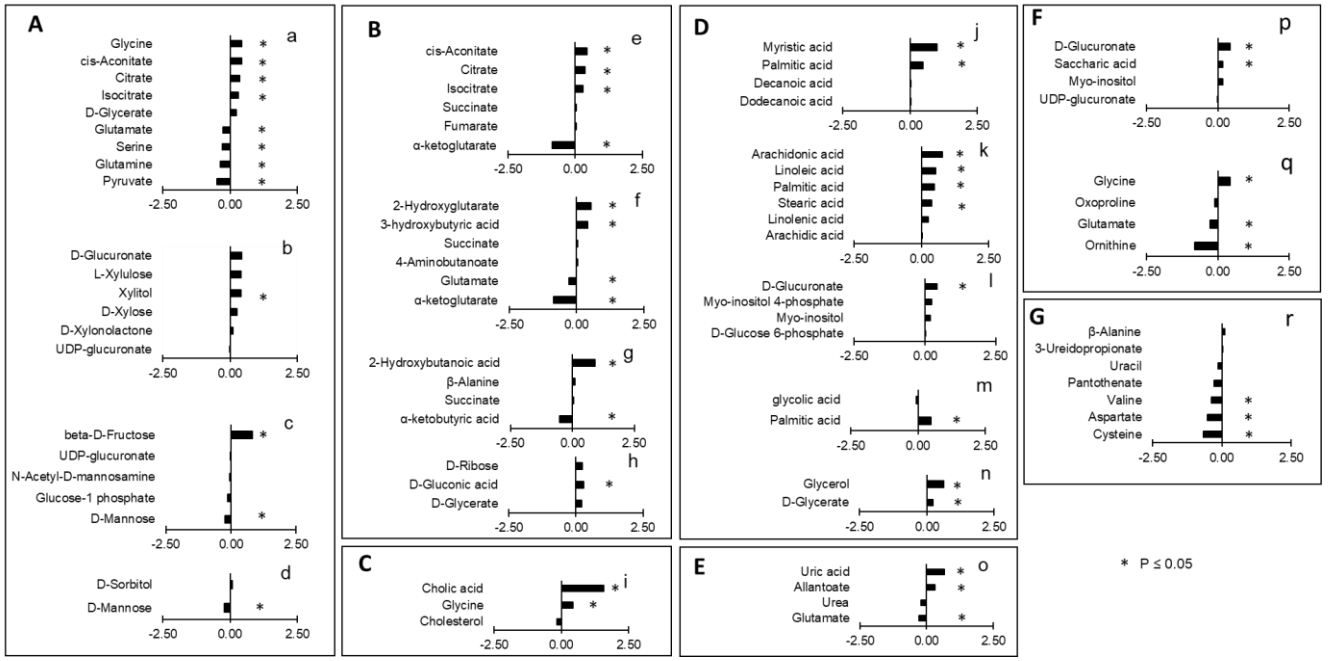
3.10. SUPPLEMENTAL MATERIAL



Supl. Figure 1. Validation statistics for fixed effects of ASCA analysis.



Supl. Figure 2. Log 2 fold change of measured metaboites belonging to AA and nitrogen metabolic pathways differing between 21 and 180 DIM: a) Alanine, aspartate and glutamate metabolism; b) Cysteine and methionine metabolism; c) Arginine biosynthesis; d) Glycine, serine and threonine metabolism; e) Arginine and proline metabolism; f) Phenylalanine metabolism; g) Tyrosine metabolism ; h) beta-Alanine metabolism ; i) Valine, leucine and isoleucine biosynthesis; j) Valine, leucine and isoleucine degradation; k) Tryptophan metabolism ; l) D-Glutamine and D-glutamate metabolism; m) Histidine metabolism; n) Phenylalanine, tyrosine and tryptophan biosynthesis; o) Thiamine metabolism, and taurine and hypotaurine metabolism.



Supl Figure 3. Log 2 fold change of measured metaboites belonging to: A) carbohydrates metabolism [a) Glyoxylate and dicarboxylate metabolism; b) Pentose and glucuronate interconversions; c) Amino sugar and nucleotide sugar metabolism; d) Galactose, and fructose and mannose metabolism]; B) energy central metabolism [e) Citrate cycle; f) Butanoate metabolism; g) Propanoate metabolism; h) Pentose phosphate pathway]; C) Bile acids metabolism; D) Lipid metabolism [j) Fatty acid biosynthesis; k) Biosynthesis of unsaturated fatty acids; l) Inositol phosphate metabolism; m) Fatty acid degradation; n) Glycerolipid metabolism]; E) Nitrogenous bases metabolism [o) Purine metabolism]; F) Redox metabolism [p) Ascorbate and aldarate metabolism; q) Glutathione metabolism]; G) Vitamins and coenzymes [r) Pantothenate and CoA biosynthesis].

Supl. Table 1. Indetification parameters, peak relative-height and fixed effects of metabolites quantified in plasma samples of NAH and NZH cows at 21 and 180 days in milk

