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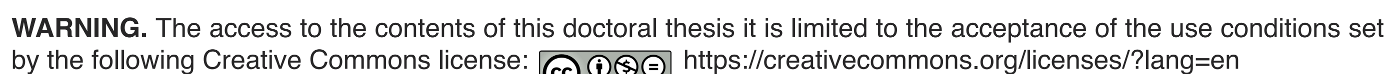
WARNING. The access to the contents of this doctoral thesis it is limited to the acceptance of the use conditions set by the following Creative Commons license:  https://creativecommons.org/licenses/?lang=en

Multiple Approaches in Studying the Biochemical Effects of Amino Acid Supplementation in Preruminant Calves

PhD Thesis

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Multiple Approaches in Studying the Biochemical Effects of Amino Acid Supplementation in Preruminant Calves

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para optar al título de Doctor

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1. Summary (English)

As the muscle system is one of the most fast-growing compartments in calves before weaning, it requires a large amount of protein deposition. Amino acids, as the basic unit of proteins, play crucial roles in the growth of animals. Supplementation of several amino acids in milk replacers has been used in calf industry for decades and several approaches have been established to assess the functionality, outcome and efficiency of this supplementation.

In this work, a "multi-omics" approach has been used, aiming to use large scale of data collected from metabolomic and proteomic analyses (known as "multi-omics" or "foodomics") to elucidate the impact of several amino acid supplementations in the milk replacer for calves. The work of this project is divided into three studies and "multi-omics" were applied in Study 1 and Study 2:

In Study 1, proteomics and metabolomics ("multi-omics") and serum clinical chemistry were used to study the skeletal muscle of calves under supplementation with several amino acids in milk replacers. Animals were divided into four groups: Ctrl, control diet without supplementation; GP, control supplemented with Glycine (G) and Proline (P); FY, control supplemented with phenylalanine (F) and tyrosine (Y); and MKT, control supplemented with methionine (M), lysine (K) and threonine (T). The integrated results indicated that MKT supplementation yielded a mild improvement of growth, increased serum creatinine and insulin, muscle Phospho-S6 / S6 ratio as well as some proteins related to muscle function, despite some of the elevations are non-significant. The metabolome analysis from skeletal muscle biopsies revealed several differences between the GP - FY groups and the Ctrl - MKT groups, suggesting a metabolic adaptation especially in GP and FY groups.

In Study 2, the same approaches to the Study 1 were used, but both plasma and skeletal muscle biopsies were included in the "multi-omics" analysis. Animals were divided into

three groups: Ctrl, control diet without supplementation; LT, control supplemented with leucine (L) and threonine (T); and Leu, control supplemented with leucine. In both supplemented treatments, the integrated results indicated that leucine and its metabolites were available in the organism. In the LT group compared to the Ctrl group, integrated metabolomics and proteomics showed that branched-chain amino acids (BCAA) degradation and mitochondrial oxidative metabolism (citrate cycle, respiratory chain) were the main activated pathways in muscle. Energy metabolic substrates would be BCAA derivatives, butyrate from ruminal production and/or lipid mobilization. The deleterious effects of activated oxidative phosphorylation were balanced by the upregulation of antioxidant proteins. An increase in protein synthesis was indicated by the increase in Phospho-S6 / S6 ratio in skeletal muscle. In the Leu group, animals suffered digestive problems at the beginning of the study and the experimental design was not correctly randomized when biopsies were obtained. The serum/plasma chemical, proteomic and metabolomic analyses showed a profile compatible with a pathological process and a biopsy-caused bias.

In Study 3, reference intervals for several biochemical parameters for unweaned calves and recently weaned piglets at different ages were calculated using large number of animals sampled at different ages from populations under different season trials. The main variable was age whereas no major trial- or sex-biased differences were noticed. This information will help veterinarians and animal science researchers to better practice their professions by providing them with more adequate diagnostic references.

2. Resumen (Español)

Como el músculo esquelético es el compartimento que se desarrolla más rápidamente antes de destete, requiere que la síntesis de proteínas funcione de manera activa. Los aminoácidos, al ser la unidad básica de las proteínas, juegan un papel muy importante en el crecimiento de los animales. La suplementación con aminoácidos en el lactoreemplazante hace décadas que se lleva a cabo en la producción de terneros, y se han establecido diversos métodos para evaluar la funcionalidad, resultado y eficiencia de esta suplementación.

En este trabajo, se ha usado un abordaje "multi-ómico" con el fin de integrar los resultados obtenidos a partir de análisis de metabolómica y proteómica (también conocido como "multi-ómica" o "foodómica") para elucidar el impacto a nivel molecular de la suplementación del lactoreemplazante con diferentes aminoácidos para la alimentación de terneros. Este proyecto está dividido en tres estudios:

En el Estudio 1, se ha utilizado una aproximación proteómica y metabolómica ("multi-ómica"), conjuntamente con parámetros séricos de bioquímica clínica para estudiar los efectos en el músculo esquelético de la suplementación del lactoreemplazante con varias combinaciones de aminoácidos. Los animales se dividieron en cuatro grupos: Ctrl, dieta control sin suplementación; GP, control suplementado con glicina (G) y prolina (P); FY, control suplementado con fenilalanina (F) y tirosina (Y); y MKT, control suplementado con metionina (M), lisina (K) y treonina (T). La integración de los resultados indica que el grupo con la suplementación de M, K y T ha mostrado una leve mejora del crecimiento, y un incremento de los niveles de creatinina e insulina en suero, de la ratio de Fosfo-S6 / S6 y de algunas proteínas relacionadas con la función muscular, a pesar de que algunos cambios no son significativos. El análisis metabolómico del músculo esquelético ha revelado varias diferencias entre GP - FY y Ctrl - MKT, sugiriendo una adaptación metabólica especialmente en los grupos GP y FY.

En el Estudio 2, se ha utilizado el mismo abordaje, incluyendo tanto plasma como biopsias de músculo esquelético en el análisis "multi-ómico". Los animales se han dividido en tres grupos: Ctrl, control sin suplementación; LT, control suplementado con leucina (L) y treonina (T); y Leu, control suplementado con leucina. En ambos tratamientos de suplementación, los resultados integrados han indicado que la leucina y sus metabolitos se encuentran disponibles en el organismo. En el grupo LT comparado con Ctrl, la integración de los resultados de metabolómica y proteómica han mostrado que la degradación de aminoácidos ramificados (BCAA) y el metabolismo oxidativo mitocondrial (ciclo del ácido cítrico, cadena respiratoria) son las vías principales activadas en el músculo. Los sustratos del metabolismo energético pueden ser derivados de BCAA, butirato procedente del rumen y/o movilización de lípidos. Los efectos deletéreos de la activación de la fosforilación oxidativa son contrarrestados por un aumento de proteínas antioxidantes. El incremento en la síntesis de proteínas está indicado por el aumento de la ratio entre Fosfo-S6 / S6 en el músculo esquelético. En el grupo de Leu, los animales sufrieron problemas digestivos al inicio del estudio, y además no fueron correctamente randomizados cuando se obtuvieron las biopsias. Los análisis de bioquímica clínica de suero, proteómica y metabolómica de músculo y sangre han mostrado un perfil que es compatible con un proceso patológico y muestran el sesgo causado por la biopsia.

En el Estudio 3, se han calculado los intervalos de referencia de varios parámetros bioquímicos para terneros lactantes y para lechones recién destetados, utilizando un elevado número de animales de diferentes edades, procedentes de ensayos llevados a cabo en diferentes estaciones. La variable principal ha sido la edad, mientras que no se han observado diferencias debidas al ensayo, la estación o el sexo. Esta información proporciona herramientas diagnósticas más adecuadas a veterinarios e investigadores en ciencia animal, y posibilitará una mejor práctica profesional.

3. Resum (Català)

Com el múscul esquelètic és el compartiment que es desenvolupa més ràpidament abans del deslletament, requereix que la síntesi de proteïnes funcioni de manera activa. Els aminoàcids, en ser la unitat bàsica de les proteïnes, juguen un paper molt important en el creixement dels animals. La suplementació amb aminoàcids en el lactoreemplaçant fa dècades que es du a terme en la producció de vedells, i s'han establert diversos mètodes per avaluar la funcionalitat, resultat i eficiència d'aquesta suplementació.

En aquest treball, s'ha fet servir un abordatge "multi-òmic" per tal d'integrar els resultats obtinguts a partir d'anàlisis de metabolòmica i proteòmica (també conegut com "multi-òmica" o "foodòmica") per esbrinar el impacte a nivell molecular de la suplementació del lactoreemplaçant amb diferents aminoàcids per a l'alimentació de vedells. Aquest projecte està dividit en tres estudis:

A l'Estudi 1, s'ha utilitzat una aproximació proteòmica i metabolòmica ("multi-òmica"), conjuntament amb paràmetres sèrics de bioquímica clínica, per estudiar els efectes en el múscul esquelètic de la suplementació del lactoreemplaçant amb diverses combinacions d'aminoàcids. Els animals es van dividir en quatre grups: Ctrl, dieta control sense suplementació; GP, control suplementat amb glicina (G) i prolina (P); FY, control suplementat amb fenilalanina (F) i tirosina (I); i MKT, control suplementat amb metionina (M), lisina (K) i treonina (T). La integració dels resultats indica que el grup amb la suplementació de M, K i T ha mostrat una lleu millora del creixement, i un increment dels nivells de creatinina i insulina en sèrum, de la ràtio de Fosfo-S6 / S6 i d'algunes proteïnes relacionades amb la funció muscular, tot i que alguns canvis no són significatius. L'anàlisi metabolòmica del múscul esquelètic ha revelat diverses diferències entre GP - FY i Ctrl - MKT, suggerint una adaptació metabòlica especialment en els grups GP i FY.

A l'Estudi 2, s'ha utilitzat el mateix abordatge, incloent tant plasma com biòpsies de múscul esquelètic en l'anàlisi "multi-òmic". Els animals s'han dividit en tres grups: Ctrl, control sense suplementació; LT, control suplementat amb leucina (L) i treonina (T); i Leu, control suplementat amb leucina. En tots dos tractaments de suplementació, els resultats integrats han indicat que la leucina i els seus metabòlits es troben disponibles en l'organisme. En el grup LT comparat amb Ctrl, la integració dels resultats de metabolòmica i proteòmica han mostrat que la degradació d'aminoàcids ramificats (BCAA) i el metabolisme oxidatiu mitocondrial (cicle de l'àcid cítric, cadena respiratòria) són les vies principals activades en el múscul esquelètic. Els substrats del metabolisme energètic poden ser derivats de BCAA, butirat procedent del rumen i / o mobilització de lípids. Els efectes deleteris de l'activació de la fosforilació oxidativa són contrarestats per un augment de proteïnes antioxidants. El increment en la síntesi de proteïnes està indicat per l'augment de la ràtio entre Fosfo-S6 / S6 en el múscul esquelètic. En el grup de Leu, els animals han patit problemes digestius al inici de l'estudi, a més no van ser correctament randomitzats quan es van obtenir les biòpsies. Les anàlisis de bioquímica clínica, proteòmica i metabolòmica de múscul i sang han mostrat un perfil que és compatible amb un procés patològic i mostren el biaix causat per la biòpsia.

A l'Estudi 3, s'han calculat els intervals de referència de diversos paràmetres bioquímics per a vedells lactants i per a garrins acabats de deslletar, utilitzant un elevat nombre d'animals de diferents edats, procedents d'assajos duts a terme en diferents estacions. La variable principal ha estat l'edat, mentre que no s'han observat diferències degudes a l'assaig, l'estació o el sexe. Aquesta informació proporciona eines diagnòstiques més adequades a veterinaris i investigadors en ciència animal, i possibilitarà una millor pràctica professional.

4. Introduction and Literature Review

4.1. Digestive Physiology of Preruminant Calf

Unlike adult ruminant animals, that are largely dependent on fermentation of digested feed to maintain normal function of life, the physiology of the newborn calves is totally nonruminant since the rumen, reticulum and omasum are undeveloped and the abomasum (or as known as true stomach) is the predominant compartment (Davis and Drackley 1998; Ellingsen et al. 2016). At this stage, the rumen, omasum and reticulum make up roughly 25%, 10% and 5% of stomach capacity, respectively while the abomasum can make up to 60% of the capacity (Jones and Heinrichs 2006). In terms of digestive physiology, the growth of calves is categorized into three phases according to previous review (Davis and Drackley 1998):

1) The phase between birth and start solid feed consumption (normally 2 – 3 weeks post-natal) is considered the first phase (Davis and Drackley 1998), where the calves are almost entirely dependent on nutrients from milk or milk replacers (MR) that are digested by enzymes from well-developed abomasum and small intestine. The colostrum, milk or MR can bypass between rumen and reticulum through esophageal groove (*sulcus reticuli*) (Sjaastad et al. 2016), a muscular structure that tightens to form a tube-like pass to lead milk go into the abomasum (Figure 1), skipping the other digestive compartments.

2) The second phase is between the calves start eating starter until are weaned. During this time, while calves still digest milk or MR in the abomasum and small intestine, the initial fermentation of starter gradually makes the development of reticulum and rumen and rapid expansion of their volume. At 3 – 4 months old, the rumen can make up to 65% of the whole stomach compartments' volume while abomasum only remains 20% (Jones and Heinrichs 2006). One of the unique characteristics during this stage is that the esophageal groove, which consists of

muscular folds, is tightened to lead milk or MR to abomasum when calf is milked, and remains loose when calf drinks water or eats dry feeds (starters or forage) (Figure 1). The dry feeds mixed with water in anaerobic environment in the rumen give bacteria perfect place to grow and as bacteria ferment nutrients, some of the most important products are volatile fatty acids (VFA), i.e.: butyrate, propionate and acetate. Apart from the VFA, the large population of rumen bacteria can also provide amino acids (AA) not only from bacterial biosynthesis, but also from microbial proteins when bacteria are washed out and been digested by the small intestine. It takes about 3 weeks for the rumen to establish sufficient bacteria to be able to supply substantial energy for the calf's growth.

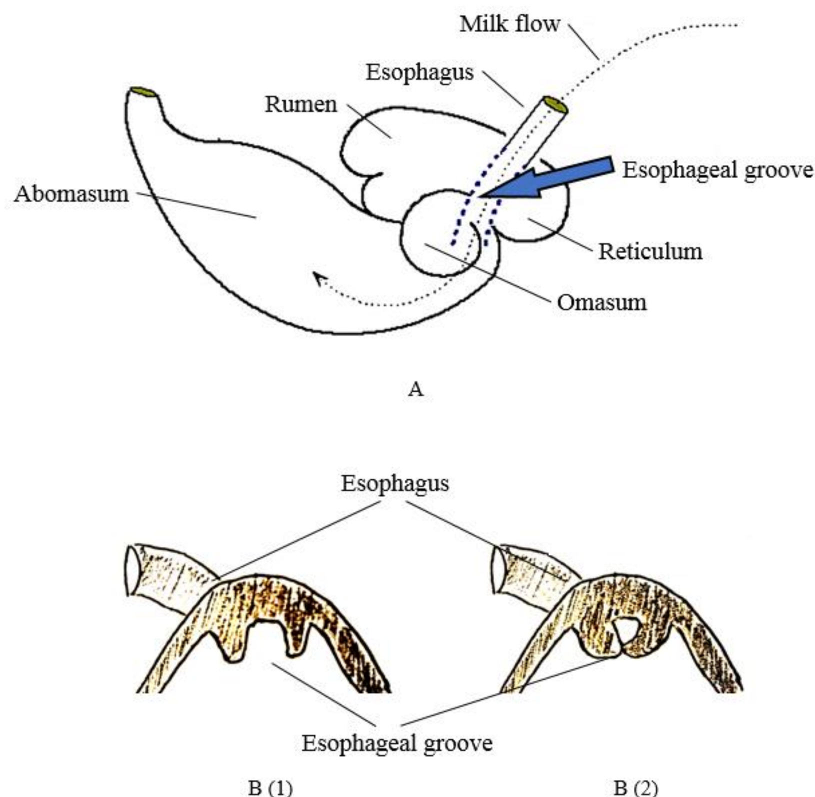

Figure 1. A. Demonstrative anatomy of digestive compartments of preruminant calf. The blue dash lines illustrate approximate location of esophageal groove. B. Demonstrative illustration of esophageal groove of calf in two states: relaxed in (1) and tightened in (2), respectively. *Source*: http://calfcare.ca/calf-feeding/the-calf%E2%80%99s-digestive-system/

3) The third phase is after weaning where solid feed is the only intake and the fermentation of dietary carbohydrates is the main energy source (Drackley 2008). In this stage, the esophageal groove disappears.

For non-ruminal digestion that occurs during the first and second phases, the enzymes in the abomasum and small intestine can efficiently digest milk proteins, fats and sugars from milk or MR made from milk protein, but are less able to digest non-milk protein or polysaccharides (Akinyele and Harshbarger 1983; Drackley 2008) even though protein digestibility goes up along the age (Davis and Drackley 1998). In the following text, we will only focus on the digestion of three major important nutrients in milk-protein liquid feed (milk, nutritional well-balanced MR or colostrum): protein, lipid and carbohydrate.

4.1.1. Milk protein

When the liquid feed enters the abomasum, the acidic condition (pH ≈ 2.0) can cause the curd formation (also known as clotting) which primarily happens with casein proteins and fat approximately 1h after feeding (Cruywagen et al. 1990; Longenbach and Heinrichs 1998). The coagulation of casein proteins is caused by the cleavage of specific peptide bond in κ-casein by rennin (chymosin) in the presence of acid (Bingham 1975), which also causes the entrapping of fat within it. The part that doesn't coagulate that contains whey proteins, lactose, soluble minerals and vitamins can pass by the abomasum and enter small intestine within 2 – 3h postprandial (Drackley 2008) without being hydrolyzed by pepsin (Boirie et al. 1997). This mechanism is very important specially for new-born calf that intakes colostrum as the whey that contains Ig can pass into small intestine prior to abomasal digestion (Cruywagen et al. 1990). The more detailed discussion on colostrum's impact to calf can be found in colostrum section (4.2.1). As the coagulum casein is trapped in the abomasum, it can be digested to polypeptides by the protease pepsin, an enzyme converted from stomach-secreted enzyme pepsinogen and activated in the presence of acid. This process is relatively slow as one study found detectable casein curd in abomasum 8h after first ingestion of colostrum (Boirie et al. 1997). One important feature in abomasal digestion of curd protein especially for colostral protein is that for

new-born calves under 2d-old, their abomasa contain the highest amounts of rennin and pepsin and later strongly decrease until 7d to become stable, this mechanism allows the new-born calves to absorb these nutrients at maximum efficiency during the perinatal period.

The fragments of casein enter the small intestine and along with other whey proteins after the pepsin digestion and they are digested into oligopeptides and free AA by pancreatic enzymes. These oligopeptides are then hydrolyzed to dipeptides, tripeptides and free AA by peptidases on the intestinal epithelial brush border as the mucosal transport mechanism only allows dipeptides, tripeptides and free AA. The absorption is through specific transport proteins on intestinal cells and, inside the enterocytes, the cytoplasmic peptidases can further hydrolyze the peptides into AA and then release AA into bloodstream.

The fate of free AA can be categorized into two mainly directions: One, participating as the precursors for synthesizing tissue proteins, enzymes and hormones. This part will be discussed in section 4.2. Two, deaminated in the liver and kidneys to keto acid and free ammonia. This process normally happens when extra AA is ingested where the keto acid can be converted to fat and carbohydrate; or be re-synthesized to AA or be oxidized. The ammonia is a waste production of AA catabolism and is toxic. For detoxification, the ammonia is converted to carbamoyl phosphate and enters urea cycle in the liver to produce urea to be released in the bloodstream and later excreted in urine by kidneys.

4.1.2. Lipid

Lipid is another important nutrient for calf as new-born calves have small amounts of body fat and relatively high energy demand in relation to live weight (LW) (Jaster et al. 1990). Moreover, in cold breeding seasons, the low temperature can alter physiological and digestive processes and, as a consequence, the demand for lipid for energy and extra

fat tissue deposit may be even higher in such conditions (Scibilia et al. 1987; Jaster et al. 1990; Jaster et al. 1992).

The fat contents of colostrum and milk from Holstein cow are 8.04 % and 3.9 %, respectively according to one study (Abd El -Fattah et al. 2012) despite the amount can vary very much depending on the season, nutrition state, etc. (McGrath et al. 2016), and generally the colostrum contains higher fat than milk (Roginski et al. 2003; McGrath et al. 2016). In milk, lipid primary consists of 98% of triglycerides (TG) while other 2% compositions are sum of diglycerides, monoglycerides, phospholipids, cholesterol, glycolipids and free fatty acids (FFA). The milk lipid absorption can be a slow process as it was previously mentioned that the milk fat is trapped within coagulum casein inside the abomasum. As a result, postprandial peak lipid absorption is at 5 – 7h later than in conventional monogastric animals (Bauchart et al. 1996).

The general lipid metabolism in preruminant is summarized on Figure 2. The TG are the major form of storage lipid in animals. Unlike adults that take VFA as primary energy source and have very low TG concentration in the blood, the preruminant calves largely depend on TG from milk for energy (Dominique and Aurousseau 1993; Yu et al. 2019). The dietary TG from milk are primary hydrolyzed by pregastric esterase (Nelson et al. 1977), a unique digestive enzyme present in saliva (Guilloteau et al. 2009), to produce diacylglycerols and FFA which are then liberated into the small intestine where diacylglycerols and remaining TG are later hydrolyzed to 2-monoaclglycerols and FFA by pancreatic lipase (Drackley 2008). In the intestinal lumen mucosal cells, FFA are re-esterified to TG and then TG are packed into chylomicrons (CM), particles that consist 87% of triglycerides and embedded with apolipoproteins (Engelking 2015). Since these particles are too big to directly enter bloodstream, CM are absorbed by lymphatic capillary (lacteal) and delivered in the lymphatic vessels and later enter veins through lymph nodes. As CM inside the bloodstream, the tissues which need TG from CM can hydrolyze TG to FA on their capillary by lipoprotein lipase, enzyme that is activated by insulin. These FA can either be stored in adipose tissues in form of TG or enter muscle to be oxidized to offer fuel. The other main form of TG is presented in body is VLDL

(Bauchart et al. 1989), they can be secreted into circulation from liver and undergo a similar fate to the CM.