Metabolite	**RI**[1]	**m/z**[2]	**BB id**	**PubChem**	**KEGG**	**Peak relative-height average mean**					**GS**[3]		**DIM**[4]		**GS × DIM**	
						21 DIM		180 DIM		SEM	*raw-P*[5]	*FDR*[6]	*raw-P*	*FDR*	*raw-P*	*FDR*
						NAH	NZH	NAH	NZH							
xylulose	544100	103	114791	135191	C00181	2.1E+03	1.4E+03	1.4E+03	1.2E+03	2.6E+02	0.079	0.581	0.170	0.302	0.424	0.695
xylose	535176	217	1808	439692	C02266	6.6E+03	6.5E+03	5.9E+03	5.0E+03	5.4E+02	0.004	0.223	0.157	0.284	0.944	0.972
xylonolactone	590775	189	17400	10264	C02341	8.4E+02	9.0E+02	7.9E+02	8.3E+02	7.5E+01	0.905	0.976	0.709	0.824	0.677	0.865
xylonic acid isomer	567437	217	5857	6912	C00379	2.0E+02	2.0E+02	2.1E+02	2.2E+02	2.6E+01	0.742	0.924	0.138	0.258	0.936	0.971
xylitol	1067809	237	100	638015	C00376	8.1E+03	9.2E+03	7.4E+03	5.6E+03	6.5E+02	0.066	0.581	0.023	0.057	0.139	0.418
valine	313502	144	3	6287	C00183	7.0E+05	4.4E+05	6.9E+05	7.8E+05	4.3E+04	0.017	0.333	0.006	0.023	0.004	0.211
uric acid	730691	441	23	1175	C00366	1.2E+04	8.6E+03	7.1E+03	5.7E+03	1.1E+03	0.013	0.278	0.005	0.020	0.379	0.664
urea	332913	189	22329	1176	C00086	1.1E+06	1.5E+06	1.3E+06	1.6E+06	1.5E+05	0.173	0.581	0.247	0.399	0.632	0.842
uracil	385735	241	1664	1174	C00106	1.6E+03	1.5E+03	1.4E+03	2.1E+03	2.0E+02	0.635	0.867	0.309	0.467	0.054	0.329
UDP-glucuronic acid	587601	217	7831	17473	C00167	4.4E+02	4.6E+02	5.2E+02	3.9E+02	5.8E+01	0.184	0.581	0.603	0.763	0.378	0.664
tyrosine	671252	218	16	6057	C00082	2.0E+05	1.9E+05	2.7E+05	2.7E+05	2.2E+04	0.397	0.738	0.003	0.015	0.687	0.868
tryptophan	780482	202	14	6305	C00078	1.5E+05	1.8E+05	2.3E+05	2.4E+05	1.5E+04	0.933	0.984	< 0.001	0.003	0.781	0.908
triethanolamine	531892	262	360127	7618	C06771	1.9E+02	1.9E+02	2.5E+02	2.0E+02	3.2E+01	0.347	0.670	0.375	0.533	0.999	0.999
trans-4-hydroxyproline	696023	333	51246	604	C02341	2.2E+04	2.6E+04	1.7E+04	1.6E+04	1.5E+03	0.908	0.976	0.002	0.013	0.349	0.655
α-tocopherol	484934	140	97	5810	C01157	5.6E+03	6.8E+03	6.6E+03	9.8E+03	6.6E+02	0.029	0.488	0.012	0.035	0.245	0.585
thymine	420133	255	1692	1135	C00178	3.8E+02	3.4E+02	3.4E+02	3.3E+02	2.8E+01	0.213	0.615	0.639	0.775	0.257	0.585
thymidine	349402	170	87703	5789	C00214	2.6E+03	2.6E+03	2.2E+03	2.3E+03	2.2E+02	0.437	0.747	0.284	0.441	0.270	0.596
threonine	408777	218	171298	6288	C00188	6.3E+04	5.0E+04	7.0E+04	8.2E+04	5.5E+03	0.289	0.622	0.011	0.032	0.056	0.329
threonic acid	497572	292	172	5460407	C01620	3.6E+03	3.8E+03	3.9E+03	3.2E+03	3.2E+02	0.164	0.581	0.950	0.977	0.066	0.329
sucrose	912704	271	16661	5988	C00089	4.0E+04	1.7E+04	4.0E+04	1.2E+04	1.7E+04	0.256	0.621	0.622	0.769	0.710	0.879

succinic acid	370608	247	161	1110	C00042	4.7E+03	4.0E+03	4.0E+03	4.3E+03	3.6E+02	0.106	0.581	0.854	0.948	0.081	0.334
stearic acid	787622	117	13	5281	C01530	1.4E+06	1.8E+06	1.4E+06	1.1E+06	9.9E+04	0.040	0.512	0.030	0.072	0.049	0.329
squalene	976237	95	23042	638072	C00751	1.1E+03	1.3E+03	1.3E+03	7.9E+02	1.8E+02	0.135	0.581	0.826	0.929	0.018	0.302
sorbitol	667922	217	162	5780	C00794	4.7E+03	4.5E+03	4.7E+03	4.0E+03	7.2E+02	0.179	0.581	0.997	0.997	0.808	0.923
serine	395020	218	25	5951	C00065	1.3E+05	8.7E+04	1.1E+05	1.5E+05	1.3E+04	0.670	0.894	0.068	0.137	0.005	0.211
salicylic acid	480699	267	3063	338	C00805	3.6E+02	4.0E+02	5.0E+02	5.3E+02	4.4E+01	0.961	0.984	0.002	0.012	0.631	0.842
saccharic acid	699211	333	11214	33037	C00818	2.7E+02	3.3E+02	2.7E+02	2.5E+02	2.7E+01	0.997	0.998	0.350	0.510	0.335	0.647
ribose	553071	217	384948	10975657	-	1.1E+04	9.3E+03	7.3E+03	9.7E+03	2.2E+03	0.880	0.976	0.800	0.905	0.252	0.585
ribonic acid	599680	292	1683	5460677	C01685	6.0E+02	3.9E+02	6.4E+02	3.4E+02	1.3E+02	0.185	0.581	0.909	0.971	0.664	0.865
ribitol	575497	217	7362	827	C00474	1.5E+03	1.8E+03	1.6E+03	1.4E+03	1.3E+02	0.413	0.738	0.281	0.439	0.256	0.585
raffinose	1120886	361	3190	439242	C00492	5.3E+02	2.3E+02	3.1E+02	1.8E+02	1.8E+02	0.466	0.760	0.996	0.997	0.921	0.971
quinic acid	632897	345	16691	6508	C00296	1.8E+02	6.0E+02	3.8E+02	3.3E+02	1.2E+02	0.289	0.622	0.350	0.510	0.106	0.364
pyruvic acid	213805	174	5864	1060	C00022	5.3E+03	9.8E+03	9.9E+03	1.1E+04	1.9E+03	0.502	0.792	0.046	0.099	0.576	0.788
putrescine	588872	174	281	1045	C00138	4.4E+02	5.7E+02	6.6E+02	6.6E+02	1.5E+02	0.817	0.958	0.296	0.452	0.416	0.695
p-tolyl glucuronide	813899	217	1688	15047	C02067	7.0E+02	6.9E+02	1.6E+03	7.4E+02	1.5E+02	0.045	0.512	< 0.001	0.004	0.003	0.211
pseudo uridine	364523	142	171970	145742	C00148	1.0E+04	1.3E+04	7.3E+03	6.4E+03	8.7E+02	0.940	0.984	0.001	0.009	0.439	0.699
proline	404121	156	2448	6931662	-	2.2E+05	1.4E+05	1.8E+05	2.3E+05	2.6E+04	0.293	0.622	0.222	0.371	0.015	0.290
pipecolinic acid	770604	159	117487	10380830	-	6.0E+03	2.4E+03	6.5E+03	5.5E+03	9.7E+02	0.011	0.266	0.041	0.092	0.113	0.376
phytanic acid	603912	299	1723	1015	C00346	6.3E+03	8.3E+03	1.0E+04	1.1E+04	8.0E+02	0.444	0.747	0.004	0.016	0.588	0.797
phosphoethanolamine	345365	314	4	1004	C00009	3.2E+02	2.7E+02	2.5E+02	2.2E+02	4.5E+01	0.238	0.621	0.361	0.522	0.679	0.865
phosphate	510327	174	2005	1001	C05332	1.1E+05	1.2E+05	1.2E+05	1.5E+05	8.4E+03	0.426	0.747	0.011	0.032	0.015	0.290
phenylethylamine	537804	218	33	6140	C00079	1.3E+03	4.3E+02	2.1E+03	5.3E+03	5.7E+02	0.246	0.621	< 0.001	0.003	0.008	0.240
phenylalanine	368081	164	1733	999	C07086	8.4E+04	8.5E+04	1.1E+05	1.1E+05	4.8E+03	0.238	0.621	< 0.001	0.004	0.371	0.664
phenylacetic acid	218927	151	31889	996	C00146	2.2E+03	2.3E+03	2.7E+03	3.1E+03	2.1E+02	0.945	0.984	0.002	0.013	0.284	0.603
phenol	654351	91	104452	68144	C05598	2.7E+03	4.0E+03	2.9E+03	5.0E+03	1.2E+03	0.218	0.615	0.604	0.763	0.782	0.908
phenaceturic acid	540818	103	360841	229	-	1.5E+04	1.6E+04	2.2E+04	1.9E+04	1.4E+03	0.149	0.581	0.005	0.019	0.502	0.764

pentose	674647	117	1680	13849	C16537	6.8E+03	7.9E+03	7.5E+03	6.0E+03	5.2E+02	0.208	0.615	0.777	0.885	0.072	0.329
pentadecanoic acid	847531	180	31571	154035	-	3.2E+04	3.9E+04	3.7E+04	3.2E+04	2.6E+03	0.403	0.738	0.751	0.867	0.278	0.603
p-cresol	280360	165	16321	2879	C01468	1.5E+04	1.1E+04	2.3E+04	2.4E+04	2.2E+03	0.185	0.581	0.002	0.012	0.327	0.646
pantothenic acid	690887	291	31356	6613	C12276	1.6E+02	1.8E+02	2.1E+02	2.1E+02	2.4E+01	0.771	0.928	0.131	0.248	0.900	0.968
palmitoleic acid	704511	311	31379	445638	C08362	3.6E+03	5.8E+03	1.7E+03	7.0E+02	4.9E+02	0.850	0.975	< 0.001	0.003	0.059	0.329
palmitic acid	713809	313	11	985	C00249	2.1E+05	2.6E+05	1.9E+05	1.5E+05	1.4E+04	0.470	0.760	0.004	0.016	0.071	0.329
oxoproline	485935	156	10	7405	C01879	1.5E+05	1.5E+05	1.8E+05	1.5E+05	8.5E+03	0.004	0.223	0.042	0.093	0.429	0.695
ornithine	527113	142	1821	6262	C00077	1.0E+05	1.1E+05	2.0E+05	1.7E+05	9.9E+03	0.135	0.581	< 0.001	0.001	0.543	0.780
oleamide	849710	144	20961	5283387	C19670	3.0E+03	3.7E+03	3.4E+03	3.4E+03	2.1E+02	0.697	0.915	0.229	0.378	0.317	0.642
octadecanol	755409	327	997	8221	-	5.5E+02	8.1E+02	7.8E+02	5.6E+02	7.2E+01	0.563	0.849	0.767	0.880	0.027	0.304
nonadecanoic acid	822782	117	46258	12591	C16535	2.1E+03	2.7E+03	1.9E+03	1.3E+03	1.9E+02	0.135	0.581	0.016	0.043	0.079	0.334
nicotinic acid	353428	180	327312	938	C00253	3.1E+03	3.9E+03	2.1E+03	2.5E+03	5.7E+02	0.521	0.802	0.065	0.132	0.921	0.971
nicotinamide	471602	179	84542	936	C00153	3.3E+03	3.8E+03	3.7E+03	3.6E+03	4.9E+02	0.801	0.951	0.515	0.666	0.697	0.869
N-acetylmannosamine	735323	319	3244	439281	C00645	3.1E+02	3.3E+02	3.2E+02	3.4E+02	3.3E+01	0.828	0.958	0.512	0.666	0.773	0.908
N-acetylglycine	356109	174	97747	10972		6.1E+03	4.8E+03	2.6E+03	2.4E+03	4.5E+02	0.059	0.581	< 0.001	0.004	0.362	0.663
myristic acid	634414	285	127	11005	C06424	9.3E+03	1.3E+04	6.8E+03	4.2E+03	9.1E+02	0.411	0.738	< 0.001	0.006	0.062	0.329
myo-inositol	730022	305	1741	892	C00137	4.7E+04	3.4E+04	3.5E+04	3.6E+04	6.4E+03	0.473	0.760	0.879	0.969	0.354	0.655
methionine sulfoxide	588656	128	372461	10062737	C15998	8.8E+03	8.7E+03	8.6E+03	7.6E+03	7.1E+02	0.286	0.622	0.923	0.971	0.869	0.966
methionine	483560	176	45	6137	C00073	3.0E+04	2.9E+04	3.7E+04	3.9E+04	2.6E+03	0.599	0.851	0.009	0.028	0.382	0.664
mannose	643848	205	390222	-	-	7.6E+04	8.8E+04	9.9E+04	9.2E+04	4.6E+03	0.233	0.621	< 0.001	0.007	0.263	0.588
maltose	946601	204	1979	439186	C00208	3.5E+03	2.9E+03	7.8E+03	3.9E+03	1.4E+03	0.132	0.581	0.219	0.370	0.777	0.908
malonic acid	305372	233	16918	867	C00383	5.8E+01	5.8E+01	6.7E+01	6.6E+01	8.9E+00	0.594	0.851	0.071	0.140	0.643	0.846
malic acid	463180	233	1391	525	C00711	3.3E+03	3.1E+03	2.7E+03	2.0E+03	7.9E+02	0.390	0.737	0.442	0.594	0.909	0.971
maleimide	662197	174	360160	5962	C00047	6.9E+03	7.4E+03	7.2E+03	1.0E+04	7.7E+02	0.168	0.581	0.040	0.091	0.089	0.345
lysine	553450	173	31632	439205	C00312	1.0E+05	9.7E+04	1.5E+05	1.3E+05	1.2E+04	0.241	0.621	< 0.001	0.007	0.960	0.983
linolenic acid	780376	108	4704	5280934	C06427	4.1E+03	2.6E+03	2.9E+03	2.8E+03	5.8E+02	0.330	0.660	0.955	0.977	0.523	0.780