Despite the preruminant calves primarily take TG as energy fuel and storage like other monogastric animals, they can also metabolize VFA such as butyrate, which can be found in milk (Guilloteau et al. 2010).

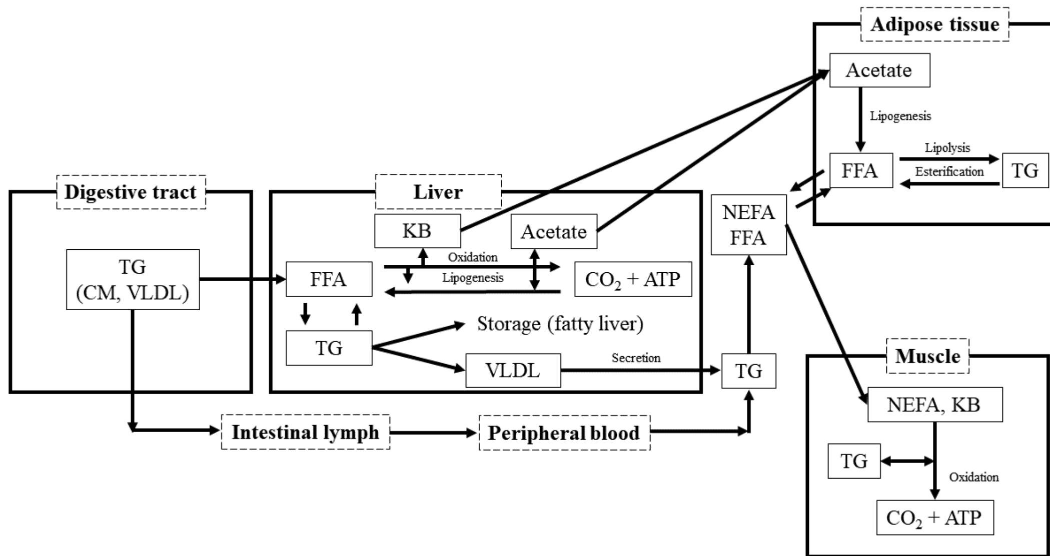

Figure 2 . Origin and fate of several lipid in non-ruminant calf. CM, chylomicrons; FFA, free fatty acids; KB, ketone body; NEFA, non-esterified fatty acid; VLDL, very-low density lipoproteins. Source: (Jean-François and Dominique 1999).

4.1.3. Carbohydrate

The digestion/metabolism of carbohydrate is quite different in preruminants compared with ruminants. One of the major reasons is that, like other monogastric animals, preruminant animals hydrolyze sugar (lactose in case of milk/MR/colostrum) to glucose and galactose in the intestine. As glucose and galactose enter the bloodstream, the liver and peripheral tissues can be exposed to large increases of insulin in order to digest them; ruminants on the other hand, are largely depend on volatile fatty acid (VFA) from the rumen to obtain energy, thus their intestinal absorption of carbohydrates is largely decreased. Meanwhile, the carbohydrate digestion in preruminant differs from other monogastric animals because preruminant can't digest saccharose due to the lack of sacharase (Drackley 2008).

As the primary carbohydrate from the milk for preruminant is lactose, lactase activity in small intestine is highest on the first day of life and keeps very high level during first 7d of life (Huber et al. 1961; Guilloteau et al. 2009). As lactose is soluble inside whey, it travels directly to small intestine and is hydrolyzed to glucose and galactose by lactase and these two monosaccharides are absorbed by brush border of epithelial cells through an Na^{+}-powered secondary active transport system (Berne and Levy 1996). These sugars later leave the epithelial cells via facilitated transport and are diffused into the mucosal capillaries.

Enzymes for starch such as amylase and maltase activities are low at birth as other sugars such as starch from starter normally are not given until 2 – 3w of age but later increase significantly in the upcoming weeks of life (Guilloteau et al. 2005) to adapt to solid feed digestion. Once the sugars enter the bloodstream, glucose is used in glycolysis or other metabolic pathways to offer fuels for tissues.

4.2. Nutrition for Preruminant Calves

Despite scientists categorized the growth of calf into three major stages as it was previously mentioned, the metabolism and the demand of nutrients could be different between day to day, this especially implies the first stage, from birth to starter intake. During these 2 – 3w of life, special attention is drawn during the first 2 – 3 days, because it is when calf starts to face the abrupt cut of nutrients from uterus and depends on nutrients from colostrum that contains high amounts of nutrients, hormones as well as immune components. After colostrum intake, and as milk or MR is the predominant nutrient source for calves until weaning, the nutritional properties for MR need to be studied to guarantee their proper growth and health. In this part, we will first focus on discussing the special nutritional and immune properties of colostrum and the way calf digests it, then, we will discuss AA in MR that are commonly studied in calf industry.

4.2.1. Colostrum's Impact on New-born Calf

Within the 3 days after the calf is born, its immune system is very vulnerable to pathogens and an immediate nutrition-rich support is needed. Colostrum has many beneficial properties compared with milk: a comparison between cow milk and colostrum indicates that the content of dry matter, protein, fat and lactose in colostrum are of 216.6%, 450.2%, 136.7% and 63.5% to those of in milk (Pecka-Kiełb 2018). Colostrum provides to the new-born calf instant nutrition and immune protection thanks to its IgG content through the passive transfer within 24h after birth (Jochims et al. 1994; Miyazaki et al. 2017).

As for the mechanism of passive transfer, for calf under 2 – 3d of life, large proteins such as Ig from colostrum are non-specifically absorbed by pinocytosis in the intestine and transferred across the cell to enter the bloodstream by exocytosis (Weaver et al. 2000). Consequently, Ig are highly elevated in the bloodstream of calf as studies show that, after colostrum intake upon birth, the levels of serum IgG are significantly elevated within 24h

compared with the levels at birth (Hadorn and Blum 1997; Egli and Blum 1998). Interestingly, since the colostral passive transfer is nonselective, some other macromolecules can also enter bloodstream in this way. One specially attention is drawn by GGT, a traditional biomarker of liver damage in case of its elevation in plasma but can also be largely elevated at 24h of age (24h after colostrum intake). This elevation can roughly be 100 folds (Hadorn and Blum 1997; Egli and Blum 1998), 150 folds (Yu et al. 2019) or even 800 folds (Thompson and Pauli 1981), and as a consequence the GGT can be used as an indicator of IgG absorption in neonatology (Miyazaki et al. 2017) but not as biomarker for liver damage. However, it's advisable to use GGT as indicator for individual rather than herd not only because variability between individuals is very high according to results in these studies, but also because low concentrations of GGT in new-born calves are linked with much higher risk of death (Thompson and Pauli 1981; Parish et al. 1997).

Since the colostrum has an important impact to calf's health, studies suggest that delay colostrum feed for one day can have disastrous impacts on calves (Hadorn et al. 1997; Hadorn and Blum 1997). These impacts are not limited to a worse blood clinical chemistry profile where hypoglycemia, low plasma IgG/GGT and higher NEFA/bilirubin were observed on the first day without colostrum. Moreover, despite colostrum is given on the 2nd day, the rise of IgG is much smaller, which implies that the closure of absorption of IgG happens around 2d of life regardless of the colostrum intake delay (Michanek et al. 1989; Michanek et al. 1990). GGT on the other hand, according to some studies, didn't rise even after colostrum intake on the 2nd day, which may suggest an even faster absorption closure for GGT (Ronge and Blum 1988; Grütter and Blum 1991). In pigs, some similar experiments were performed by withholding colostrum intake and resulted in prolonged intestinal closure (Harada and Takeuchi 2002; Sangild 2003), suggesting that the cessation of macromolecular transport is affected by several hormonal factors such as glucocorticoids, thyroxine, epidermal growth factor (EGF), insulin, secretin, etc. Indeed, due to the absence of deeper study in calves, the total mechanism of intestinal closure is not fully understood.

More interestingly, some studies have supplemented large amounts of different non-Ig proteins to colostrum and resulted in worse Ig passive transfer (Besser and Osborn 1993; Quigley et al. 1998; Davenport et al. 2000), this may be due to excessive amounts of non-Ig proteins competing with non-specific transfer.

In summary, colostrum has very complete nutritional profile and is indispensable for recent-born calf's health. Also, its absorption can be directly reflected by several blood biochemistry analytes and it is advisable to have such routine health check to guarantee the best survival quality of new-born calves.

4.2.2. Milk Replacer Nutrition for New-born Calf: A Review of Amino Acids and Functions

After the colostrum feed, the calf largely depends on nutrition from milk/MR until weaning. MR nowadays is becoming more and more popular due to the increase demanding of cow milk for human consumption and its less-costly price (Davis and Drackley 1998). MR however, can vary a lot depending on the protein sources such as milk protein (i.e. MPMR) (Terré et al. 2006; Yu et al. 2018), soybeans (Kanjanapruthipong 1998; Abe et al. 1999), plasma (Morrison et al. 2017) or wheat (Castro et al. 2016). MPMR is considered the best protein source for calves due to its best digestibility (Montagne et al. 2001; Drackley 2008; Castro et al. 2016). However, despite the advantage of using milk proteins in MR, if the AA profile is not well-balanced, certain AA can be limiting and may have negative effects in growth.

Methionine, Lysine, Threonine and Leucine: 4 Key Proteinogenic Amino Acids

Among all proteinogenic AA, methionine, lysine and threonine are the most studied. In the last decades, many studies focused on the requirements of these three (single or

combined) essential AA on different types of MRs as they are considered limiting AA especially in MR from soybean proteins (Pelaez and Walker 1979; Jenkins and Emmons 1983; Kanjanapruthipong 1998; Hill et al. 2008) when people started using non-milk proteins as alternative in the late 70s (Roy et al. 1977). Some evidences showed that supplementing these AA regardless of the MR type showed better performances (Hill et al. 2008; Yu et al. 2018) while other studies contradict part of the theory showing that no improvement were observed in MR supplemented with methionine (Castro et al. 2016) or threonine and isoleucine (Morrison et al. 2017). Despite no study was found explaining fundamental effect of these three AA to the preruminant calf, based on many other studies on other species, we hypothesize that the benefits of these three AA are mainly due to their proteinogenic and immune properties.

Methionine is a sulfur-containing AA that is the precursor of several important metabolites and other AA such as homocysteine, betaine, choline, cysteine, S-adenosylmethionine (SAM), taurine, etc. It has a relevant role in the metabolism of monocarbonate groups (Wu 2009).

Lysine is another important proteinogenic AA. It may go through several post-translational modifications (PTM) such as acetylation and methylation (Choudhary et al. 2009; Zhang et al. 2012). As it was studied using MS, 3600 lysine acetylation sites were identified on 1750 proteins, involved in important cellular process such as chromatin remodeling, cell cycle, splicing, nuclear transport and actin nucleation (Choudhary et al. 2009). Lysine is also involved in the regulation of immune system as it was shown a reduced dietary lysine is associated with worse lymphocyte proliferation in chicken (Kidd et al. 1997). Moreover, increased dietary lysine has shown an enhance effect for the NO synthesis by promoting arginine absorption as arginine and lysine share the same transport system (Wu and Meininger 2002; Luiking and Deutz 2007; Yu et al. 2018).

Among the essential AA, threonine is particularly important for the maintenance of the intestine as 30% of intestinal mucin contains threonine (Faure et al. 2005) and studies in human (Fuller et al. 1994) and pig (Stoll et al. 1998) show that up to 60% of dietary

threonine is retained by the intestine. Also, threonine directly participates in the synthesis of glycine and serine, which are also important structural AA for intestinal mucin (Li et al. 2007). Moreover, threonine and serine are correlated to the regulation of cell growth, cell proliferation, protein synthesis and promote antibody production (Duval et al. 1991; Li et al. 1999).

Leucine is one of the branched-chain AA (BCAA, together with isoleucine and valine) and, among the essential AA, it has drawn a lot of attention in recent years due to its function in promoting protein synthesis through the mTOR pathway. This effect has been described in skeletal muscle (Escobar et al. 2005; Yin et al. 2010), liver (Kanazawa et al. 2004), mammary gland (Appuhamy et al. 2012; Arriola Apelo et al. 2014) and more recently in pancreas (Cao et al. 2018; Guo et al. 2018) in different species. Leucine also enhances energy homeostasis through increasing fatty acid oxidation and it provides skeletal muscles with an increased flux of lipids, supplying energy substrates to support protein synthesis. Leucine from dietary protein can bypass metabolism in the liver, which results in a rise of plasma leucine levels and the activation of leucine signaling in peripheral tissues in response to a meal (Duan et al. 2016). Among these tissues/organs, the skeletal muscle is the most studied as it is the main site for BCAA oxidation (Bonvini et al. 2018).

Glycine and Proline: The backbones of Collagen

Glycine and proline are non-essential AA for calves. Glycine can be synthesized from threonine while proline is biosynthetically derived from arginine, an EAA only during young age for animals (Wu 2009). These two AA play a major role in the build-up of collagen, which is largely found in skin, bone, tendon as well as a small amount in muscle and other tissues. Glycine is found at every third residue (Ramshaw et al. 1998; Shoulders and Raines 2009) resulting a Gly-X-Y (Glycine-X-Y) sequence pattern where X and Y are any other AA. Among the sequence pattern, the most frequent one is Gly-Pro-Hyp (Glycine-Proline-Hydroxyproline) that ensures the highest stability of the triple-helix (Berg and Prockop 1973; Heidemann and Roth 1982). This makes glycine and proline

indispensable for the tissue as 4-hydroxyproline is also synthesized directly from proline via prolyl 4-hydroxylase.

Phenylalanine and Tyrosine: Central Role of Neural System Regulation

Phenylalanine and tyrosine are aromatic AA. Tyrosine may be synthesized from EAA phenylalanine. In many proteins, tyrosine may suffer PTMs as phosphorylation, nitrosation and sulfation. Despite in the preweaning time, growth in young calves mainly occurs in the skeleton and muscle systems (Drackley 2008), the development of neurological system is also very crucial. For phenylalanine and tyrosine, besides the role as constituents of protein, their other functions in brain are being precursors for the neurotransmitters such as catecholamines (dopamine, norepinephrine and epinephrine) (Fernstrom and Fernstrom 2007). Studies have shown that serum catecholamine concentration is correlated with availability of tyrosine, and subsequently can affect behaviors in dogs (Gazzano et al. 2018) and rats (Yeghiayan et al. 2001).

4.3. “-Omics” Approach in Farm Animal Study

As the technological tools improve dramatically, scientists in animal science also have been benefited with better ways to understand the fundamental biological processes in animals. Nowadays, the general accepted “-omics” is based on the scheme of the “-omics” cascade (Figure 3), however, this is not limited to the four major classes (genomics, transcriptomics, proteomics, metabolomics) since other realms like lipidomics (the study of lipids) and glycomics (the study of carbohydrates) may be considered a sub-branch of metabolomics and may require specific techniques for studying.

In this part, we will focus particularly in the techniques of proteomics and metabolomics as well as their applications in farm animals.

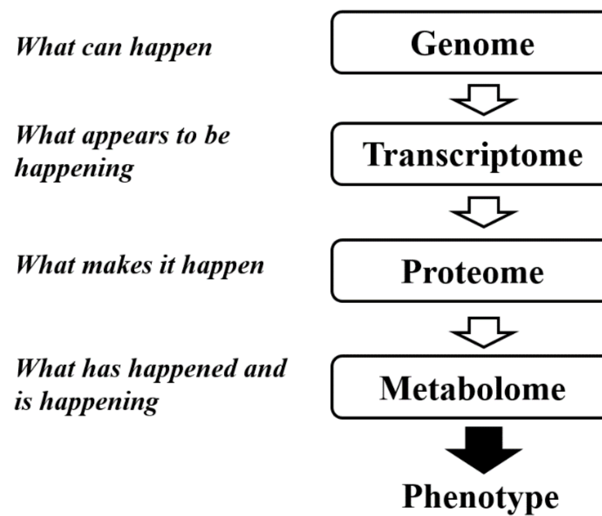

Figure 3. The "-omics" cascade comprises complex datasets that as an entity comprehensively describe the response of biological systems to disease, genetic, and environmental perturbations. The most powerful database will integrate data from all "-omics" levels. *Source:* (Dettmer et al. 2007).

4.3.1. Proteomics

Proteomics is a large-scale analysis of proteins which can greatly contribute to our understanding of gene and protein function in the post-genome era (Pandey and Mann 2000). Different from genome, which is a relatively fixed dataset, the proteome however, is not only comprised of native translation products, but also post-translational proteins (phosphorylated, acetylated, methylated, etc.), thus the proteome can be different in different stages of life, environments, etc. The history of proteomics can date back to 1970s when scientists already started to build databases of proteins based on high resolution 2-DE (O'Farrell 1975). The emerging development of proteomics started from 1990s when the technology of mass spectrometry (MS) became a powerful tool to overcome several limitations such as lack of sensitivity back in old days with 2-DE. The availability of public protein databases has led to the prosper present post-genome era.

In terms of methodology, proteomics can be categorized as bottom-up proteomics and top-down proteomics (Figure 4).

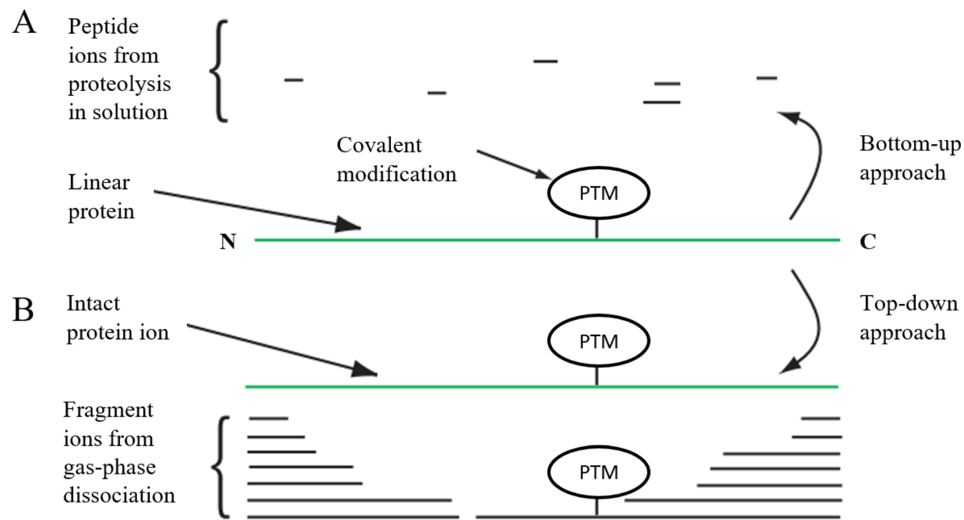

Figure 4. A) Bottom-up proteomics (BUP) and B) Top-down proteomics (TDP) approaches for protein analysis. *Source:* (Kelleher 2004). PTM, Post-translational modification.

Bottom-Up Proteomics (BUP)

This technique is considered the most common way to identify proteins and characterize AA sequences, including in veterinary science (Campos and de Almeida 2016; Bilić et al. 2018). The technical processes go from whole proteins to peptides and typically involve several steps of protein preparation – protein digestion – ions fragmentation – peptides analyses. Based on different approaches, there are mainly two sub-types: gel-based and gel-free.

1) Gel-based approaches: The 2-DE was introduced back in 1970s (O'Farrell 1975). The principle of 2-DE is based on two biochemical characteristics of proteins: isoelectric point (p*I*) and molecular mass (Chevalier 2010). In order to perform 2-DE several steps are required: sample/lysate preparation, protein quantification, clean-up, protein re-quantification, strip rehydration, isoelectrofocusing

separation (1st dimension), electrophoretic separation (2nd dimension), staining, protein/spot identification. In this way and depending on the sample type, a typical 2-DE loaded with 100 µg total protein can give a profile of more than 2000 or even up to 5000 separated protein spots and sensitively enough to detect up to <1 ng of protein per spot. Afterward, the proteins of interest can be subsequently digested and taken for MS analyses. However, some drawbacks of this technique are time/labor-consuming, bad automation, relatively low-reproducibility (Thompson et al. 2003) and lack of sensibility of extra-large/small/acidic/basic proteins (Martinez-Subiela et al. 2017). Nevertheless, 2DE remains being the best approach to study protein isoforms and some types of PTM.

2) Gel-free approaches (shotgun proteomics): Typically, the whole proteome is digested without prior protein separation and the digested peptides are separated by strong cation exchange chromatography before reversed-phase LC-MS/MS (Patel et al. 2009). There is an increasing use of this method in veterinary medicine (Campos and de Almeida 2016) as shotgun proteomics doesn't require protein pre-identification in gels and can overcome several limitations mentioned previously. Shotgun proteomics can be categorized into label-based and label-free approaches, depending on the sample preparation type.

There are several techniques available for label-based approaches (Patel et al. 2009). The most commonly used are Isobaric tag for relative and absolute quantitation (iTRAQ) and Tandem mass tag (TMT). These two techniques from different companies (SCIEX and Thermo Fisher, respectively) have very similar basis allowing the analysis of multiple sample mixtures using stable isotope labelling to provide chemically same but isotopically different internal standards for each peptide/protein for direct comparison of MS signal (Asara et al. 2008). Some of the advantages of iTRAQ and TMT are quantitative, high-coverage, high-sensitivity and good reproducibility. Disadvantages of label-based are experimental-complexity (multiple sample preparation steps), time-consuming (approximately 60h for a whole run) and potential error introduced by incomplete labeling.