linoleic acid	777414	150	165	5280450	C01595	3.6E+03	4.3E+03	3.2E+03	2.3E+03	3.0E+02	0.140	0.581	0.009	0.029	0.120	0.376
leucine	345845	158	1794	6106	C00123	5.8E+05	4.1E+05	6.2E+05	6.4E+05	3.4E+04	0.009	0.266	0.008	0.028	0.027	0.304
lauric acid	547906	117	49	3893	C02679	1.1E+04	1.3E+04	1.3E+04	1.1E+04	7.1E+02	0.127	0.581	0.618	0.769	0.028	0.304
lactulose	929908	204	6432	11333	C07064	1.1E+05	1.2E+05	9.8E+04	1.1E+05	1.9E+04	0.998	0.998	0.473	0.626	0.772	0.908
lactose	935640	191	4771	440995	C00243	3.6E+03	4.2E+03	3.3E+03	3.6E+03	6.5E+02	0.952	0.984	0.427	0.581	0.807	0.923
lactic acid	217657	191	80	612	C01432	2.4E+05	4.1E+05	2.8E+05	3.5E+05	9.3E+04	0.577	0.851	0.972	0.984	0.876	0.966
kynurenine	494077	142	4757	95562	-	3.3E+03	4.3E+03	5.9E+03	7.7E+03	6.5E+02	0.186	0.581	< 0.001	0.003	0.499	0.764
itaconic acid	769709	218	210712	-	-	3.4E+03	4.3E+03	3.7E+03	4.2E+03	3.4E+02	0.288	0.622	0.390	0.546	0.967	0.984
isothreonic acid	386511	147	101725	811	C00490	2.7E+03	3.2E+03	2.6E+03	2.8E+03	1.4E+02	0.416	0.738	0.247	0.399	0.537	0.780
isothreitol	489385	292	1679	151152	C00639	1.2E+03	1.3E+03	9.6E+02	9.3E+02	5.5E+01	0.495	0.788	< 0.001	0.005	0.644	0.846
isopentadecanoic acid	663518	117	127681	151014	-	1.2E+04	1.7E+04	1.8E+04	1.1E+04	2.1E+03	0.646	0.874	0.709	0.824	0.057	0.329
isolinoleic acid	794629	156	10647	5312483	-	5.5E+02	6.5E+02	7.5E+02	8.2E+02	8.6E+01	0.782	0.934	0.043	0.093	0.979	0.990
isoleucine	356963	158	105087	6306	C00407	4.4E+05	3.0E+05	4.5E+05	4.5E+05	2.2E+04	0.008	0.266	0.015	0.041	0.012	0.288
isogluconic acid	617338	245	32122	5318532	C00451	2.8E+02	3.5E+02	2.6E+02	2.6E+02	3.2E+01	0.609	0.858	0.173	0.303	0.550	0.780
isocitric acid	577333	277	31543	10258	-	2.8E+03	3.1E+03	2.7E+03	2.1E+03	2.5E+02	0.106	0.581	0.058	0.121	0.165	0.480
inositol-4-monophosphate	732249	202	112556	3744	-	3.7E+02	4.3E+02	2.9E+02	3.8E+02	3.6E+01	0.194	0.595	0.369	0.529	0.553	0.780
indoxyl sulfate	764586	202	724	92904	C02043	4.5E+02	5.0E+02	7.9E+02	8.0E+02	8.2E+01	0.884	0.976	< 0.001	0.005	0.931	0.971
indole-3-propionic acid	684929	202	69	802	C00954	2.4E+03	4.3E+03	3.2E+03	4.6E+03	5.3E+02	0.088	0.581	0.022	0.057	0.740	0.896
indole-3-lactate	434422	104	114270	107	C05629	8.3E+02	8.2E+02	8.1E+02	8.0E+02	1.0E+02	0.520	0.802	0.846	0.944	0.573	0.788
indole-3-acetate	443878	218	2849	12647	C00263	1.6E+03	1.9E+03	1.3E+03	1.5E+03	2.0E+02	0.758	0.924	0.141	0.260	0.865	0.966
hydrocinnamic acid	874865	128	2786	10010	C01817	1.4E+04	1.8E+04	1.4E+04	2.1E+04	2.4E+03	0.173	0.581	0.258	0.408	0.169	0.483
homoserine	664395	154	58170	6274	C00135	2.9E+02	2.3E+02	2.6E+02	2.8E+02	2.5E+01	0.171	0.581	0.471	0.626	0.058	0.329
homocystine	637795	206	12269	464	C01586	4.2E+02	4.5E+02	4.6E+02	3.9E+02	3.0E+01	0.031	0.488	0.973	0.984	0.458	0.716
histidine	751309	117	727	10465	-	4.3E+04	4.6E+04	6.1E+04	6.3E+04	3.7E+03	0.731	0.924	< 0.001	0.005	0.811	0.923
hippuric acid	557299	328	16942	439918	C03139	6.0E+04	7.2E+04	5.8E+04	6.6E+04	6.4E+03	0.448	0.747	0.952	0.977	0.677	0.865
heptadecanoic acid	227409	177	18228	757	C00160	2.6E+04	3.6E+04	1.9E+04	1.2E+04	2.3E+03	0.443	0.747	0.001	0.009	0.067	0.329

guanidinosuccinate	367302	248	385006	5257127	C00037	2.2E+02	2.4E+02	2.8E+02	3.5E+02	2.5E+01	0.268	0.622	0.002	0.012	0.347	0.655
glycolic acid	590747	357	1687	754	C03189	3.8E+03	4.5E+03	4.0E+03	4.7E+03	2.9E+02	0.215	0.615	0.313	0.469	0.418	0.695
glycine	344428	205	102844	753	C00116	4.9E+05	4.8E+05	3.2E+05	4.0E+05	4.3E+04	0.865	0.976	0.039	0.090	0.179	0.497
glycerol-α-phosphate	377308	189	394878	-	-	2.8E+03	2.8E+03	3.1E+03	5.4E+03	5.5E+02	0.283	0.622	0.012	0.035	0.042	0.315
glycerol-3-galactoside	421596	261	16952	743	C00489	5.6E+02	8.5E+02	6.3E+02	4.4E+02	9.4E+01	0.756	0.924	0.209	0.360	0.095	0.354
glycerol	600315	156	18	5961	C00064	1.4E+05	1.7E+05	1.1E+05	9.7E+04	1.2E+04	0.598	0.851	0.004	0.017	0.386	0.664
glyceric acid	529100	246	28	33032	C00025	3.4E+03	5.0E+03	3.3E+03	3.9E+03	4.3E+02	0.160	0.581	0.429	0.581	0.403	0.686
glutaric acid	665901	333	344793	94715	C00191	3.5E+02	3.4E+02	3.0E+02	3.3E+02	3.2E+01	0.635	0.867	0.608	0.763	0.217	0.551
glutamine	808788	387	360626	5958	C00092	1.9E+05	1.9E+05	2.8E+05	2.1E+05	2.0E+04	0.241	0.621	0.025	0.061	0.238	0.576
glutamic acid	594647	217	3167	65533	C00103	5.9E+04	3.6E+04	5.7E+04	5.8E+04	7.4E+03	0.070	0.581	0.034	0.081	0.085	0.342
glucuronic acid	680899	217	76	64689	C00221	7.1E+03	9.4E+03	6.9E+03	5.2E+03	1.0E+03	0.623	0.867	0.058	0.121	0.173	0.487
glucose-6-phosphate	693148	333	7501	6857417	C00800	4.7E+02	5.3E+02	5.1E+02	4.8E+02	7.8E+01	0.757	0.924	0.640	0.775	0.937	0.971
glucose-1-phosphate	690882	292	4706	128869	C00880	2.0E+03	2.5E+03	2.5E+03	2.3E+03	2.2E+02	0.889	0.976	0.187	0.324	0.301	0.617
glucose	390016	245	14755	444972	C00122	1.0E+05	1.3E+05	1.3E+05	1.3E+05	8.8E+03	0.726	0.924	0.007	0.024	0.219	0.551
gluconic acid	578299	160	3009	439650	C02095	6.6E+02	7.3E+02	1.2E+03	9.7E+02	1.5E+02	0.628	0.867	0.010	0.032	0.720	0.885
galactonic acid	641863	307	50366	439709	C02336	5.5E+02	7.2E+02	1.2E+03	9.7E+02	1.6E+02	0.751	0.924	0.010	0.030	0.545	0.780
fumaric acid	471922	217	92	222285	C00503	9.0E+02	8.8E+02	8.6E+02	8.6E+02	6.6E+01	0.271	0.622	0.938	0.977	0.436	0.699
fucose	464779	217	16903	169019	C16884	2.2E+03	3.6E+03	2.5E+03	3.1E+03	3.3E+02	0.174	0.581	0.248	0.399	0.030	0.304
fructose	507619	243	12092	8193	C02277	3.4E+04	2.5E+04	2.1E+04	1.3E+04	1.2E+04	0.686	0.907	0.626	0.769	0.693	0.869
erythritol	846510	315	45351	440043	C03546	3.0E+03	3.9E+03	3.1E+03	3.4E+03	1.5E+02	0.005	0.224	0.895	0.970	0.301	0.617
dodecanol	245118	154	1743	10935	C07272	8.2E+02	1.7E+03	1.0E+03	1.2E+03	1.8E+02	0.179	0.581	0.926	0.971	0.119	0.376
deoxycholic acid	598147	179	110341	10394	C01744	2.9E+03	6.2E+03	2.9E+03	5.5E+02	1.1E+03	0.339	0.670	0.007	0.025	0.073	0.329
dehydroabietic acid	1107794	255	95409	222528	C04483	4.2E+02	5.7E+02	5.8E+02	4.8E+02	3.4E+01	0.543	0.826	0.166	0.297	0.033	0.304
cystine	850374	239	8917	94391	C12078	5.6E+03	7.7E+03	1.0E+04	1.1E+04	8.2E+02	0.371	0.709	< 0.001	0.003	0.453	0.715
cysteine	804619	218	94	595	C01420	3.9E+03	3.5E+03	5.6E+03	6.2E+03	4.6E+02	0.584	0.851	< 0.001	0.005	0.185	0.506
cystathionine	500158	220	65	5862	C00097	1.2E+03	1.5E+03	1.5E+03	1.5E+03	9.9E+01	0.959	0.984	0.006	0.023	0.097	0.354