The label-free has become an emerging approach since no additional chemistry or sample preparation steps are required (Asara et al. 2008) thus it has overcome shortages from label-based approach. Moreover, this approach can reach a coverage of 45% (Patel et al. 2009), so more confident identifications can be achieved. However, despite label-free normally runs each analysis within very short amount of time, it needs to perform analysis one by one for larger sample amounts since it may introduce batch-biased error. Label-based approaches, on the contrary, can effectively reduce the patch-biased variability.

In conclusion, all the approaches mentioned above have their own advantages and disadvantages. A selection based on reproducibility, confidence in identification and quantification, amount of sample, finance, and sequence coverage should be considered to decide the best approach for a particular problem.

Top-Down Proteomics (TDP)

Due to the increasing demand of better understanding of protein function, and since BUP seems to have reached a bottleneck as its sequence coverage is relatively low (5 – 70%), other techniques with higher sequence coverage were necessary. In this way TDP was introduced in the later 1990s and early 2000s. In contrast to BUP, TDP directly analyzes the intact proteins by MS without previous proteolytic digestion (Siuti and Kelleher 2007), thus this not only facilitates the preparation procedure, more importantly, the intact protein information is preserved. A comparison between BUP and TDP is shown in Figure 4.

TDP is highly suitable for comprehending of PTMs, identifying families or high related gene encoding protein sequences with high identity and analyzing membrane proteins (Siuti and Kelleher 2007). Some of the limitations of TDP are low chemical resolution, harder handling with mixtures of wildtype proteins, challenging characterization of low abundant PTMs, etc. (Kelleher 2004; Siuti and Kelleher 2007). However, as instruments

become more sensitive, TDP will still have a lot of space to develop. Moreover, there is a new trending of combining BUP and TDP, which can compensate these disadvantages and ameliorate the results.

A conclusive table is made for comparison of advantages and disadvantages of both BUP and TDP (Table 1).

Table 1. Comparison of advantages of disadvantages of bottom-up proteomics (BUP) and top-down proteomics (TDP).

	Bottom-up proteomics	**Top-down proteomics**
Trypsin digestion	Yes	No
Target of analysis	Peptides	Intact proteins
Experimental complicity	Complicated	Relatively easy
LC performance	High throughput	Low throughput
MS performance	High resolution and sensibility, low sequence coverage	Low resolution and sensibility, 100% coverage
Bioinformatics	Well established	Requires more contributions

4.3.2. Metabolomics

Back to 1950s, despite human had already discovered the genes, proteins and metabolites and had some understanding of their functionality, it was considered that the biological regulation was linear, i.e., the gene codifies for the enzyme, and the enzyme regulates the metabolic pathway thus leading to the function of the organism (Hollywood et al. 2006) (shown in Figure 5A). Nowadays however, evidences demonstrate that the cellular process involves not barely “top to bottom” regulation, but also many feedback-loops between larger molecule sets since cell processes are dynamic, complex and with many interactions with other enzymes and metabolites (Figure 5B, C). As metabolites are more dynamic, diverse and sensitive to the environment than genes or proteins, a technology

that aims to a better characterization of the whole set of metabolites, i.e. the metabolomics was developed.

Metabolomics is the study of sets of small-molecules (<1500 Da) within a biological sample, which is directly related to the metabolic activity, protein activity and/or gene expression (Nicholson and Wilson 2003; Bernini et al. 2011). The minor changes in genome and epigenome can affect the proteome, and the small changes of proteome can be easily detected in the metabolome. Moreover, both physiological and environmental influences can change the overall expression of the genome, and finally affect the metabolome, making the metabolome more diverse in chemical and physical properties compared with other "-omes" (Dunn et al. 2005). Studying the metabolome can contribute to our understanding of dynamic changes in metabolism, especially in basal metabolism, environmental changes (nutrition, drugs, physical activity, stress, etc.) or the consequences of gene mutations.

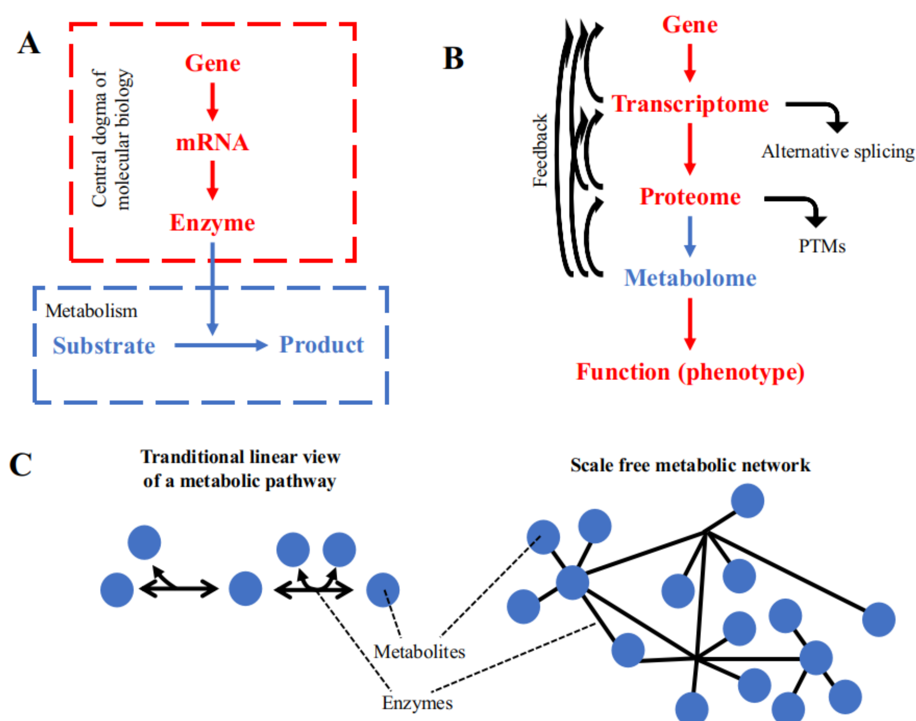

Figure 5. **A.** Traditional scheme flow of how a biological function is regulated from gene to metabolism. **B.** General regulation scheme according to nowadays "-omics" concept where a large set of genes/proteins/metabolites are involved in certain function (phenotype) with interactive feedback mechanism. **C.** Traditional model of pathway (left) and modern scale-free connective pathways. Nodes represent metabolites and lines for enzymatic reactions. *Source:* (Hollywood et al. 2006).

In this part, metabolomics will be introduced by 1) Sampling and Sample Preparation and 2) Analytic Techniques and Data Analysis.

Sampling and Sample Preparation

Metabolomics has wide applications in veterinary/animal science and several biological samples have been studied: blood (serum and/or plasma) (Beckonert et al. 2007; Baranovicova et al. 2018), mammary gland (Palma et al. 2014), skeletal muscle (Palma et al. 2016; Yu et al. 2018), meat exudate (Castejón et al. 2015), liver (Palma et al. 2016), etc.. However, special considerations should be taken when choosing sample types:

1) The physical properties: Steady sample types can make the whole study easier. Samples like serum and plasma are considered very homogenous samples due to their homeostatic properties (e.g. steady pH and temperature), thus they are less likely to introduce unnecessary variables to the study. Other sample types such as muscle and urine are considered less homogenous as the physical state, environment and sample handling can easily alter the metabolome.

2) Difficulty of preparation: Not all the sample preparations are the same. For some larger projects, big number of samples may be required and easier sample preparation can be time-saving. For example, the generic protocol for biological fluids (serum, plasma, urine, cerebrospinal fluid (CSF), synovial fluid, dialysis fluid, etc.) only includes deproteinization and metabolite extraction. Tissue samples on the other hand, may require grinding, which is time-consuming and depending on the interest of study several additional precautions are addressed which may complicate the preparation. However, some technical advances can facilitate the process such as high-resolution magic angle spinning (HR-MAS) NMR spectroscopy can measure intact tissue without any pretreatment.

3) Data availability: None of the "-omics" study can be conducted without databases. Well-studied sample types like serum and plasma tend to have a greater possibility of available databases, literature and mature protocols, while "uncommon" sample types may require scientists to do many more researches from scratch.

Sample handling is also an important topic as metabolic adaptation to the environment is very sensitive and the metabolic reaction half-lives are generally less than 1s, thus there is the potential for metabolic changes to be observed that are not related to the scientific question being studied (Dunn et al. 2005). Despite very few animal studies roll out precautions of sample handling due to the sensitivity of metabolome, a review in plants (Biais et al. 2011) calls out that recording some major environmental variables during sampling (temperature, light and season) and controlling the sample storage and transport conditions can effectively reduce the results that are not relevant to the study. This theory is also applicable for animal metabolomics as the sampling is very similar between the two kingdoms. However, special caution is needed for animal samples such as biopsy-sensitive metabolites can greatly change the concentrations in different post-biopsy ischemia interval (time between tissue is cut from the body and be properly stored at low temperature), animal welfare state (hunger, fear, illness, etc.) as well as farm temperature. The more detailed discussion will be explained at section 7.

Analytic Techniques and Data Processing

The main analytic techniques for metabolomic studies are NMR-based and MS-based, the latter requires pre-separation of metabolites using either gas chromatography after derivatization, or liquid chromatography (Beckonert et al. 2007). Both techniques have their strengths and weaknesses.

1) NMR-based: This quantitative, non-destructive, non-invasive and highly-reproducible, wide dynamic ranged and low time-consuming technique is the most used for metabolomic studies. This technique is based on that the atomic nucleus is a spinning charged particle and can be aligned either with or against an external magnetic field. The

energy difference (ΔE) from nuclei between aligned and opposed to the external magnetic field depends on the applied magnetic field strength. When the field is applied with a radiation which has the same energy as ΔE, the nuclei with lower energy state can flip to a higher one, and once radiation is removed, the nuclei undergo relaxation and returns to the original state. Meanwhile, they emit electromagnetic signals which can be detected by the sensor. In this way, the NMR spectroscopy can characterize the chemical structures depending on ΔEs from different structural properties. The ^{1}H NMR is the most used approach as hydrogen is the most abundant element on earth while other natural abundant nuclides such as ^{13}C, ^{15}N and ^{31}P are also used to provide complementary information especially for structural elucidation. Moreover, other methods such as two-dimensional (2D) NMR spectroscopy can increase signal dispersion and elucidate the connectivity between signals thus helping to identify biochemical substances (Beckonert et al. 2007). Typical 2D NMR spectroscopy includes ^{1}H-^{1}H COSY (correlation spectroscopy) and ^{1}H-^{1}H TCOSY (total correlation spectroscopy) that provide information on which hydrogens in a molecule are close in chemical bond terms and HSQC (heteronuclear single quantum correlation spectroscopy) that detects correlations between nuclei of two different types which are separated by one bond (e.g. ^{1}H-^{13}C, ^{1}H-^{31}P, etc.).

Some limitations/drawbacks of NMR-based metabolomics are: a) strong pH variations between samples can lead to signal shift in less homogenous sample such as urine and post-exercise muscle, despite this is not problematic, it should be considered in any study in which acidosis or alkalosis is induced (Beckonert et al. 2007); b) the NMR spectroscopy doesn’t have a good resolution in characterizing lipid (organic phase) and for aqueous phase, normally no more than 60 metabolites can be identified in a biological sample, thus the search of existing metabolic “dark matter” (Markley et al. 2017) can be challenging.

For data process, collected ^{1}H NMR spectrum data (.1r) can be processed by Chenomx, a powerful commercial software that can identify and quantify metabolites, predict compound chemical structure and chemical shift in NMR spectrum. Based on its large metabolite library, its assignment is made by fitting the chemical shifts in the spectrum and quantification is based on the assigned peak area referring to the internal standard

concentration. Apart from the Chenomx library, additional assignment can be made by searching from literature or online repository such as BioCyc, ChEBI, HMDB, KEGG, MetaboLights (Haug et al. 2013), PubChem, etc.

2) MS-based: Despite NMR spectroscopy is still widely used, the number of MS-based metabolomic publications has overtaken the NMR-based ones from 2009-2010 (Emwas 2015; Markley et al. 2017) since it overcomes several drawbacks presented by NMR spectroscopy. Herein we will explain the MS-based metabolomic approach and its several advantages over NMR.

The MS coupled with other techniques such as gas chromatography (GC), liquid chromatography (LC), ultra/high-performance LC (UPLC/HPLC), thin-layer-chromatography (TLC), electrospray ionization (ESI), etc. has gained increasing popularity in recent years. Some common platforms include GC-MS, LC-MS, HPLC-MS, UPLC-MS, DFI-MS/MS and TLC/GC-FID. One of the main advantages in MS-based over NMR-based is the ability of characterizing lipid (lipidomics) as NMR-based has very low organic resolution. Using LC-MS techniques, a “shotgun” lipidomics approach is available to analyze lipid chasses and mass fragment and thus identifying lipid types; alternatively, separating lipid classes individually and identifying fatty acids using GC-MS allows better identifications (Psychogios et al. 2011). One human serum metabolome study revealed that only 49 aqueous metabolites were identified by NMR while a minimum of 96 and up to 3381 different types of metabolites were identified with an MS platform (shown in Figure 6) (Psychogios et al. 2011). Also, MS-based shows advantage over NMR-based targeted metabolomics as this approach usually focuses on the characterization of known metabolites and the preparation of samples can be adjusted to reduce the effects of interference from associated metabolites (Emwas 2015). Moreover, many labs start working on both metabolomics and proteomics and only MS can cover both approaches. For the above reasons, there is a rapid increase of MS-based metabolomic studies.

MS identification has a very similar process to NMR except that some MS-specific databases are available: the most common ones are METLIN (Colin A. Smith et al. 2006) and MassBank (Horai et al. 2010). A detailed overview of libraries and their comparison has been reviewed by others (Vinaixa et al. 2016).

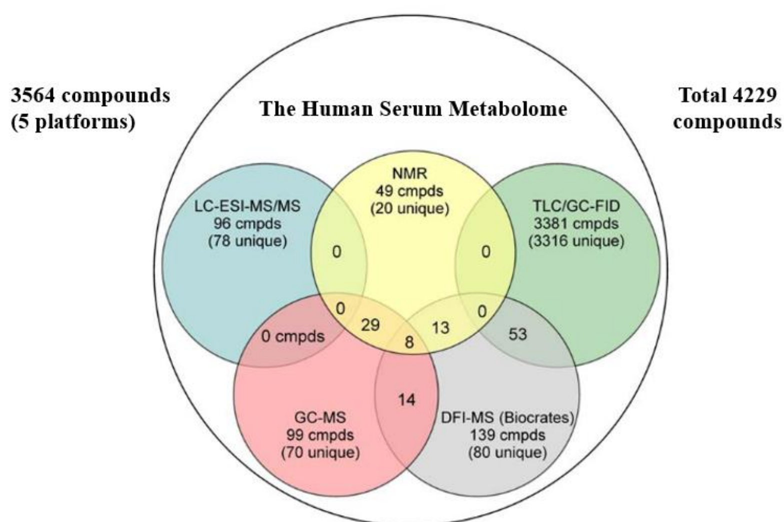

Figure 6. Venn diagram showing the overlap of human serum metabolites detected by global NMR, GC-MS, LC/GC-FID, LC-ESI-MS/MS and MS/MS methods compared to the detectable serum metabolome. cmpds: compounds. *Source:* (Psychogios et al. 2011).

3) Hybrid NMR/MS: In recent years, the call of merging NMR spectroscopy and MS approaches for metabolomic study has been emerging in science and it is aimed to take advantage of strengths from both sides. The hybrid NMR/MS metabolomics is mainly used in known metabolite validation and unknown metabolite structural construction (Bingol and Brüschweiler 2015), however, it is rarely used in farm animal science or veterinary.

A comparison between NMR-based and MS-based metabolomics is summarized in Table 2.

Table 2. Comparison of NMR-based and MS-based metabolomics.

	NMR-based	MS-based
Sensitivity	Low but can be improved with higher magnetic field, dynamic nuclear polarization, cryo- and microprobes	High and detection limit reaches nanomolar
Automation and high-throughput	Good	Poor to moderate
Analytic strength	Very robust for metabolites in aqueous phase, but not for those in organic phase	Generally robust for all kinds of metabolites, but also depending on the method
Analytic focus	Untargeted	Untargeted and targeted
Reproducibility and recovery	Very high reproducibility, non-destructive, non-invasive recoverable approach	Moderate reproducibility, destructive approach
Number of detectable metabolites (urine as example)	Normally < 60	Normally > 50, up to ~3000 depending on the method
Platforms	NMR spectrometer with different magnetic strengths and/or probes (^{1}H, ^{13}C, ^{15}N and ^{31}P)	GC-MS, LC/GC-FID, LC-ESI-MS/MS, etc.
Sample preparation	Generally easy, for tissue samples may require extract except HR-MAS NMR	Requires several steps, more complicated in multi-platforms.
***In vivo* study application**	Yes	No
Space requirement and affordability	Normally requires big, separated space, higher magnetic strength requires even bigger room, generally expensive	Normally compact and can be fitted on lab bench top, generally cheaper than NMR spectrometer

4.4. Reference Interval (RI) Establishment

Despite many techniques are established for a better monitoring of animal health, the basic clinical biochemistry of blood is still the most used, quickest and most economical way for fast physiological assessment and medical diagnosis. The reference values, sometimes known as “normal values”, are traditionally poorly defined and are not determined by a uniform process. This problem is even commoner in veterinary pediatric science where many reference values are not available, or set with small populations from one farm, which may lead to non-reliable RI and misdiagnosis. Thus, better population-specific reference values are needed for young animals.

According to Clinical and Laboratory Standards Institute (CLSI), the RI is defined as the interval between and including two numbers, i.e. an upper and lower reference limit, which are estimated to enclose a specified percentage (usually 95%) of the values of a subjected population. The calculation of RIs is a systematic process that takes into account the various influences on the measured laboratory test results (CLSI 2010). However, the determination of RI specific for young animals may meet several challenges not only because the small population from single farm, but also because health is a relative condition lacking a universal definition (CLSI 2010) and several factors may strongly alter the final value.

For most analytes, the lower and upper reference limits are assumed to demarcate the estimated 2.5th and 97.5th percentiles of the underlying distribution of values, respectively (CLSI 2010). There are normally two methods for RI determination: nonparametric method and parametric method.

Nonparametric Method: Calculation

The nonparametric method is widely used as it is a simpler approach as well as it is recommended by CLSI. This method does not require a Gaussian distribution, outlier removal and is calculated based on large sets of number ($n \geq 120$ is recommended (Reed et al. 1971)).

For the calculation, r represents the rank of an observation within a total population of n where the smallest is ranked $r = 1$ and the largest $r = n$. The lower reference limit r_1 (2.5th percentile) corresponds to the observation $r = 0.025 * (n + 1)$ while the upper reference limit r_2 (97.5th percentile) corresponds to the observation $r = 0.975 * (n + 1)$. Since the calculated values of r_1 and r_2 are often not integers, the limits are calculated by interpolating between the data points corresponding to the ranks on either side of r_1 and r_2. As an example from data published previously on calf RI (Yu et al. 2019), the $n = 185$ for serum SOD in calves at 2 weeks old, thus according to calculation:

$$r_1 = 0.025 * (185 + 1) = 4.65 \approx 5$$

$$r_2 = 0.975 * (185 + 1) = 181.35 \approx 181$$

The values of interpolated 5th is 0.4 U/L and 181st is 0.84 U/L, thus the serum RI of SOD for calves at 2 weeks is [0.4 – 0.84] U/L.

Treatment of Outliers and Data Transformation for Parametric Method

The nonparametric method is a handy tool for calculating the RI, however, many times farms or clinics cannot gather enough population (up to 120) (Egli and Blum 1998; Mohri et al. 2007; Pérez-Santos et al. 2015). In these cases, the parametric method is an alternative. As the Gaussian population is required in the first place, distribution test such as Anderson-Darling or Shapiro-Wilk test are needed to test the normality. If no Gaussian

distribution is observed, a transformation is needed. The most commonly used is Box-Cox transformation explained by G. E. P. Box and D. R. Cox (Box and Cox 1964). For practical use, R package "AID" (Osman Dag, Ozgur Asar 2019) or Microsoft Excel add-on "Reference Value Advisor" (Geffré et al. 2011) recommended by the American Society for Veterinary Clinical Pathology (ASVCP) can easily perform such transformation. Afterwards, distribution normality test is still needed.

Meanwhile, for some "unfixable" non-Gaussian distribution, possible outliers are the main factor that cause observed measurements be less homogeneous. However, despite outliers are known to be aberrant observations due to mistake, the emphasis should be on retaining rather than deleting them (CLSI 2010). While each lab may decide which measurement to be retained or deleted (example in a previous study (Yu et al. 2019)), statistical tools such as Tukey (Tukey 1977) and Dixon (Dixon 1953) can also aid for better detection.

Parametric Method: Calculation

The parametric method can be categorized into standard method and robust method. For typical 95% prediction interval in standard method, it is calculated as follow:

$$Lower\ limit\ =\ mean\ -\ t\cdot\sqrt{\frac{n+1}{n}}\cdot sd$$

$$Lower\ limit\ =\ mean+\ t\cdot\sqrt{\frac{n+1}{n}}\cdot sd$$

Where, the t is the 97.5% quantile of a student t-distribution with $n-1$ degrees of freedom.