cyanoalanine	772979	218	18481	439258	C02291	1.4E+03	1.1E+03	1.2E+03	1.4E+03	1.2E+02	0.276	0.622	0.347	0.510	0.067	0.329
creatinine	502599	115	31	588	C00791	3.7E+05	4.3E+05	4.2E+05	3.7E+05	1.7E+04	0.085	0.581	0.380	0.536	0.033	0.304
conduritol-β-expoxide	701723	318	34065	9989541	-	1.6E+03	1.3E+03	1.1E+03	9.1E+02	2.5E+02	0.346	0.670	0.151	0.275	0.886	0.966
citrulline	621404	157	1712	9750	C00327	2.3E+04	2.4E+04	3.0E+04	2.7E+04	1.6E+03	0.140	0.581	0.008	0.028	0.546	0.780
citric acid	615341	273	17009	311	C00158	1.7E+05	2.0E+05	1.7E+05	1.2E+05	1.6E+04	0.156	0.581	0.017	0.045	0.050	0.329
cholic acid	1109517	253	110403	221493	C05463	2.7E+04	5.3E+04	2.2E+04	5.0E+03	8.7E+03	0.310	0.636	0.002	0.011	0.040	0.315
cholesterol	1078536	129	19	5997	C00187	1.5E+05	2.1E+05	1.8E+05	2.2E+05	1.5E+04	0.048	0.512	0.088	0.169	0.880	0.966
cerotinic acid	1033286	145	17982	10469	-	2.5E+02	3.3E+02	4.4E+02	3.9E+02	3.5E+01	0.985	0.998	0.007	0.026	0.292	0.612
capric acid	452386	229	50422	2969	C01571	2.9E+03	3.7E+03	3.3E+03	3.3E+03	1.7E+02	0.522	0.802	0.214	0.364	0.118	0.376
β-glycerolphosphate	574470	243	22021	2526	C02979	6.2E+02	6.7E+02	5.7E+02	7.6E+02	5.2E+01	0.253	0.621	0.254	0.405	0.020	0.304
β-alanine	435564	248	148	239	C00099	3.2E+03	3.3E+03	2.8E+03	3.3E+03	2.8E+02	0.896	0.976	0.660	0.788	0.037	0.304
azelaic acid	610551	317	329430	19347555	C08261	5.8E+02	6.8E+02	5.6E+02	5.6E+02	5.2E+01	0.994	0.998	0.428	0.581	0.897	0.968
aspartic acid	480387	232	79	5960	C00049	1.1E+04	9.1E+03	1.4E+04	1.4E+04	9.8E+02	0.090	0.581	< 0.001	0.004	0.155	0.460
asparagine	553743	231	369588	6267	C00152	8.8E+03	8.2E+03	1.0E+04	9.7E+03	6.7E+02	0.217	0.615	0.035	0.083	0.578	0.788
arachidonic acid	834339	91	6529	444899	C00219	1.6E+03	2.1E+03	1.2E+03	9.5E+02	1.6E+02	0.650	0.874	0.003	0.015	0.217	0.551
arachidic acid	856421	117	291	10467	C06425	5.1E+03	5.9E+03	6.0E+03	4.8E+03	2.7E+02	0.002	0.223	0.709	0.824	0.082	0.334
aminomalonate	455754	218	413	100714	C00872	1.9E+04	2.3E+04	1.7E+04	1.5E+04	1.7E+03	0.574	0.851	0.041	0.092	0.207	0.551
α-ketoglutarate	507392	198	294	51	C00026	2.1E+03	2.2E+03	4.2E+03	3.6E+03	5.2E+02	0.302	0.633	0.002	0.011	0.997	0.999
α-aminoadipic acid	573295	260	125502	92136	C00956	1.6E+03	1.2E+03	1.9E+03	2.0E+03	1.6E+02	0.047	0.512	< 0.001	0.004	0.034	0.304
allantoic acid	726050	259	117021	203	C00499	5.6E+04	7.6E+04	5.3E+04	5.2E+04	4.1E+03	0.311	0.636	0.016	0.043	0.106	0.364
alanine	240378	116	18223	5950	C00041	5.9E+05	4.3E+05	4.1E+05	6.3E+05	3.5E+04	0.905	0.976	0.502	0.659	< 0.001	0.021
aconitic acid	586815	229	29	643757	C00417	6.9E+02	7.3E+02	5.8E+02	4.7E+02	5.4E+01	0.146	0.581	0.012	0.035	0.494	0.764
9-myristoleate	630100	117	97452	-	-	6.1E+03	9.1E+03	5.8E+03	3.7E+03	8.3E+02	0.865	0.976	0.009	0.029	0.026	0.304
6-deoxyglucose	575986	117	342712	93579	-	3.8E+03	7.0E+03	4.4E+03	5.9E+03	8.3E+02	0.140	0.581	0.674	0.799	0.090	0.345
5-methoxytryptamine	864466	174	284	1833	C05659	3.5E+03	1.4E+03	6.5E+02	1.6E+03	9.1E+02	0.934	0.984	0.297	0.452	0.221	0.551
5-hydroxynorvaline	777606	290	31552	1826	C05635	6.6E+02	6.4E+02	8.7E+02	7.3E+02	4.4E+01	0.036	0.511	0.002	0.012	0.746	0.897