The robust method on the other hand, is recommended especially for small sample sizes according to CLSI. However, to be able to use robust method, data symmetricity needs to be tested for the first place. The robust method involves iterative process (Horn and

Pesce 2005), in which the initial location (center) is estimated by the median and the initial scale (spread) by the median absolute deviation about the median (MAD) (CLSI 2010). However, it is always recommended using larger datasets since as the amount of observations increases, the confidence interval narrows.

5. Background and Objectives

The line of research of our group is the search for biomarkers in animal science and veterinary medicine, and the application of "-Omics" technologies to veterinary problems and challenges. Previously to the present Thesis, research on stress and welfare biomarkers in pigs and cows was undertaken (Marco-Ramell et al. 2011; Marco-Ramell et al. 2012; Marco-Ramell et al. 2014; Marco-Ramell et al. 2016). Later, under the ANEMOMA project, this search was extended to the involvement of the central nervous system in pigs (Valent et al. 2017; Valent et al. 2019). The present work is based on AMINOCRET, a project funded by the Ministerio de Economia y Competitividad (AGL2015-68463-C2-2-P). This is a project coordinated with IRTA (Institut de Recerca i Tecnologia Agroalimentaria), with Dr Marta Terré, as Principal Investigator in the IRTA subproject.

The general goal of AMINOCRET was aimed to identify candidate AA that may be limiting for calf growth, as well as to evaluate the potential functionality of some AA thus improving the growth efficiency and muscle mass development in the preweaning time of calves. "-Omics" technologies such as Proteomics and Metabolomics were used to get further insight into the biological mechanisms underlying these effects.

This general goal was divided into three specific objectives:

1) Study the effects of supplementing the MPMR used for calf nutrition with a combination of Methionine, Lysine and Threonine (MKT), Phenylalanine and Tyrosine (FY), and Glycine and Proline (GP). Blood samples and skeletal muscle biopsies were obtained.
2) Study the effects of supplementing the MPMR used for calf nutrition with a combination of Leucine and Threonine (Leu/Thr) or Leucine alone (Leu). Blood samples and skeletal muscle biopsies were obtained.

During the development of this work, many clinical chemistry values for several parameters were obtained from Studies 1 and 2, as well as other studies in the AMINOCRET project. These data together with a collaboration on recently weaned piglets with Dr Susana Martín (Departament de Ciència Animal i dels Aliments, Facultat de Veterinària, UAB) allowed us to include a new objective:

3) Establishment of reference intervals in clinical chemistry for lactating calves and post-weaned piglets.

Metabolomic studies were carried out in collaboration with *ITQB NOVA, Universidade Nova de Lisboa, Portugal* (Dr. Manolis Matzapetakis) and proteomic studies were performed in collaboration with *Faculty of Veterinary Medicine, University of Zagreb, Croatia* (Dr Peter David Eckersall, Dr Josipa Kuleš, Dr Nicolas Guillemin and Dr Anita Horvatić).

6. Results

Two scientific articles have been published up to this moment:

In the first publication, we randomly chose 8 animals per treatment from Study 1 and studied the dietary AA supplementation impact to the skeletal muscle metabolome, we also analyzed the performance data, blood clinical biochemistry as well as AA profile, aiming to establish a reliable and reproducible way to evaluate skeletal muscle metabolome for animal nutrition studies.

In the second publication, since we had obtained clinical biochemistry values for many parameters for all studies at different animal ages, we collected analytes' data from a large population from calves and piglets. After careful inspection, we found out that all animals were healthy and the major differences within the same analyte were due to the age of the animal. Thus we used all these data to establish robust reference intervals primarily using non-parametric method. This will benefit the veterinarian in assessing young animals' health in farm.

The rest of the Results of this Thesis are described as Annexes, following the rules of the "Normativa UAB: Estudis de doctorat de conformitat al RD 99/2011".

In this manner, results corresponding to the three described objectives, will be grouped as follows:

- Objective 1: Results: Publication 1 (6.1) and Annex 3 (9.1)
- Objective 2: Annex 1 (9.1) and Annex 2 (9.2)
- Objective 3: Results: Publication 2 (6.2)

6.1. Publication 1: *Skeletal Muscle Metabolomics and Blood Biochemistry Analysis Reveal Metabolic Changes Associated with Dietary Amino Acid Supplementation in Dairy Calves*

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Skeletal muscle metabolomics and blood biochemistry analysis reveal metabolic changes associated with dietary amino acid supplementation in dairy calves

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The effects of different amino acid (AA) supplementations of milk protein-based milk replacers in pre-ruminant calves from 3 days to 7 weeks of age were studied. Animals were divided into 4 groups: Ctrl) Control group fed with milk protein-based milk replacer without supplementation; GP) supplementation with 0.1% glycine and 0.3% proline; FY) supplementation with 0.2% phenylalanine and 0.2% tyrosine; MKT) supplementation with 0.62% lysine, 0.22% methionine and 0.61% threonine. For statistical analysis, *t*-test was used to compare AA-supplemented animals to the Ctrl group. At week 7, body weight and average daily gain (ADG) were measured and blood samples and skeletal muscle biopsies were taken. Blood biochemistry analytes related to energy metabolism were determined and it was shown that MKT group had higher serum creatinine and higher plasma concentration of three supplemented AAs as well as arginine compared with the Ctrl group. GP group had similar glycine/proline plasma concentration compared with the other groups while in FY group only plasma phenylalanine concentration was higher compared with Control. Although the AA supplementations in the GP and FY groups did not affect average daily gain and metabolic health profile from serum, the metabolome analysis from skeletal muscle biopsy revealed several differences between the GP-FY groups and the Ctrl-MKT groups, suggesting a metabolic adaptation especially in GP and FY groups.

Traditionally, bovine milk is considered to be the standard for feeding dairy cattle[1] and before 1956, whole milk was the main feed for calves[2]. Due to the increasing demand for dairy products, many dairy cattle farms started feeding calves with commercial milk replacers (MRs)[3]. MR quality can vary according to protein source. In milk protein-based MR (MPMR), the protein source originates from milk protein derivatives, and they are considered to have the best quality as they are easier to be digested by young calves[4]. However, due to differences in the levels of certain amino acids (AAs) between MRs and bovine milk, MR AA composition may be inadequate for optimal calf growth, as has been described for infant formulas and human milk[5,6].

Among the AAs required for pre-ruminant calf's growth, methionine (M), lysine (K) and threonine (T) are the most studied due to their low concentration in both milk and milk replacers[7–10]. There are indications of improved average daily gain (ADG) and feed efficiency intake in MPMR with MKT supplementation[10] but other studies suggested no performance benefits comparing MPMR supplemented with methionine[1], or with threonine and isoleucine[11].

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Group	Initial BW (kg)	BW at biopsy (kg)	Initial age (days)	Age at biopsy (days)	ADG (g/d)
Ctrl	44.2 ± 1.66	77.8 ± 1.73	3.3 ± 0.77	48.5 ± 1.04	740 ± 17.5
GP	47.2 ± 1.74	81.9 ± 2.46	2.4 ± 0.26	48.1 ± 0.83	759 ± 23.6
FY	42.3 ± 2.40	75.6 ± 2.58	2.6 ± 0.38	47.5 ± 0.96	740 ± 17.9
MKT	44.6 ± 2.70	80.8 ± 2.75	2.4 ± 0.65	47.6 ± 0.94	798 ± 26.9

Table 1. Performance data (Mean ± SE) in calves fed with different AA supplementations. Ctrl: Control milk replacer; GP: Milk replacer supplemented with glycine and proline; FY: Milk replacer supplemented with phenylalanine and tyrosine and MKT: Milk replacer supplemented with lysine, methionine and threonine (n = 8 per group). ADG = Average daily gain; BW = Body weight. Results were analysed by *t*-test for comparisons between Ctrl and treatment groups.

In humans the comparison of human milk amino acid profile with that of infant formula has been used to propose AA supplementation or ingredient composition in infant formula[5,6]. The comparison of cow milk AA profile[12] with the skimmed-milk based MR (used as a control in the present study) evidenced than some AAs are deficient in MR compared with cow milk. For instance, proline (P), tyrosine (Y) and phenylalanine (F) were 7.6, 36.9, and 17.5% lower in the control MR in comparison with bovine whole milk[12]. Furthermore, these AAs may be interesting because of their role in the organism, for example proline and glycine (G) are very abundant in collagen[13], the main structural protein of connective tissue. Phenylalanine and tyrosine play a major role in the neural system as well as the hormonal regulation of metabolism as they are precursors for the synthesis of catecholamines (epinephrine, norepinephrine and dopamine) and thyroid hormones[13]. Moreover, a recent study concludes that phenylalanine and tryptophan could be limiting growth in calves fed following an enhanced-growth feeding program[14].

Even though many studies described the growth effects of AA supplementation, the effect on tissue composition has rarely been addressed, particularly from the metabolomics perspective. The hypothesis of the present work is that inclusion of these AAs would be able to modify the metabolome of the skeletal muscle since they affect growth, are important components of collagen or are precursors of anabolic or catabolic hormones. The metabolome, the complete set of small-molecules found within a biological sample, is directly related to the metabolic activity, protein activity and/or gene expression[15,16]. The skeletal muscle is suitable for metabolomic approaches, as several studies have been applied not only in differentiating meat characters of different cattle breeds[17], geographic origins[18,19], post mortem periods[20] and sheep breeds and farming conditions[21], but also in evaluating meat conservation and aging through meat exudate[22].

The objective of this study is to determine the effects of a MPMR-based diet supplemented with three groups of amino acids (FY, GP, MKT) fed to dairy calves from 3 days up to 7 weeks of age on the ADG and blood biochemical analytes. The second goal is, by using the NMR-metabolomic technology, to monitor the impact of AA supplementations of MPMR on the skeletal muscle metabolome, hence asserting their importance for the dairy industry.

Results

Performance data. Initial and biopsy time body weight and ages were measured for the four groups in the study, as shown in Table 1. No differences were observed between groups, although a statistical tendency for higher ADG was observed in MKT group compared with Ctrl ($p = 0.096$) and FY groups ($p = 0.097$).

Blood profile and multivariate analysis of data. At the end of the treatment (week 7), several serum biochemical analytes related to health and nutritional status as well as plasma free AA profile were determined (Table 2).

For serum biochemistry analytes, there were no differences between Ctrl and the GP or FY group, whereas MKT group presented higher values of serum creatinine compared to the Ctrl group ($p = 0.023$) as well as a tendency for higher total protein ($p = 0.059$). As for the plasma AA concentration, the three supplemented AAs were higher in the MKT group than in the Ctrl group, as well as the concentration of plasma arginine. In the case of FY group, the concentration is significantly higher for phenylalanine supplemented compared with Ctrl group. In the case of GP, there were no significant differences compared to the Ctrl group.

Multivariate analysis of the combined data from serum biochemistry and plasma AA using PCA agreed with the univariate results since the different treatment groups did not show major differences (Fig. 1). Pairwise PLS-DA could only discriminate between the Ctrl and the MKT treatment (NC = 2, $R^2Y = 0.91$, $Q^2 = 0.60$). The VIP variables with values higher than 1.5 and therefore most responsible for the difference were, in decreasing VIP order, methionine, threonine, arginine, creatinine and lysine.

Muscle tissue metabolomics profile. *NMR spectra analysis and compound identification.* Prior to the metabolomics analysis of the muscle tissue extracts, the assignment of the features of the NMR spectrum to specific compounds was made. Selected spectra were assigned using database assisted spectral deconvolution with the Chenomx software platform using its internal database as well as the human metabolite database data (http://www.hmdb.ca/). 2D TOCSY spectra and literature reports[21,22] were also used to verify the identity of other metabolites. A total of 70 compounds could be successfully identified (Supplementary Table S1). From these, a subset of 36 metabolites was selected for quantification using Chenomx. The selection of this group was made based on the ability to reliably quantify them, their relevance from chemometric multivariate analysis (see chemometric

Concentration (Mean ± SE)	Ctrl	GP	FY	MKT
Serum biochemical profile				
ALT (U/L)	10.4 ± 0.7	11.1 ± 1.5	9.2 ± 0.5	10.2 ± 1.2
AST (U/L)	37.5 ± 3.2	39.6 ± 5.7	33.5 ± 4.9	42.2 ± 5.9
Cholesterol (mg/dL)	64.9 ± 8.2	65.2 ± 6.5	68.0 ± 12.7	72.7 ± 6.6
Creatinine (mg/dL)	0.60 ± 0.04[a]	0.68 ± 0.05[a]	0.57 ± 0.03[a]	0.71 ± 0.02[b]
GGT (U/L)	18.5 ± 2.0	14.2 ± 1.1	16.6 ± 1.5	17.9 ± 0.6
Glucose (mg/dL)	86.3 ± 2.8	92.2 ± 4.3	96.9 ± 6.1	92.8 ± 5.4
Haptoglobin (mg/mL)	0.12 ± 0.01	0.13 ± 0.01	0.18 ± 0.06	0.16 ± 0.04
IGF-I (ng/mL)	264.4 ± 35.9	282.2 ± 31.6	245.9 ± 28.9	318.5 ± 33.3
Insulin (μg/L)	0.75 ± 0.13	1.04 ± 0.20	0.85 ± 0.11	1.07 ± 0.22
NEFAs (mM)	0.15 ± 0.03	0.16 ± 0.03	0.13 ± 0.02	0.15 ± 0.02
TGs (mg/dL)	19.91 ± 5.23	20.99 ± 4.59	16.20 ± 1.87	23.51 ± 5.37
TP (g/dL)	4.40 ± 0.11	4.42 ± 0.12	4.57 ± 0.15	4.75 ± 0.13
Urea (mg/dL)	10.54 ± 0.65	13.40 ± 1.44	9.84 ± 0.96	11.63 ± 1.07
Plasma AAs profile				
Alanine (μM)	236.9 ± 16.6	228.9 ± 18.1	230.8 ± 30.8	259.6 ± 13.9
Arginine (μM)	223.7 ± 17.4[a]	264.2 ± 23.1[a]	236.7 ± 23.6[a]	291.9 ± 18.7[b]
Asparagine/Serine (μM)	177.9 ± 14.0	195.4 ± 18.9	189.2 ± 23.8	190.6 ± 10.3
Cysteine (μM)	39.0 ± 5.5	38.3 ± 5.9	38.9 ± 6.78	40.4 ± 5.9
Glutamic acid (μM)	77.8 ± 5.2	68.0 ± 6.8	65.2 ± 5.5	75.1 ± 6.5
Glycine (μM)	326.8 ± 26.1	372.1 ± 25.2	340.6 ± 19.9	368.3 ± 15.3
Histidine/Glutamine (μM)	487.1 ± 30.8	504.0 ± 34.8	485.7 ± 49.1	503.9 ± 24.6
Isoleucine (μM)	137.0 ± 11.0	130.7 ± 12.7	134.7 ± 15.8	129.56 ± 6.9
Leucine (μM)	195.20 ± 19.64	181.37 ± 21.66	188.57 ± 28.64	172.05 ± 8.31
Lysine (μM)	220.3 ± 29.6[a]	231.4 ± 24.3[a]	240.8 ± 43.4[a]	304.3 ± 18.8[b]
Methionine (μM)	36.4 ± 6.1[a]	35.4 ± 6.6[a]	36.1 ± 7.4[a]	73.6 ± 5.1[b]
Phenylalanine (μM)	56.4 ± 6.5[a]	57.5 ± 6.5[a]	79.0 ± 7.7[b]	52.2 ± 3.7[a]
Proline(μM)	137.4 ± 11.3	163.7 ± 15.0	141.3 ± 23.2	147.3 ± 10.9
Threonine (μM)	270.9 ± 10.9[a]	276.6 ± 24.3[a]	270.3 ± 25.9[a]	386.7 ± 26.7[b]
Tryptophan (μM)	49.7 ± 4.4	56.0 ± 6.3	52.2 ± 8.2	54.8 ± 3.6
Tyrosine (μM)	66.7 ± 10.3	69.6 ± 13.8	86.8 ± 8.6	67.2 ± 7.0
Valine (μM)	280.7 ± 18.7	273.4 ± 26.5	272.1 ± 35.3	267.5 ± 10.2

Table 2. Serum biochemical analytes and plasma AA concentrations in calves fed with different AA supplementations. Key: Ctrl: Control milk replacer; GP: Milk replacer supplemented with glycine and proline; FY: Milk replacer supplemented with phenylalanine and tyrosine and MKT: Milk replacer supplemented with lysine, methionine and threonine (n = 8 per group). ALT = alanine aminotransferase; AST = aspartate aminotransferase; GGT = gamma glutamyltransferase; IGF-I = insulin-like growth factor 1; NEFAs = non-esterified fatty acids; TGs = triglycerides; TP = total protein. Results were analysed by *t*-test for comparisons between Ctrl and treatment groups. Values in a row with different superscripts are significant different ($P < 0.05$).

section) and their relevance to the biochemical analytes of this study. The assignment of the selected metabolites on a representative spectrum is shown in Fig. 2.

Univariate and multivariate analyses. Upon inspection, two samples (one from GP and one from FY) were found to have very low concentrations. This implies a putative extraction problem and were discarded from the analysis. A total of 30 samples remained and were subsequently used. The mean concentrations of the 36 quantified metabolites corrected for tissue mass are shown in Table 3. According to the *p* values from the *t*-test, no differences between Ctrl and the MKT group could be identified, whereas the GP group had higher carnosine and nicotinurate levels than the Ctrl group. In the FY group, a lower concentration of cadaverine and higher concentration of nicotinurate were found.

Multivariate analyses of the tissue metabolite concentrations, using PCA (Fig. 3a) were consistent with the univariate results. As in the case of the blood profile, the different treatment groups did not show any global differences. Pairwise PLS-DA could only discriminate between the Ctrl-FY groups (NC = 3, $R^2Y = 0.92$, $Q^2 = 0.58$) and FY-MKT groups (NC = 3, $R^2Y = 0.86$, $Q^2 = 0.45$). The VIP variables with values higher than 1.4 reveal that the most responsible for the differentiation were, in decreasing VIP order, cadaverine and nicotinurate in Ctrl - FY comparison and cadaverine and NAD^+ in FY-MKT comparison.

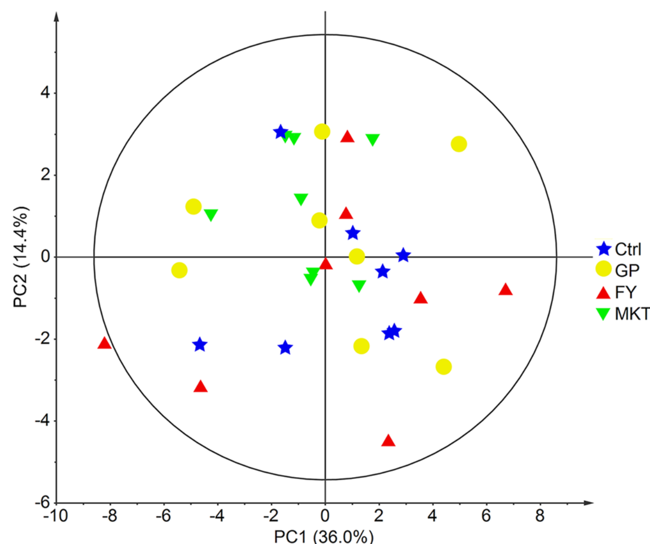

Figure 1. PCA of combined serum biochemical analytes and plasma AAs. Data from dairy calves fed with control milk replacer (Ctrl); milk replacer supplemented with glycine and proline (GP); milk replacer supplemented with phenylalanine and tyrosine (FY) and milk replacer supplemented with lysine, methionine and threonine (MKT) (n = 8 per group). Ellipse Hotelling's T2 (95%).

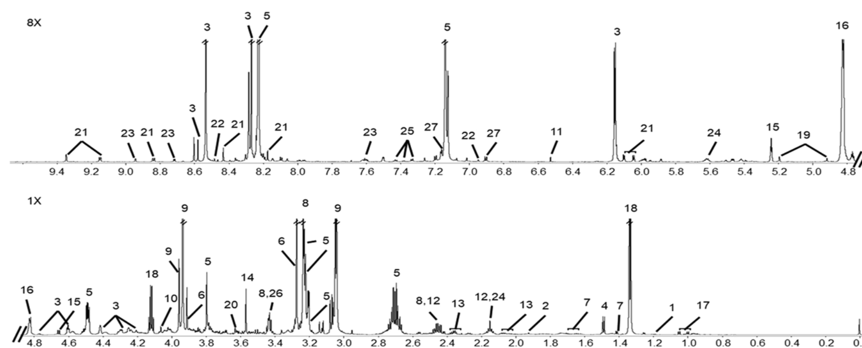

Figure 2. Representative 800 MHz 1H-NMR spectrum of calf muscle aqueous fraction. 1X zoom from 0.0–4.8 ppm and 8X zoom from 4.8–9.4 ppm. Assignments of the 36 metabolites that were quantified: 1: 3-Hydroxybutyrate; 2: Acetate: 3: ADP/AMP/ATP; 4: Alanine; 5: Anserine/Carnosine; 6:Betaine; 7: Cadaverine/Lysine; 8: Carnitine; 9: Creatine/Creatine Phosphate; 10: Creatinine; 11: Fumarate; 12: Glutamine; 13: Glutamate; 14: Glycine; 15: Glucose/Glucose-6-Phosphate; 16: Residual H_2O (not quantified); 17: Isoleucine/Leucine/Valine; 18: Lactate; 19: Mannose; 20: Myo-Inositol; 21: $NAD^+/NADP^+$; 22: NADH/NADPH; 23: Nicotinurate; 24: O-Acetylcarnitine; 25: Phenylalanine; 26: Taurine; 27: Tyrosine.