5-hydroxy-3-indoleacetic acid	536657	174	1698	138	C00431	7.4E+02	4.0E+02	3.1E+02	8.2E+02	2.1E+02	0.743	0.924	0.897	0.970	0.104	0.364
5-aminovaleric acid	542795	179	31555	127	C00642	2.5E+03	3.3E+03	2.5E+03	1.6E+03	3.2E+02	0.399	0.738	0.045	0.097	0.037	0.304
4-hydroxyphenylacetic acid	325027	233	85123	10413	C00989	1.2E+03	1.8E+03	7.8E+02	8.7E+02	1.5E+02	0.169	0.581	0.002	0.013	0.535	0.780
4-hydroxybutyric acid	488730	304	1842	119	C00334	2.6E+03	2.9E+03	2.0E+03	1.9E+03	2.3E+02	0.704	0.917	0.004	0.016	0.849	0.960
4-aminobutyric acid	554966	161	107897	111	C02642	1.3E+02	1.7E+02	1.6E+02	1.3E+02	3.4E+01	0.821	0.958	0.656	0.788	0.421	0.695
3-ureidopropionate	278632	191	145501	92135	C01089	4.3E+02	5.2E+02	4.6E+02	4.8E+02	3.8E+01	0.766	0.927	0.592	0.760	0.739	0.896
3-hydroxybutyric acid	521554	247	32000	1662	C03761	5.6E+05	5.5E+05	3.8E+05	4.5E+05	4.7E+04	0.886	0.976	0.065	0.132	0.238	0.576
3-hydroxy-3-methylglutaric acid	404807	141	107144	18647689	-	3.3E+02	2.3E+02	3.6E+02	3.6E+02	4.2E+01	0.099	0.581	0.073	0.142	0.116	0.376
3-aminoisobutyric acid	800205	204	100875	16048618	C05401	2.6E+03	1.2E+03	7.5E+02	9.1E+02	2.8E+02	0.146	0.581	0.002	0.012	0.008	0.240
3-4-hydroxyphenylpropionic acid	309837	102	10827	64956	C05145	2.5E+02	3.2E+02	3.5E+02	3.5E+02	2.6E+01	0.734	0.924	0.003	0.015	0.354	0.655
2-ketoisocaproic acid	310761	89	208	70	C00233	1.2E+04	8.4E+03	9.5E+03	9.5E+03	9.9E+02	0.078	0.581	0.920	0.971	0.076	0.334
2-ketobutyric acid	243116	89	105066	58	C00109	3.3E+03	2.9E+03	4.3E+03	4.6E+03	4.1E+02	0.588	0.851	0.017	0.043	0.209	0.551
2-hydroxyglutaric acid	506306	247	2000	43	C02630	1.1E+03	1.1E+03	8.0E+02	6.7E+02	1.0E+02	0.325	0.657	0.002	0.011	0.539	0.780
2-hydroxybutanoic acid	258161	131	40	440864	C05984	2.2E+04	1.9E+04	9.9E+03	1.2E+04	2.3E+03	0.451	0.747	0.003	0.015	0.281	0.603
2-deoxytetronic acid	433456	189	1208	150929	-	7.1E+02	9.5E+02	7.2E+02	7.1E+02	3.5E+01	0.444	0.747	0.071	0.140	0.063	0.329
2-aminobutyric acid	285825	130	160842	6657	C02721	9.3E+04	7.2E+04	7.6E+04	6.9E+04	8.4E+03	0.132	0.581	0.415	0.576	0.135	0.414
2,8-dihydroxyquinoline	626989	290	104444	97250	C06342	2.7E+02	2.6E+02	1.8E+02	1.8E+02	2.3E+01	0.256	0.621	0.003	0.014	0.573	0.788
1-monostearin	959214	203	648	24699	D01947	3.7E+03	4.5E+03	5.6E+03	5.5E+03	3.2E+02	0.959	0.984	< 0.001	0.002	0.374	0.664
1-monopalmitin	900644	371	391871	-	-	6.0E+03	8.4E+03	9.3E+03	9.7E+03	5.6E+02	0.255	0.621	< 0.001	0.003	0.258	0.585
1-kestose	1123027	361	14692	440080	C03661	5.5E+02	2.3E+02	4.3E+02	1.7E+02	2.0E+02	0.281	0.622	0.890	0.970	0.888	0.966
1,5-anhydroglucitol	632918	191	171285	64960	C07326	6.7E+03	7.2E+03	5.3E+03	3.4E+03	8.0E+02	0.063	0.581	0.016	0.043	0.324	0.646
1,2,4-benzenetriol	521803	239	26704	10787	C02814	1.3E+02	2.1E+02	1.9E+02	1.4E+02	4.4E+01	0.830	0.958	0.912	0.971	0.334	0.647

1: Target retention index in the BinBase database system (Fiehn Lab), where RI is obtained as the conversion of absolute retention times to relative retention times based on a set of pre-defined internal standards, using Fiehn retention indexes; 2: m/z value used to quantify peak height for each BinBase database entry; 3: genetic strain; 4: days in milk; 5: Raw P value according to ANOVA; 6: FDR-adjusted P value.

Suppl. Table 2. Top 20 features identified by MEBA

Ranking	Metabolite	Hotelling-T^2
1	alanine	21.96963
2	valine	15.22464
3	isoleucine	14.96326
4	arachidic acid	13.37323
5	oxoproline	12.52577
6	squalene	12.21192
7	leucine	11.8687
8	p-tolyl glucuronide	11.71774
9	erythritol	11.6995
10	serine	10.49169
11	proline	9.82223
12	pipecolinic acid	9.38211
13	creatinine	9.3576
14	lauric acid	8.73514
15	β-glycerolphosphate	8.67303
16	octadecanol	8.61977
17	stearic acid	8.58904
18	phosphate	8.33654
19	α-aminoadipic acid	8.22099
20	pentose	8.13426

4. DISCUSIÓN GENERAL Y CONCLUSIONES

4.1. DISCUSIÓN GENERAL

La respuesta productiva de la vaca lechera está afectada por la interacción genotipo × ambiente, y esta interacción se explica en parte por diferencias en el metabolismo energético y lipídico durante la transición de acuerdo con el ambiente en el que se encuentran y al genotipo del animal (Roche et al., 2006; Lucy et al., 2009; Chagas et al., 2009). En este trabajo 4demostramos que la etapa de lactancia, la estrategia de alimentación y el efecto del genotipo implican también cambios en el metabolismo de los AA y el metabolismo redox. Más aún, nuestros resultados sugieren que existen diferencias en la capacidad de adaptación metabólica entre distintas líneas genéticas Holstein, dadas por diferencias en el metabolismo de los AA ramificados (leucina, valine, isoleucina) y una mayor sensibilidad redox en los animales NZH vs. NAH. A su vez, mediante análisis de PCA, demostramos que los animales NZH tienen trayectorias metabólicas (analizadas en forma integral) con una mayor resistencia al cambio a lo largo de la lactancia en comparación con las vacas NAH.

Consistente con trabajos previos, los animales NZH tuvieron una menor PL y mayor concentración de sólidos lácteos en comparación con la línea NAH (Harris y Kolver, 2001) asociado al menor peso vivo y posiblemente al menor consumo de materia seca (Sheahan et al., 2011). Si bien no hay indicios de una menor movilización de reservas corporales en las vacas NZH, tuvieron un mejor estatus energético (menor glucosa e insulina) y mejores valores del índice PI (desbalance fisiológico, Bjerre-Harpøt et al., 2012), particularmente en la estrategia de alimentación que maximizó el pastoreo en la dieta. Sugiriendo que efectivamente estos animales tienen una mejor capacidad de adaptación en la medida que se maximiza el pastoreo en la estrategia de alimentación.