Chemometric analysis. To assess if additional regions of the NMR spectra contained information that could be used to discriminate between the experimental groups, multivariate analysis of the full spectra (chemometric) was also employed. As shown in Fig. 3b, no clear separation or clustering could be observed. This is consistent with the multivariate analysis based on the metabolite concentrations. However, PLS-DA could produce a model with acceptable statistical parameter for the comparison of the FY and Ctrl groups (NC = 3, $R^2Y = 1.00$, $Q^2 = 0.44$). A close look at the VIP data reveals that most compounds with VIP scores over 2.5 are energy-related metabolites, AA, AA derivatives as well as some unassigned compounds (Supplementary Table S2). After a visual inspection of binned spectra, an increase in the total area between 1.422–1.467 ppm and 1.632–1.760 ppm was identified in MKT group (Supplementary Fig. S1). According to the assignment, it was found that these regions mainly contain lysine/cadaverine/arginine/leucine/ornithine as well as some other unassigned metabolites.

Comparative analyses of metabolites between AA-treatments. While the focus of this work is on the differences between the treatments and the Ctrl group, the identification of differences among treatments has the potential to provide insights into the processes affected by the modifications in feed composition. Therefore, a one-way ANOVA with Tukey post-hoc test was used to identify the most significant changes among AA groups. The results of this analysis are summarized in the scheme shown in Fig. 4, where the upper portion shows the results for blood and the lower those for muscle tissue (complete information in Supplementary Tables S3 and S4).

Metabolites (μMol/100 g muscle ± SE)	Ctrl	GP	FY	MKT
3-Hydroxybutyrate	0.27 ± 0.03	0.29 ± 0.04	0.35 ± 0.03	0.26 ± 0.03
Acetate	0.89 ± 0.11	0.86 ± 0.06	0.90 ± 0.05	0.83 ± 0.08
ADP+ATP+AMP	30.59 ± 3.72	33.92 ± 2.25	29.45 ± 6.25	26.93 ± 4.60
Alanine	10.88 ± 1.32	10.45 ± 1.37	10.17 ± 1.18	10.76 ± 1.06
Anserine	19.36 ± 1.68	22.72 ± 2.30	20.82 ± 2.12	18.99 ± 2.55
Aspartate	0.76 ± 0.08	0.64 ± 0.10	0.85 ± 0.10	0.78 ± 0.12
Betaine	26.61 ± 2.83	29.65 ± 2.63	27.63 ± 2.10	21.04 ± 3.81
Cadaverine	2.01 ± 0.27[a]	1.72 ± 0.30[a]	0.97 ± 0.15[b]	3.10 ± 0.70[a]
Carnitine	27.52 ± 1.62	32.75 ± 2.83	30.42 ± 1.99	24.84 ± 2.24
Carnosine	159.96 ± 7.79[a]	185.24 ± 8.17[b]	190.04 ± 14.08[a]	140.55 ± 15.61[a]
Creatine	281.90 ± 27.39	317.50 ± 24.28	293.29 ± 24.86	254.32 ± 30.07
Creatine phosphate	74.41 ± 14.75	62.08 ± 20.60	83.93 ± 17.42	65.62 ± 14.77
Creatinine	11.48 ± 2.31	10.20 ± 3.08	12.67 ± 2.02	12.21 ± 2.55
Fumarate	0.36 ± 0.08	0.45 ± 0.10	0.27 ± 0.05	0.33 ± 0.06
Glucose	11.06 ± 2.78	12.76 ± 2.37	9.20 ± 1.69	9.25 ± 1.52
Glucose-6-phosphate	12.45 ± 4.90	18.18 ± 4.40	7.52 ± 3.07	6.24 ± 1.72
Glutamate	9.54 ± 0.63	11.08 ± 1.14	9.89 ± 0.72	9.67 ± 1.58
Glycine	24.68 ± 3.23	23.46 ± 3.35	22.73 ± 3.97	22.99 ± 2.95
Isoleucine	1.05 ± 0.13	0.80 ± 0.13	1.01 ± 0.18	0.85 ± 0.08
Lactate	203.66 ± 47.73	300.35 ± 65.52	208.37 ± 61.25	169.21 ± 25.05
Leucine	1.43 ± 0.21	1.19 ± 0.20	1.39 ± 0.28	1.16 ± 0.13
Mannose	2.79 ± 0.64	3.32 ± 0.56	1.66 ± 0.41	1.76 ± 0.23
Myo-inositol	4.82 ± 0.35	4.94 ± 0.52	4.43 ± 0.32	4.56 ± 0.43
NAD^+	2.63 ± 0.12	2.88 ± 0.19	3.05 ± 0.18	1.94 ± 0.32
NADH	1.00 ± 0.17	0.69 ± 0.10	0.82 ± 0.20	0.79 ± 0.18
NADP	0.10 ± 0.01	0.12 ± 0.01	0.11 ± 0.01	0.11 ± 0.01
Nicotinurate	1.47 ± 0.08[a]	2.14 ± 0.22[b]	1.84 ± 0.07[b]	1.45 ± 0.15[a]
O-Acetylcarnitine	4.57 ± 1.38	3.64 ± 0.53	4.36 ± 0.53	2.95 ± 0.32
Phenylalanine	0.48 ± 0.05	0.42 ± 0.04	0.58 ± 0.07	0.40 ± 0.04
Pyruvate	1.20 ± 0.19	1.40 ± 0.20	1.05 ± 0.11	1.01 ± 0.09
Taurine	19.87 ± 1.34	21.94 ± 2.00	23.03 ± 1.00	18.58 ± 1.96
Tyrosine	0.72 ± 0.08	0.64 ± 0.07	0.89 ± 0.09	0.65 ± 0.08
Tryptophan	0.19 ± 0.02	0.19 ± 0.02	0.17 ± 0.02	0.19 ± 0.02
Valine	2.38 ± 0.27	1.99 ± 0.20	2.18 ± 0.33	1.85 ± 0.20

Table 3. Mean concentrations of quantified metabolites from semitendinosus muscle of calves fed with different AA supplementations. Key: Ctrl: Control milk replacer (n = 8); GP: Milk replacer supplemented with glycine and proline (n = 7); FY: Milk replacer supplemented with phenylalanine and tyrosine (n = 7) and MKT: Milk replacer supplemented with lysine, methionine and threonine (n = 8). Results were analysed by *t*-test for comparisons between Ctrl and treatment groups. Values in a row with different superscripts are significant different ($P < 0.05$).

Finally, when performing a PCA analysis based on the metabolites cadaverine, carnitine, carnosine, NAD^+ and nicotinurate, Ctrl and MKT groups tend to appear on one side while GP and FY groups on the other side (Fig. 5).

Discussion

The supplementation of MR with AAs is an important topic in pre-ruminant nutrition. In the present work, the impacts of supplementing MR with three different AA combinations (GP, FY and MKT) on weight gain, blood biochemistry profile and muscle metabolome were analysed after feeding the calves with control or supplemented MR for 7 weeks. The effectiveness of the supplementation has been evaluated by quantifying the plasma free AA profiles. Methionine/lysine/threonine, as well as phenylalanine, increased in the plasma of the supplemented groups as expected (MKT and FY, respectively). On the contrary, there were no significant differences in glycine/proline, and in tyrosine, probably due to the non-essential character of these AA or a difference in doses or intestinal absorption. An increase in plasma arginine has also been observed in the MKT group. A plausible explanation is that arginine and lysine share the same transport system for entering into the cell[23]. Thus, an increase in plasma lysine could compete with arginine for transport into the muscle leading to a concomitant increase in plasma arginine.

The profile of serum biochemical analytes considered as markers of the nutritional status of the animal (glucose, urea, creatinine, cholesterol, TGs, NEFAs and total protein) did not show major differences between groups.

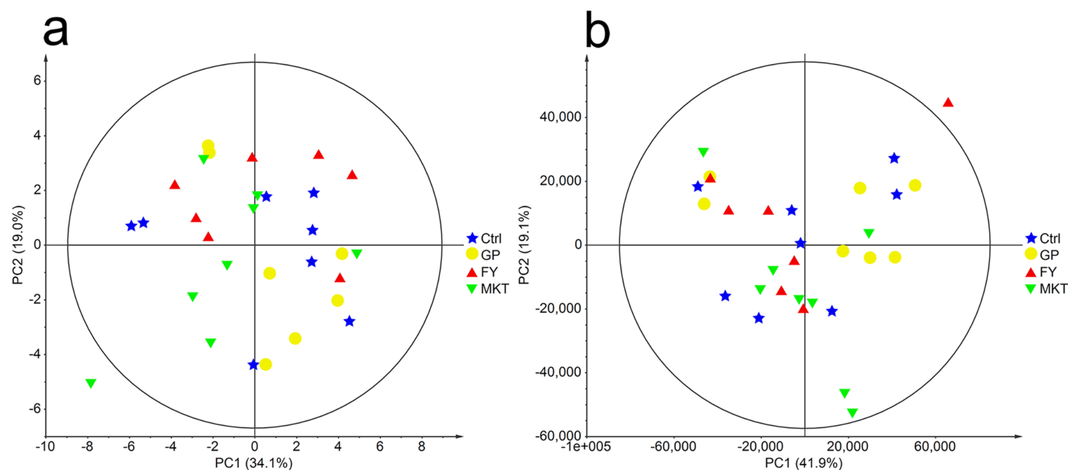

Figure 3. PCAs of **a** Quantified muscle metabolites and **b** Chemometrics data from muscle biopsies. Data from dairy calves fed with control milk replacer (Ctrl, n = 8); milk replacer supplemented with glycine and proline (GP, n = 7); milk replacer supplemented with phenylalanine and tyrosine (FY, n = 7) and milk replacer supplemented with lysine, methionine and threonine (MKT, n = 8). Ellipse Hotelling's T2 (95%).

	Ctrl	GP	FY	MKT
Ctrl				*Threonine*** *Methionine***
GP	Nicotinurate*			*Threonine*** *Methionine***
FY				*Creatinine** *Threonine*** *Methionine*** Phenylalanine*
MKT		*Carnitine* *Carnosine* *NAD*+** *Nicotinurate**	Cadaverine* *Carnosine** *NAD*+** *Phenylalanine*	

Blood profile

Muscle profile

Figure 4. Schematic overview of the differential metabolite profiles in blood (right-up corner) and muscle (down-left corner) between groups. Data from dairy calves fed with control milk replacer (Ctrl); milk replacer supplemented with glycine and proline (GP); milk replacer supplemented with phenylalanine and tyrosine (FY) and milk replacer supplemented with lysine, methionine and threonine (MKT). Blood analytes are shown in the upper-right corner, and muscle metabolites are shown in the down-left corner. Parameters at higher concentration in the row-group vs the column-group are presented in underlined font, whereas parameters at higher concentration in the column-group vs the row-group are presented in italic font. (*$P < 0.05$, **$P < 0.01$, no asterisk: statistical tendency $0.05 \le P \le 0.1$).

The only significant difference was observed in calves with MKT treatment, which showed higher serum values for creatinine versus the Ctrl group in the *t*-test and versus the FY group in the Tukey HSD test. These results suggest an increase in protein anabolism since blood creatinine concentration is positively related to muscular mass[24]. This conclusion may be reinforced by the observed tendency towards an increase in total protein in the MKT group versus the Ctrl group ($p = 0.059$). The enzymes ALT, AST and GGT were analysed for their role in the hepatic metabolism of AA and as markers of liver function, and no differences were observed between groups. Haptoglobin, marker of inflammation, and insulin and IGF-I, markers of metabolism and growth, were not altered either by AA supplementation.

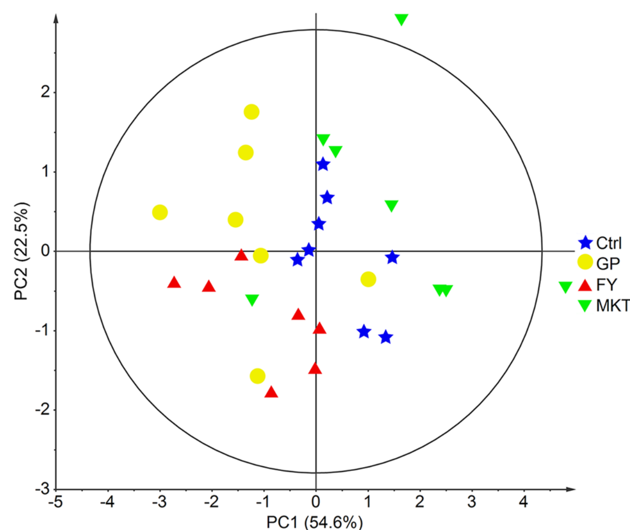

Figure 5. PCA based on 5 quantified muscle metabolites (cadaverine, carnitine, carnosine, NAD^+ and nicotinurate). Data from dairy calves fed with control milk replacer (Ctrl, n = 8); milk replacer supplemented with glycine and proline (GP, n = 7); milk replacer supplemented with phenylalanine and tyrosine (FY, n = 7) and milk replacer supplemented with lysine, methionine and threonine (MKT, n = 8). Ellipse Hotelling's T2 (95%).

The semitendinosus muscle NMR-metabolome was then characterized. From a glimpse of the results of muscle profile (Fig. 5), a remarkable finding is that the most distinguishable groups from Ctrl were FY and GP, in contrast with the ADG and serum analysis where MKT was the most divergent. Comparing Ctrl and MKT groups, there were no differences from the *t*-test and Tukey HSD pairwise comparisons of quantified muscle metabolites. Furthermore, the multivariate PLS-DAs of quantified metabolites and chemometrics results did not show an acceptable discrimination between Ctrl and MKT groups.

On the other hand, when comparing quantified metabolites in GP and FY groups versus Ctrl, the *t*-test identified nicotinurate being higher in FY and GP, carnosine being higher in GP and cadaverine being lower in FY. Differences between all groups are shown in Fig. 4, which summarizes the results from the ANOVA analysis with Tukey HSD test over the whole dataset. As explained in the figure, it is evident that GP and FY are different from MKT. Thus, cadaverine was lower in FY versus MKT, carnosine was higher in FY versus MKT. Nicotinurate was higher in GP versus Ctrl and MKT, and NAD^+ was higher in GP and FY versus MKT. Furthermore, PLS-DAs of quantified metabolites and chemometrics shows an acceptable discrimination test between Ctrl-FY and FY-MKT comparison and PCA analysis based on the metabolites cadaverine, carnitine, carnosine, NAD^+ and nicotinurate, grouped Ctrl and MKT group on one side and GP and FY groups on the other side (Fig. 5). Finally, when the surroundings of the two chemical shifts of cadaverine were analysed (between 1.422–1.467 ppm and 1.632–1.760 ppm), an increase in the total area in MKT group was observed versus both GP and FY groups. The increased total areas in MKT group may due to higher concentration of lysine and/or any of these other metabolites.

In summary, the main differential muscle metabolites were cadaverine, carnosine, carnitine, nicotinurate and NAD^+. Considering the physiological interpretation of these results, cadaverine is the product of decarboxylation of lysine. Given the fact that MKT group was supplemented with lysine, this may be the reason for higher cadaverine levels.

Carnosine is a dipeptide abundant in skeletal muscle of mammals[25–27]. Amongst other biological functions, it is a powerful antioxidant[28,29]. It is interesting to notice that the conversion of proline into hydroxyproline during collagen synthesis requires the action of proline hydroxylase, an enzyme that needs molecular O_2 as substrate. Likewise, the conversion of phenylalanine into tyrosine requires oxygen as a substrate for phenylalanine hydroxylase. This situation may indicate that the muscle cell in the groups supplemented with GP or FY could be under a situation of oxidative damage. The increase in carnosine concentration could alleviate this condition.

Carnitine is involved in the transport of acyl groups to the mitochondria, and thus in oxidative metabolism. This metabolite was also increased in GP. Similarly, nicotinurate, one of the metabolites of NAD^+, is increased in the GP versus Ctrl and MKT groups. NAD^+ is the final product of the oxidative respiratory chain and it has increased concentrations in both GP and FY groups.

Altogether, these results suggest a higher importance of aerobic metabolism in GP and FY groups.

Despite the fact that this study reveals the metabolomic changes due to the AA supplementation adaptation, two main limitations need however to be addressed. Firstly, this study is only focused on muscle metabolome, thus the general metabolomic profile is not presented. These results may be extended by analyzing the urine and/or serum metabolome. Secondly, only the aqueous fraction was analysed in the NMR. As such, organic phase remained unstudied. As a result, the lipid metabolism is not included and may omit some important information. Addressing these two points are important follow-up studies.

Conclusions

To the best of our knowledge, this work is the first to report a NMR metabolomics study of skeletal muscle of calves with diets differing in AA supplementation. Herein we conclude that the AA supplementation added to the MR in the conditions given in the present work does not have a marked influence in the ADG in calves from 3 days to 7 weeks of age. Nevertheless, calves supplemented with MKT had higher serum creatinine concentration, considered an indicator of increased muscular mass. These differences between MKT and the Ctrl group are however not reflected in the metabolomics analysis of skeletal muscle, as all profiles are very similar. Nevertheless, the metabolomic analysis of skeletal muscle revealed several differences between the GP/FY groups and the Ctrl/MKT groups, suggesting a metabolic adaptation especially in the GP ad FY groups. In conclusion, our results support the performance improvement after MKT supplementation in MR compared with Ctrl and GP and FY combinations, and reveal that different AA profile of calf MR can modify protein and muscle metabolism. This study may help the dairy industry to take metabolic factors into account when supplementing relevant AAs in MR.

Methods

Animals and housing. Animals were managed according to the recommendations of the Animal Care Committee of *Institut de Recerca i Tecnologia Agroalimentàries* (IRTA) under the approval research protocol FUE-2017-00587321, authorization code 9733. Thirty-two Holstein male calves (weighing 44.6 ± 1.07 kg of BW at the age of 2.7 ± 0.27 days) were obtained from Granja Murucuc (Gurb, Barcelona, Spain) and housed individually at IRTA Torre Marimon (Caldes de Montbui, Barcelona, Spain). Animals used in the component here described were a part of a larger study with a higher number of animals that aimed to evaluate calf performance. The sample size was chosen based on the previous similar studies[18,19,21,30–32].

Diet treatments and performance recordings. MR was offered in 3-L nipple bottles twice a day, and all animals followed the same MR feeding program. Calves were fed on 4 L/d commercial milk replacer at 12.5% Dry Matter (DM). Milk replacer (Nukamel, Weert, The Netherlands) contained 25.4% CP (based on skimmed milk protein, and whey protein concentrate) and 20.3% fat from experimental days 1 to 4. Subsequently, the volume of MR was increased to 5 L/d from day 5 to 7, followed by 6 L/d from days 8 to 14, and 6 L/d at 15% DM concentration from day 15 to 49. At day 50, MR was reduced to one feeding per day of 3 L at 15% DM until day 56 when animals were fully weaned.

Calves were divided in four different treatments according to AA supplementation in the MR formula (which replaced whey protein concentrate in the control MR). All AAs were provided by Livzon Group (Fuxing Pharmaceutical, Ningxia (China)). Treatments were previously defined in a list, which indicated the MR treatment in each individual calf box. Then, as calves were arriving to the facilities, they were allocated correlatively to the individual boxes with a predetermined treatment. In this way, the only identification of the calves was the eartag number, which allowed the blinding of researchers and care-takers to treatment allocation. The four treatments were:

A (Ctrl): Control group with no AA supplementation
B (GP): supplementation with 0.1% glycine plus 0.3% proline in the MR formula
C (FY): supplementation with 0.2% phenylalanine plus 0.2% tyrosine in the MR formula
D (MKT): supplementation with 0.62% lysine plus 0.22% methionine plus 0.61% threonine in the MR formula

Ingredients and chemical composition are shown in Table 4. All animals had free access to water, and a pelleted concentrate starter feed (Pinallet, Cardona, Spain) was limited to minimize the impact of concentrate feeding on amino acid absorption: 100 g/day from day 1 to day 17 of study, 200 g/d from days 18 to 21, 300 g/d from day 22 to day 24. Subsequently, 400 g/d were used from day 25 to day 28, 500 g/d, from day 29 to day 31, 600 g/d from day 32 to day 33 d, 700 g/d at day 34, 800 g/d at day 35, 1,000 g/d from day 36 to day 42, 1,200 g/d from day 43 to day 49, and *ad libitum* afterwards until 63 d of study, when the study finished. Information about composition and AA concentration in the starter is provided in Table 5. Chopped barley straw was offered *ad libitum* during this study (Table 6). Calves were weighed weekly with an electronic scale (Mobba SC-01, Badalona, Spain) until the end of the study.

Blood and muscle sample collection. At the 7th week of the study, blood samples were obtained from jugular vein 2 h after the morning MR feeding, and were kept in 10 ml Vacutainer tubes without anticoagulant to obtain the serum for biochemistry profiles or with lithium heparin for plasma AA analyses. Serum and plasma were obtained by centrifugation at 1,500 *g* for 10 min and stored in aliquots at −20 °C until further analysis.

At 47.9 ± 0.46 days of age, a biopsy was obtained from semitendinosus muscle. Briefly, animals were injected with xylazine intravenously at the dose of 0.005 mL/kg BW, once the animals were sedated, the hind leg was shaved and disinfected with iodine povidone. Then, 2 mL of 0.02 g/mL lidocaine were subcutaneously injected as a local anaesthetic. A 3-cm cut in the skin and subcutaneous layer was done with a scalpel, and a 2-cm zone of the muscle was delimited with suture, and it was dissected with curve scissors. Finally, the muscular, subcutaneous and cutaneous layers were sutured. Muscle samples were immediately frozen into liquid nitrogen and subsequently kept at −80 °C until further analysis.

Quantification of plasma AAs. Plasma AAs were measured by HPLC following a modified protocol[33]. At the day of assay, 100 µl of thawed plasma were transferred to a 2.0 mL micro-centrifuge tube and 10 µl of 10 mM DTT in PBS (pH 7.4) and 5,79 µl 2.5 mM GABA as internal standard were added. The solution was vortexed for 30 s and an equivalent volume of 10% sulfosalicylic acid was added to precipitate the proteins, followed by mixing for 2 min. The samples were centrifuged at 14,000 g for 10 min at 4 °C and the supernatant collected[34].

Ingredients (g/kg DM)	Ctrl	GP	FY	MKT
Whey protein concentrate 35[a]	150	150	157	156
Skimmed milk powder	390	400	400	400
Fatted whey 50[b]	390	390	388.5	390
Whey protein concentrate 60[c]	40	26	20.5	9.5
Premix[d]	30	30	30	30
Proline	0	3	0	0
Glycine	0	1	0	0
Phenylalanine	0	0	2	0
Tyrosine	0	0	2	0
Lysine	0	0	0	6.2
Methionine	0	0	0	2.2
Threonine	0	0	0	6.1
Composition, (g/kg DM, unless otherwise stated)				
DM (g/kg)	979	974	981	980
CP	254	255	251	254
EE	202	204	202	202
Lactose	448	454	456	444
Ashes	70	73	70	69

Table 4. Ingredients and chemical compositions of milk replacers. Key: Ctrl: Control milk replacer; GP: Milk replacer supplemented with glycine and proline; FY: Milk replacer supplemented with phenylalanine and tyrosine and MKT: Milk replacer supplemented with lysine, methionine and threonine. CP = crude protein; DM = dry matter; EE = ether extract. [a]Contains 35% protein in the whey. [b]Contains 50% protein in the whey. [c]Contains 60% protein in the whey. [d]Mineral and vitamin composition: Vitamin A 25,000 IU/kg; Vitamin D3 4,500 IU/kg; Vitamin E 300 mg/kg, Vitamin C 300 mg/kg, Vitamin B1 16 mg/kg, Vitamin B2 10 mg/kg, Vitamin B6 10 mg/kg, Vitamin B12 80 mg/T, Vitamin K 5.5 mg/kg, Folic acid 1 mg/kg, Pantothenic acid 23 mg/kg, Niacin 50 mg/kg, Fe 150 mg/kg, Zinc 170 mg/kg, Copper 10 mg/kg, Manganese 40 mg/kg, Iodine 1.3 mg/kg, Selenium 0.4 mg/kg.

Composition	
DM (%)	87.55
CP (% DM)	17.85
Detailed Amino Acid Composition (g/kg DM)	
Aspartic acid	1.55
Glutamic acid	3.33
Serine	0.85
Histidine	0.41
Glycine	0.76
Threonine	0.7
Arginine	1.17
Alanine	0.79
Tyrosine	0.58
Valine	0.77
Methionine	0.21
Phenylalanine	0.82
Isoleucine	0.66
Leucine	1.35
Lysine	1.06
Hydroxyproline	0.043
Proline	1.05
Tryptophan	0.19

Table 5. Chemical omposition and aminoacid content in the starter feed. CP = crude protein; DM = dry matter.

For derivatization, the AccQ Fluor (Waters, Milford, MA, USA) method was used following the instructions provided by the manufacturer. A total of 10 µL standard solution or supernatant of deproteinized plasma sample was taken into a 1.5 mL micro-centrifuge tube, buffered with 70 µL Waters AccQFluor Borate Buffer and derivatized by addition of 20 µL of AccQ-Fluor reagent, vortexed and heated for 10 min at 55 °C.

Composition	
DM (%)	83.8
CP (% DM)	3.8
ADF (% DM)	52.9
NDF (% DM)	79.7
Ash content (% DM)	6.4

Table 6. Chemical composition of the barley chopped straw. CP = crude protein; DM = dry matter; ADF = Acid Detergent Fibre; NDF = Neutral Detergent Fibre.

HPLC was performed on an Elite LaCHrom (Hitachi, Tokyo, Japan) equipped with an UV detector (Hitachi L-24200, Tokyo, Japan) with a Novapak C18 column (300 mm × 3.9 mm) from Waters. The flow rate was 1.0 ml/min and the column temperature was kept at 28 °C. The injection volume was 10 µL and the detection wavelength was set at 254 nm. The solvent system consisted of two eluents: (A) AccQ > Tag eluent A concentrate (10%, v/v) and water (90%, v/v) and (B) 60% acetonitrile/40% water. The following gradient elution was used: 0 to 0.50 min, 100% A; 0.5 min, 98% A to 2% B; 20 min, 93% A to 7% B; 32 min, 91.5% A to 8.5% B; 40 min, 82% A to 18% B; 47.5 min: 79% A to 21% B; 55 min: 60% A to 40% B; 60 min: 100% B. Pure AAs were used as standards and GABA was used as internal control (Sigma, St. Louis, MO, USA). Standards were prepared at 2.5 mM stock solutions in 0.1 M HCl and was serially diluted from 500 µM to 7.81 µM to perform the standard curve in order to quantify AA of plasma samples. EZChrom Elite system V3.1.7 software (Agilent, Santa Clara, CA, USA) was used for system control and data acquisition. Validation of the technique showed a good precision (Coefficients of Variation from 1% to 5% for all AA), good linearity ($R^2 > 0.998$) except for serine ($R^2 = 0.990$) and cysteine ($R^2 = 0.989$) and a limit of quantification lower than 10 µM (except for serine (11 µM) and histidine/glutamine (21 µM).

Determination of serum biochemical analytes. Serum biochemistry analytes were analysed with the Olympus AU400 analyser with following techniques and OSR reagents (Olympus System Reagent)[35,36]: glucose (HK method), urea (GLDH method), creatinine (Jaffé method), cholesterol (CHOP-PAP-method), triglyceride (GPO-PAP method), total protein (Biuret method), ALT (International Federation of Clinical Chemistry (IFCC) method), AST (IFCC method) and GGT (IFCC method). NEFAs were determined with NEFA-C reagent (Wako Chemicals GmbH, Neuss, Germany). Quality control protocols were based on the daily quantification of two control sera of low and high concentration (Control serum I and Control serum II, Beckman Coulter) Haptoglobin was determined by a colorimetric method (Tridelta, Ireland). Insulin and IGF-I were determined by ELISA (Bovine Insulin ELISA from Mercodia, Uppsala, Sweden and Human IGF-I ELISA E20 from Mediagnost, Reutlingen, Germany). This reagent is suitable for bovine samples, as declared by the manufacturer, but the cross-reactivity was not assessed by the authors.

Intra-assay Coefficients of Variation (CV) were: glucose (1.7%), urea (2.1%), creatinine (1.4%), cholesterol (1.1%), triglycerides (3.0%), total protein (0.9), ALT (1.7%), AST (1.0%), GGT (0.6%), NEFAs (2.7%), Haptoglobin (4.1%), insulin (4.1%) and IGF-1 (4.4%).

Muscle extraction and NMR data acquisition. Muscle tissues were transferred from −80 °C freezer to dry ice. All muscle tissues were ground into fine powder while mixed with liquid nitrogen in a mortar and pestle. The extraction of the aqueous metabolites was performed using a modified chloroform/methanol method previously described[21]. In brief: approximately 0.5 g of muscle tissue was mixed with 1.2 mL of chloroform/methanol (1:2 v/v) in a glass tube and was vortexed for 1 min. Then an extra 0.4 mL of chloroform was added to compensate the evaporated chloroform and the tube was vortexed for an additional 1 minute. Afterwards 0.4 mL of double distilled water was added and the tube vortexed again for 1 min. The homogenate was centrifuged at 1935 *g* for 20 min at 4 °C. 1000 µL of the top (methanol/water) fraction was transferred into a 2 mL polypropylene tube and dried in a centrifugal vacuum concentrator. To resuspend the dried sample for NMR analysis, 600 µL of phosphate buffer in D_2O (150 mM; pH 6.6 (pD 7.0); with 100 µM of TSP-D4 (deuterated Trimethylsilylpropanoic acid)) were added, followed by 1 min vortexing and 10 min centrifugation at 14,100 g at room temperature. The supernatants were transferred into NMR borosilicate tubes rated for 800 MHz (New Era, Vineland, NJ, USA).

1D ^{1}H NOESY spectra were collected on an 800 MHz NMR spectrometer equipped with a room temperature HCN inverse Z-gradient probe (Bruker Biospin, Billerica, MA, USA). The pulse sequence used for 1D gradient NOESY with water pre-saturation was "noesygppr1D" with the Bruker standard acquisition parameters for profiling (spectral width: 16393.443 Hz, recycling delay: 4 s, acquisition time: 1.9988480 s, mixing time 10 ms). 96 scans and 8 dummy scans were used for each spectrum resulting in a 10-min experiment. For select samples, additional homonuclear and heteronuclear spectra (^{1}H J-resolved, ^{1}H-^{1}H COSY, and ^{1}H-^{13}C HSQC) spectra were also collected to assist with compound identification.

NMR data processing and analysis. Spectra were processed with 1 Hz exponential appodization, no additional zero filling and polynomial baseline correction with the TopSpin 3.2 software (Bruker Biospin, Billerica, MA, USA). Compound quantification in reference to the TSP internal standard was performed with the Chenomx 8.1 NMR Suit (Edmonton, Alberta, Canada). The limit used for compound quantification in Chenomx was set at 0.002 mM.

Metabolomics data have been deposited to the EMBL-EBI MetaboLights database (DOI: 10.1093/nar/gks1004. PubMed PMID: 23109552) with the identifier MTBLS720. The complete dataset can be accessed here https://www.ebi.ac.uk/metabolights/MTBLS720.

Statistical analysis. *Performance and blood data*. The univariate analysis was carried out with the SPSS 24 (IBM, Armonk, NY, USA). The significance level was declared at $P < 0.05$ and a tendency was considered at $0.05 \leq P \leq 0.1$. Descriptive data are presented with the means and the standard error (mean ± SE). Shapiro-Wilks test was used to assess normality of data. Means were compared to the Ctrl group by a *t*-test and for pairwise comparisons, ANOVA with Tukey HSD post hoc was applied. The multivariate analyses were performed by SIMCA 13.0.3 (Umetrics AB, Umeå, Sweden) after applying auto-transform to reduce data skewness and UV scaling to reduce relative importance of large values.

Muscle data. For Chemometric statistical analysis, processed spectra were loaded into "R" where peak alignment was performed with SPEAQ. The data were then exported into a coma-separated value (CSV) file and loaded into SIMCA 13.0.3. All spectra were treated with a log transform and by Pareto scaling. For the whole groups, unsupervised Principal Components Analysis (PCA) was performed to detect outliers, patterns and trends. For pairwise Partial Least Square Discriminant Analyses (PLS-DAs), $Q^2 > 0.4$ were selected as acceptable models and Variable Importance in Projection (VIP) scores were used to identify metabolites responsible for the separation. These metabolites together with others that are relevant for the project were then quantified using database assisted spectral deconvolution that was performed using Chenomx. The resulting concentration were normalized based on the mass tissue used for extraction and then further performed univariate analysis by SPSS 24 and multivariate analysis by SIMCA 13.0.3 after applying auto-transform and UV scaling.

Availability of Data and Materials

All data generated or analysed during this study are included in this published article and its Supplementary files.

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Author Contributions

K.Y. performed the experimental metabolomics work and data analysis, sample measurements and participated in the manuscript writing. M.M. directed and participated in the metabolomic analysis. D.V. participated in sample measurements. Y.S. participated in sample measurements. A.A. contributed to the manuscript writing and metabolomics study design. M.T. participated in the experimental design and was in charge of the animal feeding trial and revised the manuscript. A.B. participated in the experimental design, supervised the analytical work and revised the manuscript. All authors read and approved the final manuscript.

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6.2. Publication 2: *Age-Related Serum Biochemical Reference Intervals Established for Unweaned Calves and Piglets in the Post-weaning Period*

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Age-Related Serum Biochemical Reference Intervals Established for Unweaned Calves and Piglets in the Post-weaning Period

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The purpose of the present study is to establish the influence of age on serum biochemistry reference intervals (RIs) for unweaned calves and recently-weaned piglets using large number of animals sampled at different ages from populations under different season trials. Specifically, milk replacer (MR)-fed calves from April–July 2017 ($n = 60$); from December 2016–March 2017 ($n = 76$) and from April–August 2018 ($n = 57$) and one group of healthy weaned piglets ($n = 72$) were subjected to the study. Serum enzymes and metabolites of calves at age of 24 h (24 h after colostrum intake), 2, 5, and 7 weeks from merged trials and piglets at 0, 7, and 14 days post-weaning (at 21, 28, and 35 days of age) were studied. The main variable is age whereas no major trial- or sex-biased differences were noticed. In calves, ALT, AST, GGT, GPx, SOD, NEFAs, triglycerides, glucose, creatinine, total protein, and urea were greatly elevated ($p < 0.001$) at 24 h compared with other ages; glucose, creatinine, total protein, and urea constantly decreased through the age; cholesterol's lowest level ($p < 0.001$) was found in 24 h compared with other ages and the levels of haptoglobin remained unchanged ($p > 0.1$) during the study. In comparison with the adult RIs, creatinine from 24 h, NEFAs from 2 w, GGT from 5 w, and urea from 7 w are fully comparable with RIs or lie within RIs determined for adult. In piglets, no changes were noticed on glucose ($p > 0.1$) and haptoglobin ($p > 0.1$) and there were no major changes on hepatic enzymes (ALT, AST, and GGT), total protein, creatinine and urea even though several statistical differences were noticed on 7 days post-weaning. Cholesterol, triglycerides, NEFAs, cortisol and PigMAP were found increased ($p < 0.05$) while TNF-alpha was found less concentrated ($p < 0.001$) at 0 days post-weaning compared with other times. Moreover, the RIs of creatinine and GGT are fully comparable with RIs or lie within RIs determined for adult. In conclusion, clinical biochemistry analytes RIs were established for unweaned calves and recently-weaned piglets and among them some can vary at different ages.

Keywords: serum clinical biochemistry, reference interval, non-parametric, unweaned calf, weaning piglet, age

INTRODUCTION

Serum biochemical reference intervals (RIs) play an important role in assessing animal health. However, for farm animals, the majority of RIs are set for adult individuals. Similar to human medicine, where pediatric RIs are readily available, there is a necessity of robust and specific RIs for young animals in veterinary science, since their physiology is different from adults and it may be markedly affected by milk-feeding and weaning. The use of adult RIs intervals may be inadequate since young animals may metabolize in a different way. Furthermore, young animals must be closely monitored since they are more vulnerable to diseases, and their diets may have a bigger impact on the health condition in comparison with the adult. Thus, many of the RIs publications in veterinary science established for adults (1, 2) may be misleading (3) and the establishment of RIs in young animals is needed to adequately discriminate between healthy and diseased individuals.

In newborn calves, their growth involves several morphological, metabolic and physiological changes (4) and these changes may be reflected by metabolites and enzymes' levels in blood (5, 6). Calves undergo metabolic and digestive tract physical changes during the weaning process (in the dairy industry it occurs between 6 and 9 weeks of age) (7). At birth, most of the nutrients (glucose, amino acids, and long-chain fatty acids) from milk are digested in the abomasum and absorbed in the small intestine. Thus, young calves are non-ruminant. As calf starts to consume solid feeds (like concentrate feeds), the rumen starts to develop. At weaning, the rumen is mostly developed and it supports part of the nutrient absorption from solid feed fermentation. This different nutrient supply at weaning also entails a shift in the hepatic function from a glycolytic to glucogenic liver altering metabolites and enzymes levels in blood.

In swine industry, an "early weaning" is commonly used with the objective of reducing and improving the sow productive cycle. Normally weaning occurs abruptly at 21–28 days of age and the piglet needs to be adapted to eat a novel food (usually solid diet) after the separation from the sow. In contrast to the natural weaning which gradually process until 17 weeks of age (8), early weaning may become stressful for piglets. The immediate post-weaning period is critical for the piglet health since at that moment they are very susceptible to gastrointestinal problems and infections.

In both species, the early stage age involves several biochemical and metabolic changes that may be fundamentally different from the adult ones. Moreover, since young animals are more vulnerable to certain pathogens, basic health laboratory routine check based on serum biochemical analytes is essential.

In the past 20 years, neonatal clinical biochemistry gradually has been established and the biochemical RIs on unweaned cattle calves of different breeds and ages up to 3 months of old (3, 9–11) and for piglets younger than 1 month of age (12, 13) have been published. In all these studies, several noticeable biochemical changes along the age were reported. Nevertheless, these studies have two main drawbacks. First, RIs have been calculated from small populations (<35), and this does not meet the requirement of non-parametrical RI. As it is recommended in Clinical and Laboratory Standards Institute (CLSI) Guideline, a non-parametric method is recommended due to its simplicity and reliability (14), thus a sufficient population for RI establishment could be more trustworthy. Second, many RIs are calculated from individual experiments, and since factors like diet, geography, season, sex, and breed may affect the RI, thus the obtained value may not applicable in other conditions.

In this study, the first objective is to determine clinical biochemistry values from a large set of healthy unweaned calf populations from 3 different nutritional programs and breeding seasons at 24 h, 2 weeks, 5 weeks, and 7 weeks of age; the second objective is to determine healthy recent-weaned piglets' clinical biochemistry values from both sexes at 0 days, 7 days, and 14 days post-weaning and the third objective is to evaluate all the values and establish RIs. The purpose is to obtain RIs useful for the veterinary practitioner and for research.

MATERIALS AND METHODS

Calf Housing and Diet Management

Male Holstein calves were managed according to the recommendations of the Animal Care Committee of Institut de Recerca i Tecnologia Agroalimentàries (IRTA) under the approval research protocol FUE-2017-00587321, authorization code 9733. Holstein male calves were obtained from Granja Murucuc (Gurb, Barcelona, Spain) and housed individually at IRTA in Torre Marimon (Barcelona, Spain). Plastic ear tag identification with the animal's number was used. In all cases, animals were under veterinary control and no health problems were observed during the experimental period. The total number of individuals included in the study was $n = 193$. Animals belonged to three trials.

In Trial 1 (T1), 60 new-born calves (age = 0 h) were fed with colostrum within the first 6 h after birth. At 24 h of age, blood samples were taken. Then, at age of 3 ± 1.3 days old, animals were fed with a MR and blood samples were collected at 2 and 5 weeks of age 4 h after the morning feeding. The T1 was carried out between April and July 2017.

In Trial 2 (T2), 76 calves (average age = 3 ± 1.7 d) were fed with MR and blood samples were collected at 2, 5, and 7 weeks of age 4 h after the morning feeding. The T2 started in December 2016 and ended in March 2017.

In Trial 3 (T3), 57 calves (average age = 5 ± 2.2 d) were fed with MR and blood samples were taken at 2, 5, and 7 weeks of age 4 h after the morning feeding. The T3 was carried out between April and August 2018.

All animals followed the same MR feeding program that consisted on feeding 2-L of MR (Nukamel, Weert, The Netherlands) twice a day containing at 12.5% concentration of MR powder (500 g/d) for the first 4 days, subsequently MR was increased to 5 L/d of MR at 12.5% concentration of MR powder (625 g/d) for the next 3 days, then MR was increased to 6 L/d of MR at 12.5% concentration of MR powder

(750g/d) for the next 7 days and then 2 meals of 3 L at 15% concentration (900 g/d) until 49 d of age. Then, MR was limited to a single offer of 3 L also at 15% (450 g/d) until end of the study. Animals also had access to water and chopped barley straw *ad libitum*. Chemical composition and ingredient of the MRs and the starter intake information are shown in **Tables S1, S2**.

Piglet Housing and Sampling

A total of 336 commercial crossing piglets ([Large White x Landrace] x Pietrain), male and female 21-d old piglets of 4–7 kg of body weight (BW) were weaned. The animals were moved from the farrowing to the nursery unit (within the same commercial farm without transport) in the morning on the day of weaning. Plastic ear tag identification with the animal's number was used. In the nursery room, pigs were then allocated in 24 pens (14 piglets/pen). Each pen has had a commercial non-lidded hopper and a nipple waterer to ensure *ad libitum* feeding and free water access. The experimental procedure has been approved by ethical committee (CEEAH) of Universitat Autònoma de Barcelona (Registration n°:1406 of the Departament de Medi Ambient i Habitatge of the Generalitat de Catalunya).

At weaning, animals were offered the same basal pre-starter diets following the same specification. The pre-starter and starter diets were fed *ad libitum* for fourteen consecutive days (P1) and 21 consecutive days (P2), respectively. A basal diet was formulated to contain 2,470 kcal NE/kg; 20.5% CP/kg and 1.35% Dig Lys for P1 and 2,400 kcal NE/kg; 18.1% CP/kg and 1.20% Dig Lys for P2. Diets were formulated to meet the requirements for growth of newly weaned piglets (15). No antibiotics and ZnO were included in the basal diets.

Blood samples from 3 piglets per pen (a total of 72 animals) were collected avoiding cross-contamination between pens at 8 am at week 3 of age (day 0 after weaning), week 4 (day 7 after weaning), and week 5 (day 14 after weaning). Piglets were individually weighted before weaning and distributed for a balanced body weight within each pen. Piglets were sorted by descending BW within each pen and the one in the middle (median), the ones immediately above and below the median were picked out to be sampled. The same animals per pen were sampled on weeks 4 and 5, respectively.