Independientemente de la línea genética, nuestros resultados indican que los cambios homeorréticos durante la transición implican fuertes cambios en el metabolismo proteico en general y en varias vías metabólicas de AA, lo que resulta consistente con trabajos reportados previamente (Sadri et al., 2020). Así, las mayores

concentraciones de 3-metilshitidina observadas previo al parto, indican que todas las vacas estaban movilizando proteína lábil siendo mayor en los animales en PMAX y en la línea genética NAH (Houweling et al., 2012), sugiriendo que la movilización muscular y adiposa no ocurren al mismo tiempo (van der Drift et al., 2012). En el mismo sentido, los resultados metabolómicos sugieren que el balance de los AA fue menor durante la lactancia temprana (menores concentraciones plasmáticas de AA y de varios intermediarios del ciclo de la urea) que, durante la lactancia media, probablemente para sostener la alta producción de leche como consecuencia del BEN (Bell et al., 2000). En este sentido, se ha demostrado que durante la lactancia temprana aumenta la absorción hepática de AA y disminuye la actividad del ciclo de la urea (Graber et al., 2010).

La mayor concentración plasmática de urea observada para los animales NZH vs. NAH podría ser consecuencia de una mayor actividad del ciclo de la urea asociado a un menor grado de infiltración de lípidos en el tejido hepático (Graber et al., 2010) así como también a un mejor balance de AA, consistente con la menor concentración plasmática de 3-metilhistidina observada previo al parto (van der Drift et al., 2012). A su vez, el mejor estatus energético y menor desbalance fisiológico observado en los animales NZH parece haber estado asociado a menores concentraciones plasmáticas de los AA ramificados (valine, leucina, isoleucina) durante el inicio de la lactancia. Una menor concentración de estos AA podría estar asociada a una mayor sensibilidad a la insulina durante la lactancia temprana en comparación con las vacas NAH, previamente demostrado (Chagas et al., 2009). En este sentido, las altas concentraciones de AAR en los animales NAH es posible que sea causa y/o consecuencia —aún no está claro— de una mayor movilización de reservas corporales a través de una menor sensibilidad a la insulina, altas concentraciones de estos AA han sido asociadas con el desarrollo de la resistencia a la insulina. Recientemente se han reportado resultados similares al comparar vacas obesas vs. normales (Sadri et al., 2020) y al comparar animales con RFI contrastantes, observándose que la menor sensibilidad a la insulina observada en los animales menos eficientes estuvo asociada a una mayor concentración plasmática de estos AA (Cantalapiedra, com. pers.). Si bien no se conocen con certeza los mecanismos que vinculan la resistencia a la insulina con

las altas concentraciones de AAR, se ha sugerido que una activación crónica de mTOR a través de los AAR que podría estimular una sobre-fosforilzación del substrato del receptor de insulina 1 (IRS1) generando una menor sensibilidad a esta hormona (Wullschleger et al., 2006; Asghari et al., 2017).

Además de las diferencias previamente discutidas en relación con el metabolismo energético y proteico, es posible que una mejor capacidad de adaptación metabólica de los animales NZH también incluya una mayor sensibilidad del metabolismo redox. Así, los animales NZH no solo presentaron indicios de un mayor daño oxidativo de lípidos y proteínas reflejado en mayores concentraciones de TBARS y carbonilos respectivamente (Celi y Gabai, 2015), sino también parecen haber tenido una mejor respuesta antioxidante (alta actividad SOD durante más tiempo, mejor recuperación de la concentración de α-tocoferol durante la lactancia) (Bernabucci et al., 2005). Si bien la literatura en este tema es escasa, Marco-Ramell et al. (2012) reportaron resultados similares, encontrando mayores daños oxidativos y respuesta antioxidante en los animales adaptados durante siglos a las condiciones de montaña.

Finalmente, en el presente trabajo los animales NZH se caracterizaron por tener un menor desplazamiento metabolico durante la lactancia que les permitió recuperarse mas rápidamente y llegar a la lactancia media con un estado cercano al estado inicial (-45 DIM), mientras que los animales NAH en lactancia media tuvieron una situación muy similar a la observada al inicio de la lactancia (momento de alta demanda nutricional y alta carga metabólica) caracterisitico del BEN. En este sentido, los animales NZH demostraron mayor resistencia al cambio frente a un evento de perturbación como el caso de la transición, sugiriendo una mayor resiliencia en términos metabólicos (Friggens et al., 2017). Los resultados de nuestro trabajo resultan alentadores en el uso de este enfoque para estudiar cualitativamente los cambios homeorréticos del metabolismo en forma integrada, asimismo, consideramos que se debe profundizar en la selección de qué variables usar.

4.2. CONCLUSIONES GENERALES

Los resultados de este trabajo demuestran que, durante la transición de la vaca lechera, los procesos homeorréticos incluyen drásticos cambios en el metabolismo

proteico y redox que se caracterizan por la movilización de musculo esquelético y disminución del ciclo de la urea, así como por el aumento del daño oxidativo de lípidos y proteínas. A su vez, la maximización de la actividad del pastoreo incrementa la expresión de las vías homeorréticas lo que se ve reflejado en un mayor aumento de los biomarcadores plasmáticos de movilización de tejido adiposo y muscular, y daño oxidativo. Sin embargo, la respuesta adaptativa en términos metabólicas está afectada por el genotipo lechero, así los animales NZH parecen tener una mejor capacidad de adaptación que los NAH, que en términos integrales implica una trayectoria metabólica aparentemente con mayor resistencia al cambio. Las diferencias adaptativas entre estas dos líneas genéticas Holstein parecen residir en una menor movilización de músculo esquelético, menor sensibilidad a la insulina posiblemente asociado a menores concentraciones plasmáticas de los aminoácidos ramificados durante la lactancia temprana, así como a una mayor sensibilidad del metabolismo redox al comparar los animales NZH (más adaptados) vs. NAH (menos adaptados).

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