Sample Collection and Analysis

Blood samples obtained from jugular venipuncture were collected in 10 ml Vacutainer tubes without anticoagulant. Sera were obtained by centrifugation at 1,500 g for 10 min and transferred to new tubes to be stored in aliquots at −80°C until further analysis. At the day of analysis, aliquots were thawed. Measurement of serum clinical biochemistry analytes was performed on the Olympus AU400 analyser except cortisol and TNF-alpha. The protocols for the use of Olympus System Reagents (OSRs) and other commercial reagents were in reference of previous studies (16, 17). Quality control protocols were based on the daily quantification of two control sera of low and high concentration (Control serum I and Control serum II, Beckman Coulter). For cortisol and TNF-alpha in piglets, two commercial ELISA kits were used: Cortisol ELISA Kit (DRG, Marburg, Germany) and Porcine TNF-alpha Quantikine ELISA Kit (R&D Systems, Abingdon, UK). The analytes, methods, reagents, and coefficients of variation (CV%) are shown in **Table S3**.

RI Establishment and Statistics

The RI establishment was in accordance with American Society for Veterinary Clinical Pathology (ASVCP) guidelines (18). These guidelines mirror the CLSI recommendations. All RIs were generated by Reference Value Advisor (19), an add-in program in Excel (Office 365, Microsoft, Redmond, WA, USA). For population amounts over 120, non-parametric RIs were directly used since this method doesn't assume any specific shape for data distribution. For populations <120 (24 h of age for calves and piglet samples), non-parametric RIs were not able to be computed. The criteria of outlier removal are following: (a) For interquartile range (IQR) = Q_3(third quartiles)—Q_1(first quartiles), values that exceed interquartile fence set at $Q_1-3*IQR$ and $Q_3+3*IQR$ are considered outliers; (b) Without removing certain outliers, if Reference Value Advisor computes result with good distribution and/or symmetricity, then the outliers are retained; (c) If no normal distribution and/or symmetricity are observed even outliers are removed and data are power transformed, such population is calculated with Cook's distance and Cook's outliers are detected and deleted. After the removal of outliers, populations were recalculated for RIs. Once the RIs were established, one-way analysis of variance (ANOVA) and Tukey's HSD with Bonferroni correction test, boxplots with difference annotations and distribution histograms were made by "R" (20) under RStudio environment (21) using the combinations of "multcompView" (22), "nortest" (23), "ggplot2" (24), "gridExtra" (25), and "ggpubr" (26) packages and values that exceed interquartile fence set at $Q_1-3*IQR$ and $Q_3+3*IQR$ were considered outliers.

RESULTS

For calves and piglets, the frequency of distribution histograms of all analytes from all the individuals (both trial-wise and merged-data-wise) were visualized before the RIs were established (shown in **Figures S1, S2**). The distribution from the different sets of animals overlapped and differences between groups were not significant, indicating that no year season, experimental or sex-biased distributions were found and in consequence, all the individuals of the same age were included in the same reference population. There were only two exceptions identified in calves: AST and cholesterol at week 2, where T3's population displayed a non-overlapping distribution curve.

For calves, the methods of RI calculations of 24 h was parametric since population consisted in 30 individuals and from 2 w non-parametric method was used as the populations are over 120.

For piglets, since the population size didn't reach 60, parametric method was used for all ages and analytes except one non-parametric method was suggested using by the software on AST at 7 d due to unfixable normality of distribution.

A summary of the results of both species at different ages are shown in **Tables 1**, **2**, respectively. **Figures 1**, **2** show the pattern of age-related differences for all parameters. The *post-hoc* multi-comparison results are shown in **Tables S4, S5**. Missing and undetectable samples were discarded in this study.

DISCUSSION

A table with RI for calves and piglets has been designed for veterinary use with comparison of results in adults obtained from the literature (**Tables 3**, **4**).

RIs for Unweaned Calves

It was noticed that the all enzymes (ALT, AST, GGT, GPx, and SOD) not only showed the highest concentrations at 24 h of age, but also significantly higher than any other times of age. The high concentration in newborn animals is an indication that these enzymes are absorbed from colostrum, at least in the case of GGT and AST (9, 32). The most extreme elevation occurred with GGT, which was 20 folds higher at 24 h than at 2 w and further decreases at 5 w and 7 w. Even though the GGT level is relatively low in adult cow's serum, its level in colostrum can peak-up to ∼800-fold greater than in the serum of the same cow (33). GGT can increase its activity in calves' serum as early as 6 h after colostrum intake (intake at birth) (34) and similar to our results, it has been described that it takes approximately up to 5 weeks for GGT to decline to low level (adult's level) after the intake of colostrum (3, 11, 33). Other studies found that lower concentrations of GGT in new-born calves are linked with much lower levels of globulins/total protein and higher risk of death (33, 35); moreover, one study showed 0% sensitivity of GGT when it comes to the detection of hepatic diseases before 6 w old (36) since the highly concentrated colostrum-derived GGT presented in the serum can mask the level of GGT that is derived from liver damage. Indeed, a better characterization of GGT levels for new-born calves may help to monitor calves' health to prevent early-age death but not used to detect hepatic diseases. It is also relevant to notice the high individual variability of GGT values in 24 h old calves, probably indicating differences in colostrum intake, absorption and/or metabolization.

After 24 h of age, ALT's level reached its lowest at 2 w and gradually went uphill afterwards while AST stayed relatively steady and low levels from 2 to 7 w. However, at 7 w of age, both levels of ALT and AST were still lower compared with adult's RI. Other studies with longer terms showed that AST reaches adult's level around 8–10 w of life (9, 10). According to previous study along with GGT and other enzymes (36), AST's sensitivity to liver damage is 80% for calves <6 w old thus AST alone should not be considered clinical useful in diagnosing hepatic diseases. In addition, despite the statistical differences in AST throughout 2–7 w, none of their pair-wise comparison fold changes were larger than 1.15 or <0.85, thus biologically these were not considered major differences. For ALT, its characterization in cow is seldom studied because is not so commonly used for hepatic disease diagnosis since its activity is low and isn't massively liberated into serum during hepatic diseases in large animal (2).

SOD and GPx also showed rather stable levels from 2 to 7 w. Unfortunately, we didn't find any RIs of GPx for adult; and for SOD only RI of cow during transition period (60 d before until 100 d after milk) was found. Both mineral dependent enzymes [Mn, Cu and Zn for SOD (37) and Se for (2, 38)] are important enzymes whereas SOD can dismutate two O_2^- molecules to H_2O_2 and O_2 while GPx can catalyze H_2O_2 to H_2O (2). Study of SOD in RBC suggested that there is no such variation in the activity between cattle and human (39). Even though there are very few information about SOD and GPx, a better characterization may contribute to the monitor of minerals and oxidation in animal's body.

NEFAs and TGs were also elevated at 24 h because colostrum is rich in fat (40); their concentrations dropped at 2 w compared to 24 h and started increasing moderately with age until 7 w. While the level of NEFAs is almost comparable with adult's RI roughly from 5 w, TG's level is still beyond the adult's RI upper limit at 7 w. TGs can richly appear in chylomicrons, which are secreted by the intestine after fat-containing meal in preruminant calves. The TGs from chylomicrons can be hydrolyzed to NEFAs and then be transported to tissue for fat storage or for oxidation to produce energy (41); on the other hand, serum NEFAs can increase in case of fasting or negative energy balance. Indeed, the growth of calf requires a large amount of energy from fat and evidences have already shown that extra fat can increase BW gains (42, 43). An adult's-like RI in NEFAs from 5 w and a rather higher levels of TGs throughout the study indicate that the calves demanded high amount of energy for growth and no negative energy balance was noticed. Another form of TGs is very-low-density lipoprotein (VLDL) but it is generally low in bovine compared with in human (41).

Cholesterol had a very similar trend with one study (9) where the lowest concentration was found at 24 h and the concentrations started to increase smoothly. Even though it was previously described that colostrum contains higher cholesterol concentration compared with milk (44) and the serum cholesterol concentration is proportional to the colostral cholesterol concentration (45), the increase in total cholesterol along with increases in NEFAs and TGs probably indicate a major lipid mobilization within tissues after 24 h.

Glucose showed significant drop throughout age. The glucose supply from mother in neonate stops abruptly immediately after birth and the suckling neonate is entirely dependent on glucose supply via colostrum/milk, meanwhile the neonate needs to rapidly turn on pathways of endogenous glucose production as well as for fatty acid oxidation to maintain its organs and cells' functionality (46). The decrease of glucose and increase of NEFAs and TGs during the first 7 w may be the consequence of the high fat and relatively low carbohydrate content of colostrum/milk (47, 48). Despite the constant fall of serum glucose throughout the study, the RIs obtained from last measurement was still higher than the adult's one.

Moreover, creatinine, TP and urea also decreased throughout age until 7 w. Creatinine is a breakdown product of creatine (Cr) and creatine phosphate (PCr) in muscle and is cleared by the kidney. In human neonatology, it was well explained that the kidneys in fetus don't play a major role in maintaining

TABLE 1 | Summary of statistical values for clinical biochemistry analytes from calves of 24 h (24 h after colostrum taking), 2, 5, and 7 weeks of age.

	Analyte	ALT	AST	Cholesterol	Creatinine	GGT	Glucose	GPx	Haptoglobin	NEFAs	TGs	TP	SOD	Urea
	Unit	**U/L**	**U/L**	**mg/dL**	**mg/dL**	**U/L**	**mg/dL**	**U/L**	**mg/mL**	**mmol/L**	**mg/dL**	**g/dL**	**U/mL**	**mg/dL**
24 h	Mean	12.1	87.5	36.1	1.22	1459.8	125.8	737.1	0.16	0.41	39.1	6.02	0.90	19.4
	Median	10.8	85.0	33.6	1.20	1303.0	119.4	632.0	0.14	0.40	38.2	6.15	0.53	19.3
	SD	4.4	20.4	11.1	0.17	831.3	28.5	347.4	0.05	0.15	19.7	0.71	0.89	5.6
	Minimum	6.8	53.0	20.6	0.96	288.0	68.5	343.1	0.09	0.19	13.5	4.42	0.05	8.7
	Maximum	22.3	134.0	67.4	1.64	3170.0	211.1	1650.4	0.32	0.77	87.0	7.43	2.79	34.9
	N	29	30	30	30	28	30	28	28	30	30	30	30	30
	Method	Trans. Std.	Trans. Std.	Trans. Std.	Trans. Std.	Trans. Std.	Untrans. Std.	Trans. Std.	Trans. Std.	Untrans. Std.	Trans. Std.	Trans. Std.	Trans. Std.	Trans. Std.
2 w	Mean	6.8	36.6	71.5	1.00	100.8	110.6	505.0	0.14	0.10	19.6	5.11	0.31	15.2
	Median	6.6	38.0	78.3	0.99	76.0	110.6	452.4	0.13	0.09	16.0	5.10	0.25	13.9
	SD	1.7	9.3	29.3	0.20	70.6	22.1	194.0	0.12	0.06	12.1	0.63	0.21	7.9
	Minimum	3.3	0.0	11.5	0.54	12.0	65.4	231.6	0.06	0.03	5.5	3.75	0.02	6.3
	Maximum	12.1	60.0	145.0	1.71	348.0	222.5	1610.7	1.48	0.31	81.8	7.14	1.33	92.7
	N	188	188	188	188	188	188	186	190	188	188	188	185	188
	Method	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP
5 w	Mean	8.3	42.2	82.1	0.83	24.5	101.8	421.1	0.14	0.15	21.9	4.99	0.23	13.9
	Median	8.1	42.0	77.0	0.82	23.0	101.4	383.0	0.12	0.14	18.1	4.99	0.20	13.7
	SD	2.1	15.0	29.4	0.16	9.5	18.4	157.6	0.18	0.06	12.0	0.50	0.14	3.9
	Minimum	4.3	19.0	30.6	0.50	7.0	43.8	242.2	0.06	0.05	7.4	3.77	0.02	6.3
	Maximum	19.5	178.0	187.7	1.40	54.0	157.9	1525.3	2.21	0.30	71.7	6.49	0.94	27.3
	N	193	193	193	193	193	193	192	193	193	193	193	191	193
	Method	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP
7 w	Mean	9.5	37.5	65.5	0.67	16.8	93.0	437.1	0.13	0.18	24.1	4.80	0.23	11.2
	Median	9.4	36.5	64.9	0.66	16.0	93.3	413.2	0.12	0.17	21.5	4.70	0.20	10.9
	SD	2.4	10.8	23.0	0.12	4.4	15.3	128.0	0.06	0.07	14.3	0.54	0.13	2.9
	Minimum	4.7	16.0	18.8	0.43	8.0	59.2	262.4	0.07	0.05	6.1	3.66	0.01	5.8
	Maximum	19.0	77.0	133.4	1.03	31.0	147.8	1330.8	0.56	0.40	101.9	6.47	0.66	18.8
	N	134	134	134	134	134	134	133	134	134	134	134	130	134
	Method	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP

SD, Standard Deviation; N, Number of animals; ALT, Alanine Aminotransferase; AST, Aspartate Aminotransferase; GGT, Gamma-Glutamyl Transferase; GPx, Glutathione Peroxidase; NEFAs, Non-Esterified Fatty Acids; TGs, Triglycerides; TP, Total Proteins; SOD, Superoxide Dismutase.
Methods: NP, Non-parametric; Trans. Std., Transformed data using standard method; Untrans. Std., Untransformed data using standard method.

fetal homeostasis (49, 50); also, the high blood concentration of creatinine comes from maternal transfer and endogenous Cr and PCr degradation and it does not indicate fetal renal failure. It was noticed that at 2 and 5 w the median creatinine levels were around the bottom line of the adult's RI while at 7 w it was below [median and SD at 7 w was 0.66 ± 0.12 mg/dL and adult's RIs are 1.0–2.0 mg/dL according to Kaneko et al. (2) and 0.9–1.4 mg/dL according to Pérez-Santos et al. (11)], comparing to another similar study (11), where mean creatinine laid within normal adult's RI from 6 d until 90 d of age. Yet we didn't find any other issues related to the muscle or health in these calves and a possible reason of this is due to diet/season/genetic variabilities between the farms. TP was also elevated at 24 h due to passive transfer of protein which mainly contains immunoglobulins from colostrum (32, 51, 52). This result is similar to those were reported in previous studies (11, 53). Urea had a similar change pattern to the TP along the time. It is the product of protein breakdown (52) and is synthesized by the liver and finally excreted by the kidney, which is directly influenced by the total protein level.

A special attention was paid to markers of stress since assessing a good welfare status is one of the main problems in calf production. The levels of haptoglobin didn't change along the time, similar to one previous study (54) and are lying within the health adult's RI range (10 mg/dL) (27). It is important to have specific RIs for haptoglobin in young calves since it is an acute-phase protein used as biomarker of inflammatory and infectious diseases (55), very common in young calves. The individuals with high haptoglobin values identified as outliers may be individuals with subclinical diseases, which were recognized as healthy in a clinical inspection. The antioxidant enzymes GPx and SOD, markers of oxidative stress (56) had higher values at 24 h, but the RIs are similar afterwards.

As it was previously mentioned, on week 2 we found trial-biased distributions (**Figure S1**) on AST and cholesterol, where T3 showed lower concentration range compared with others.

TABLE 2 | Summary of statistical values for clinical biochemistry analytes from piglets at 0, 7, and 14 days post-weaning (at 21, 28, and 35 days of age).

	Analyte	**ALT**	**AST**	**Cholesterol**	**Cortisol**	**Creatinine**	**GGT**	**Glucose**	**Haptoglobin**	**NEFAs**	**PigMAP**	**TGs**	**TNF-alpha**	**TP**	**Urea**
	Unit	**U/L**	**U/L**	**mg/dL**	**ng/L**	**mg/dL**	**U/L**	**mg/dL**	**mg/mL**	**mmol/L**	**g/L**	**mg/dL**	**pg/mL**	**g/dL**	**mg/dL**
0 d	Mean	32.8	78.4	125.5	74.9	0.80	46.0	84.9	0.50	0.40	3.26	43.2	54.3	3.78	12.1
	Median	31.3	63.0	122.0	60.2	0.79	41.0	80.8	0.41	0.40	2.88	40.9	48.8	3.68	11.7
	SD	9.4	47.5	41.8	50.1	0.15	16.5	17.8	0.39	0.14	1.22	13.1	22.3	0.64	3.6
	Minimum	10.2	20.0	51.4	2.0	0.57	11	49.0	0.05	0.15	1.22	22.6	23.1	2.34	6.7
	Maximum	63.7	242.0	272.2	202.2	1.16	89	141.4	1.68	0.76	5.88	85.0	110.6	5.42	25.2
	N	66	68	61	71	54	65	57	61	61	71	59	67	58	57
	Method	Trans. Std.	Trans. Std.	Trans. Std.	Trans. Std.	Untrans. Rob.	Trans. Std.	Untrans. Rob.	Trans. Std.	Trans. Std.	Trans. Std.	Trans. Std.	Trans. Std.	Trans. Std.	Trans. Std.
7 d	Mean	24.8	45.8	53.1	19.0	0.94	40.9	79.1	0.62	0.18	0.99	35.5	103.2	3.49	18.2
	Median	22.8	37.0	52.0	15.6	0.93	40.0	78.9	0.49	0.15	0.94	31.7	101.7	3.46	17.2
	SD	8.8	22.3	14.3	12.4	0.16	13.9	12.4	0.52	0.11	0.31	11.2	27.7	0.48	6.9
	Minimum	8.2	15.0	27.0	1.1	0.66	13	55.3	0.07	0.04	0.43	18.8	41.4	2.39	4.3
	Maximum	53.3	125.0	95.5	52.5	1.51	74	107.6	2.39	0.58	2.24	73.3	165.7	4.57	41.3
	N	67	67	65	68	65	67	64	66	62	69	65	72	65	64
	Method	Trans. Std.	NP*	Trans. Std.	Trans. Std.	Trans. Std.	Trans. Std.	Trans. Std.	Trans. Std.	Trans. Std.	Trans. Std.	Trans. Std.	Trans. Std.	Trans. Std.	Trans. Std.
14 d	Mean	35.5	65.6	69.7	21.2	0.85	50.9	83.0	0.57	0.11	0.74	38.8	98.1	3.84	14.7
	Median	28.7	48.0	62.4	18.9	0.82	49.0	85.8	0.30	0.10	0.70	38.0	92.5	3.74	12.9
	SD	23.6	45.7	36.9	16.7	0.17	17.4	16.5	0.66	0.03	0.28	10.0	42.8	0.69	7.5
	Minimum	11.4	23.0	37.5	1.6	0.53	17	34.5	0.05	0.05	0.32	20.2	43.6	2.76	4.6
	Maximum	177.9	260.0	303.9	83.8	1.26	112	126.9	3.05	0.21	2.13	68.3	292.0	6.36	38.5
	N	64	62	62	65	57	66	60	61	55	63	60	71	59	57
	Method	Trans. Std.	Trans. Std.	Trans. Std.	Trans. Std.	Trans. Std.	Trans. Std.	Trans. Std.	Trans. Std.	Trans. Std.	Trans. Std.	Trans. Std.	Trans. Std.	Trans. Std.	Trans. Std.

SD, Standard Deviation; N, Number of animals; ALT, Alanine Aminotransferase; AST, Aspartate Aminotransferase; GGT, Gamma-Glutamyl Transferase; NEFAs, Non-Esterified Fatty Acids; PigMAP, Pig Major Acute Phase Protein; TGs, Triglycerides; TNF-alpha, Tumor Necrosis Factor alpha; TP, Total Proteins.

Methods: NP, Non-parametric method; Trans. Std., Transformed data using standard method; Untrans. Rob., Untransformed data using robust method.

**Non-parametric method was used due to unfixable distribution [p-value (Anderson-darling) < 0.05 and p-value (symmetry test) < 0.01] both before and after transformation. This method was recommended by the software.*

This reason may be because the average age in T3 is ∼2 d older than other two. This difference indicates that only 2-days age difference can cause mean concentration difference in some parameters, thus special care should be taken when comparing local clinical values with RIs in literature.

RIs for Post-weaning Piglets

No major changes were noticed on hepatic enzymes (ALT, AST, and GGT), glucose and TP. Cholesterol, NEFAs and TGs showed their highest values just after weaning (0 d). This may be due to the sudden dietary change from high fat content of milk to less-digestible, more-complex solid feed that piglets eat after weaning (57).

Throughout the ages, all medians of urea were within adult's RI while all medians of creatinine were <0.8 folds to the adult's RI lower fence. Both analytes showed highest concentrations at 7 d post-weaning. The increase in urea and creatinine only at 7 d post-weaning may be due to transient dehydration after the dietary change. These pattern changes are very similar to one study between 5-day-old unweaned piglets and 30-day-old post-weaning piglets (weaned at 28 d of life) supplemented with iron in solid diet (12). However, since this report only measured 5 d and 30 d, different from our opinion, the author's explanation of the increases was due to the increase of muscle mass and helped by the gradual supplementation of enriched solid diet.

Special interest has been devoted to markers of stress and inflammation. Thus, cortisol was measured as the paradigmatic stress hormone and found higher just after weaning, similar to other studies (58–60), this probably is due to the stress associated to separation of the mother and change of diet; it's also interesting to notice that from 7 d post-weaning, the mean concentrations of piglets are comparable to healthy adults' one (31), which suggests that in this study the diet change only caused a transient stress to the animals. The acute phase proteins haptoglobin and Pig-MAP were also measured; whereas haptoglobin RIs were not influenced by age and weaning, Pig-MAP was higher at 3 weeks-old just after weaning. Absolute values for haptoglobin were similar to those reported at 3-week-old (13). In that study, reference values for both acute phase proteins were established for pigs older than 4 weeks. Pig-MAP is also considered a stress marker, which increases in stressful conditions like transport (61–63). Finally, the cytokine TNF-alpha is frequently used as a marker of infection, especially in gastrointestinal disorders which often affect piglets at weaning (31, 64, 65). For this reason, it is

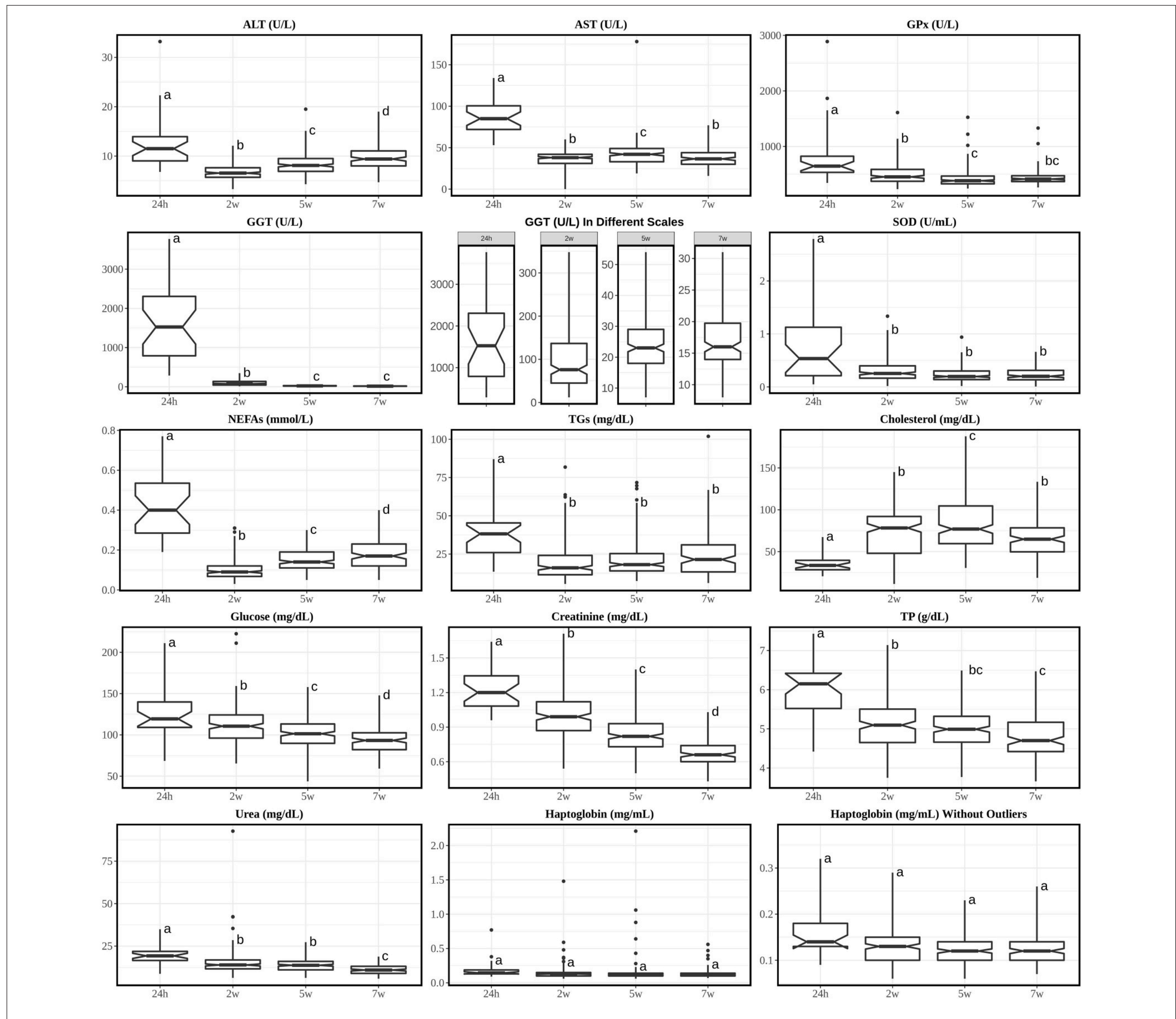

FIGURE 1 | Boxplots of analytes with Tukey's HSD *posthoc* with Bonferroni correction from calves' sera at 24 h, 2 w, 5 w, and 7 w of age. Annotations over the boxes with different superscripts are significantly different ($p < 0.05$). Interquartile fence was set between Q1-3*IQR and Q3+3*IQR. 24 h, calves at 24 hours of age; 2 w, calves at 2 weeks of age; 5 w, calves at 5 weeks of age; 7 w, calves at 7 weeks of age. Analytes were alphabetically plotted according to the orders of discussion.

interesting to have RIs available in healthy animals. A moderate increase in TNF-alpha was shown at 7 and 14 days after weaning, probably due to the change of diet. This increase in serum may be secondary to the transient response in gene expression of inflammatory cytokines in the gut after weaning (66).

Similar to the calves, creatinine from 0 d post-weaning (3 w of age) was also comparable to the adult's RIs. GGT's RI is also comparable to adult's one from 7 d of age while for calves it takes 5 weeks to drop into adult's RI (2).

CONCLUSIONS

Specific information about reference intervals for young animals is a requirement for the adequate clinical and nutritional management of the individuals. Even in farm animals, where collective evaluation of health is frequent instead of an individual assessment, the availability of specific RIs for young ages is essential to for an accurate diagnosis of health problems. In this sense, most important life periods in these two farm animal species: in cattle, colostrum uptake and suckling is a critical period during which the calf starts growing and obtains the immune defenses necessary for a sustained growth. In piglets, the post-weaning period is the most critical due to the high incidence of gastrointestinal and respiratory disorders. In this work, we have used a large number of individuals to obtain RIs specific for calves and piglets at these critical stages, and the most relevant parameters related to these stages evaluated.

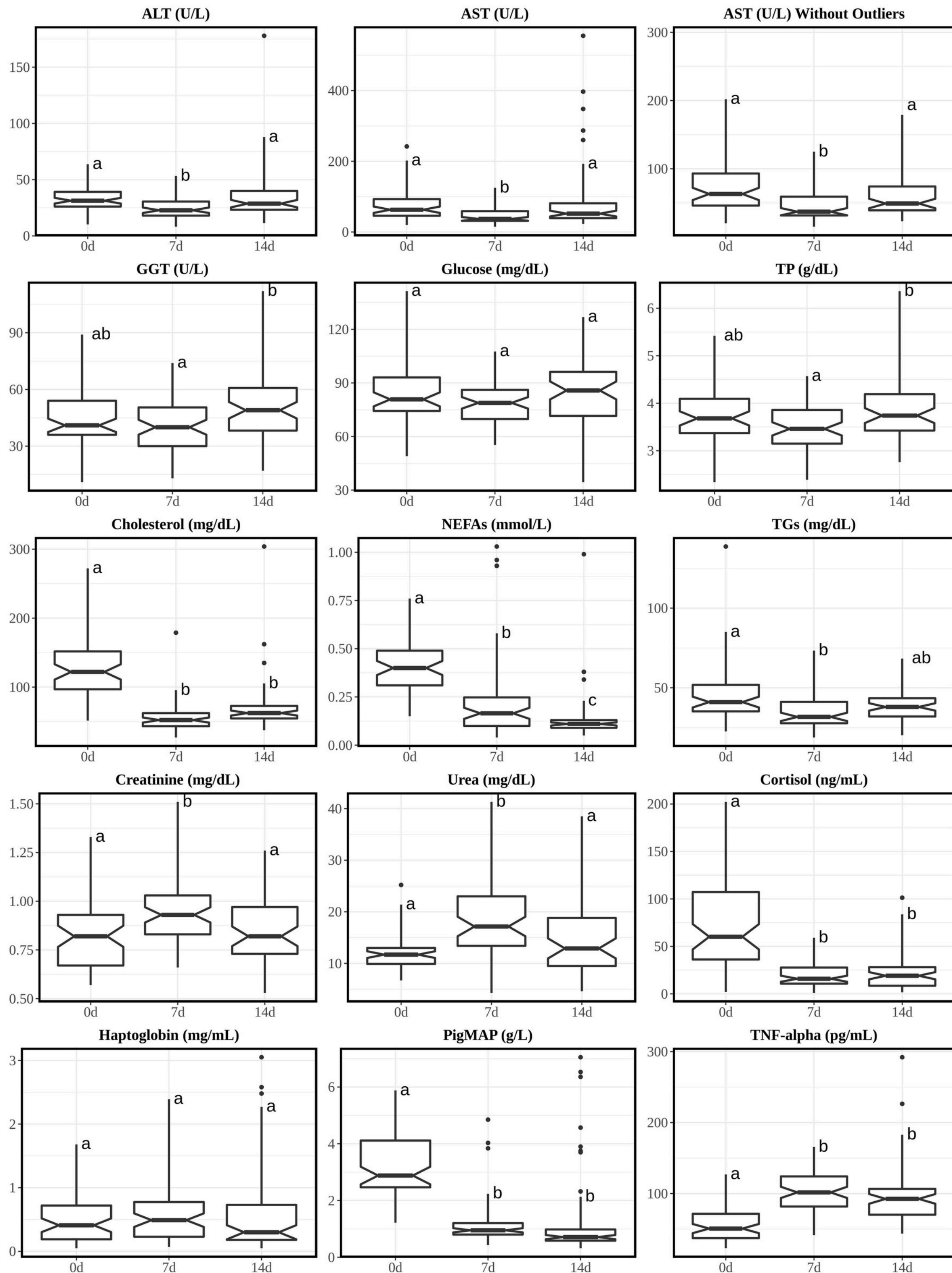

FIGURE 2 | Boxplots of analytes with Tukey's HSD *posthoc* with Bonferroni correction from piglets' sera at 0, 7, and 14 days post-weaning (age: 21, 28, and 35 days, respectively). Annotations over the boxes with different superscripts are significantly different ($p < 0.05$). Interquartile fence was set between Q1-3*IQR and Q3+3*IQR. 0d, piglets at the 0 day of weaning (right after weaning); 7d, piglets at the 7th day of weaning; 14d, piglets at the 14th day of weaning. Analytes were alphabetically plotted according to the orders of discussion.

TABLE 3 | Reference intervals (RIs) of clinical biochemistry analytes from calves of 24 h of age (24 h after colostrum taking) 2, 5, and 7 weeks of age and RIs of adult cattle from literature.

Analyte	Unit	24 h	2 w	5 w	7 w	Adult	
ALT	U/L	6.3–24.7	4.0–11.1	4.8–13.3	5.2–15.3	11–40	(2)
AST	U/L	49.6–133.4	19.5–55.0	22.0–63.3	19.0–63.3	78–132	(2)
Cholesterol	mg/dL	20.8–67.5	20.8–128.9	33.9–136.4	25.4–118.3	80–120	(2)
Creatinine	mg/dL	0.93–1.65	0.64–1.51	0.56–1.20	0.47–0.99	1.0–2.0	(2)
GGT	U/L	0.0–3201.8	16.5–262.5	12.0–48.6	11.0–27.6	6.1–17.4	(2)
Glucose	mg/dL	75.3–193.3	70.3–152.9	63.0–144.1	63.4–123.0	45–75	(2)
GPx	U/L	363.7–2116.5	270.5–1023.7	255.5–842.3	288.7–711.0	-	
Haptoglobin	mg/dL	0.09–0.31	0.07–0.36	0.07–0.46	0.08–0.38	< 1.0 §	(27)
NEFAs	mmol/L	0.09–0.72	0.04–0.27	0.07–0.29	0.06–0.35	0.07–0.46	(28)
SOD	U/mL	0.05–4.57	0.04–0.84	0.05–0.62	0.03–0.57	(12.71 ± 0.34) *	(29)
TGs	mg/dL	11.3–93.7	6.6–57.5	8.2–58.8	6.7–58.5	0–14	(2)
TP	g/dL	4.58–7.52	3.95–6.54	3.94–5.93	3.87–6.13	6.74–7.46	(2)
Urea	mg/dL	9.2–32.5	7.5–30.4	7.7–23.8	6.8–18.3	20–30	(2)

ALT, Alanine Aminotransferase; AST, Aspartate Aminotransferase; GGT, Gamma-Glutamyl Transferase; GPx, Glutathione Peroxidase; NEFAs, Non-Esterified Fatty Acids; TGs, Triglycerides; TP, Total Proteins; SOD, Superoxide Dismutase.

§Mean concentration obtained from 10 healthy cows.

**Cow in transition period (60 d before until 100 d after milk).*

TABLE 4 | Reference intervals (RIs) of clinical biochemistry analytes from piglets at 0, 7, and 14 days post-weaning (PW) (at 21, 28, and 35 days of age) and RIs of adult pig from literature.

Analyte	Unit	0 d PW	7 d PW	14 d PW	Adult	
ALT	U/L	16.5–54.2	11.4–47.1	13.6–92.7	31–58	(2)
AST	U/L	27.5–230.4	15.7–122.9	26.6–223.9	32–84	(2)
Cholesterol	mg/dL	58.9–224.3	28.7–85.5	39.8–141.2	36–54	(2)
Cortisol	ng/mL	6.9–206.5	1.4–50.8	1.0–66.7	(29.7 ± 1.0)	(2)
Creatinine	mg/dL	0.50–1.09	0.69–1.34	0.55–1.22	1.0–2.7	(2)
GGT	U/L	18.7–85.1	15.3–70.8	23.1–92.7	10–60	(2)
Glucose	mg/dL	44.0–117.8	56.4–106.3	46.6–113.6	85–150	(2)
Haptoglobin	mg/dL	0.03–1.55	0.08–2.34	0.05–0.18	0.02–3.00 §	(30)
NEFAs	mmol/L	0.15–0.71	0.04–0.48	0.05–0.18	-	
PigMAP	g/L	1.33–6.14	0.51–1.73	0.36–1.39	0.46–2.36 §	(30)
TGs	mg/dL	23.9–76.5	21.0–65.9	22.2–62.0	-	
TNF-alpha	pg/mL	22.0–108.4	51.9–162.4	46.3–202.6	< 10 *	(31)
TP	g/dL	2.67–5.27	2.59–4.52	2.79–5.57	7.90–8.90	(2)
Urea	mg/dL	7.1–21.6	6.1–33.7	5.1–36.0	10–30	(2)

ALT, Alanine Aminotransferase; AST, Aspartate Aminotransferase; GGT, Gamma-Glutamyl Transferase; NEFAs, Non-Esterified Fatty Acids; PigMAP, Pig Major Acute Phase Protein; TGs, Triglycerides; TNF-alpha, Tumor Necrosis Factor alpha; TP, Total Proteins.

§RIs made for mixed-age pigs at 5 w old obtained from 10 commercial farms.

**Undetectable TNF-alpha level for 6 healthy pigs. The lower limit for detection is > 10 pg according to manufacturer.*

The first set of analytical parameters are related to nutrition and metabolism, i.e., indicators of carbohydrate, lipid, and protein metabolism. Many health problems in young animals arise from nutritional unbalances and management procedures.

Secondly, enzymatic markers of oxidative stress were also evaluated. Oxidative stress is a relevant issue in cattle (67) and, specifically, neonates are particularly susceptible because of the challenge due to the transition from the hypoxic intrauterine environment to extrauterine life, susceptibility of neonates to infection, and limited antioxidant protection (68). In piglets, several diseases with severe consequences on health occur concomitantly with oxidative stress (56).

Third, markers of stress (cortisol) and inflammation/infection have been also evaluated. In this sense, the TNF-alpha cytokine and the acute phase proteins Pig-MAP and haptoglobin have been analyzed for piglets and calves, respectively.

This information will help veterinarians and animal science researchers to better practice their

professions by providing them with more adequate diagnostic tools.

ETHICS STATEMENT

Male Holstein calves were managed according to the recommendations of the Animal Care Committee of Institut de Recerca i Tecnologia Agroalimentàries (IRTA) under the approval research protocol FUE-2017-00587321, authorization code 9733. Piglets' procedure has been approved by ethical committee (CEEAH) of Universitat Autònoma de Barcelona (Registration no: 1406 of the Departament de Medi Ambient i Habitatge of the Generalitat de Catalunya).

AUTHOR CONTRIBUTIONS

KY performed the manuscript writing, RI establishment and statistics analyses. FC provided statistical advices. Handling of animals and sample collection was performed by DS-O for piglets and MT for calves. LA, RP, and YS performed sample analyses and AB conducted in the conceptualization, supervised the analytical work, and revised the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2019.00123/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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7. General Discussion

In this section, we will discuss: 1) the applications of "multi-omics" in animal science, which contains the results from Study 1 and Study 2; 2) the nutritional aspects of AA supplementation studied in the present work; and 3) the importance of RI establishment. Finally, some comments on the future of "-omics" in veterinary and animal science will be considered.

Results reported as Annexes are also included in the Discussion.

7.1. “Multi-Omics” approach in animal science

In the present work, “-omics” techniques have been established to study the proteome and metabolome of skeletal muscle to better understand the effects of AA supplementation in preweaning calves. On the other hand, since blood is a very homogenous sample type and is easy for sampling, characterizing the plasma/serum metabolome and proteome can not only reflect the nutritional availability (e.g. level of AA, glucose, FAs, etc.), but also mirror the general function of other organs. Thus we have characterized the skeletal muscle metabolome and proteome of animals from Study 1. We later used metabolomic and proteomic approaches on both skeletal muscle and blood to understand the effects of AA supplementation in Study 2.

7.1.1. Proteomics in skeletal muscle and serum in preruminant calves

AA supplementation were chosen depending on the expected results on animal performance. Thus, the preferred combinations were MKT since they are EAA and could be limiting in certain conditions (Kanjanapruthipong 1998; Drackley 2008; Hill et al. 2009), and LT/Leu as leucine directly acts on muscle protein synthesis, (Wu 2009; Escobar et al. 2010; Laplante and Sabatini 2012).

Results from proteomics indeed indicated that different adaptations occur depending on the combination of supplemented AA. Supplementation with leucine was supposed to have the higher impact on muscle and the proteomic results indeed confirmed this assumption. As it has been described in Annex 1, an increase in BCAA catabolism, mitochondrial oxidative metabolism and antioxidant proteins has been observed.

Supplementation with MKT in skeletal muscle caused few changes in some proteins related to muscle function (increase in MYH6, MYH7, PDLIM7). Although small, these

changes together with the increased Phospho-S6 to S6 ratio (Ruvinsky and Meyuhas 2006) suggested than indeed there was a positive effect of MKT supplementation, according to the mild positive effect on the animal performance.

In contrast, supplementation with FY had almost no effect on the muscle proteome, in correspondence with the biological function of these AA (Wu 2009).

An unexpected result was the identification of several isoforms of SerpinA3 in all the serum proteomic analysis. This was the only protein which was detected in all experiments, either in muscle (MKT and FY) or plasma (LT and Leu). Tendencies were different, since it was found in higher abundance than in the Ctrl group in some cases (MKT and Leu) or lower abundance in others (FY and LT). Since multiple biological functions have been described for SerpinA3 (Billingsley et al. 1993; Kalsheker 1996; Rau et al. 2007), an explanation to these changes is still lacking.

It is interesting to notice that the problem concerning the Leu group was detected in our laboratory before having the information about the animals. The proteomic result, especially in plasma, identified a profile typical of a pathology: increase in acute phase proteins SAA-1,2,3 (Simojoki et al. 2011), fibrinogen as inflammatory marker (Conner et al. 1988; Malyszko et al. 1996) and complement components (Walport 2001; Carroll 2004). When people in the farm were asked, our suspicion was confirmed. Likewise, the differences between the biopsies observed after by PCA analysis in metabolomics were also later confirmed by the distribution of individuals among the biopsy days.

7.1.2. Applications of metabolomics in skeletal muscle and serum

As it was described previously, our preferred combinations were MKT and LT, and results from skeletal muscle and serum metabolomics indicated several expected adaptations in LT but not MKT.

The most obvious changes with LT/Leu groups are the several elevated BCAA as well as their metabolites (acetoacetate and 2-oxoglutarate) in muscle, and the general observation confirmed that a metabolic relationship within BCAA exists and that BCAA is primarily metabolized in the skeletal muscle (Pedroso et al. 2015; Holeček 2018). The serum metabolomic profile also confirms the general elevated BCAA catabolism in LT, but as Leu suffered digestive symptoms, thus the elevation was not obvious. More interestingly, several AA involved in aminoacyl-tRNA biosynthesis showed significant increases and/or significant positive correlations with leucine. This mechanism of leucine being a "trigger" of AA flow and enhancing protein synthesis is not fully understood (Duan et al. 2016; Solon-Biet et al. 2019). In the LT experiment, threonine was not elevated in muscle nor in serum, but serine, which is obtained from threonine, was significantly elevated.

It is also interesting to note that, although not all muscle metabolites were identified, the chemometric data may also provide valuable information and worth further study. For example, several unidentified peak areas that may contain lysine, cadaverine, arginine, leucine and ornithine, as well as some other unassigned metabolites, were significantly elevated. In consequence, it would be interesting to quantify such metabolites to better characterize the effects of AA supplementation.

Attention is drawn by the Leu group as several sugars (glucose, glucose-1-phosphate, glucose-6-phosphate and mannose) together with lactate were decreased in skeletal muscle while some sugars (glucose, galactose, fructose and mannose) were elevated in serum. As it was previously described that the Leu group was biased due to the unrandomized biopsy, the coincidences between the lower ischemia interval during biopsy and lower sugar metabolisms (Baines 2009; Kalogeris et al. 2012) in the muscle; and between the higher sugar availability in the serum and higher local temperature (Suarez and Barrett-Connor 1982; Antunović et al. 2002; Sakatani et al. 2012) follow the physiological logic and are within our expectations.

One of the goals of the present project was to integrate the results from metabolomic and proteomic approaches to one specific condition. In the Study 1 (MKT or FY supplementation) the apparent connection between metabolomic and proteomic results was negligible, due to the few changes found in the proteomic analysis. Nevertheless, in Study 2 (LT supplementation), results from both approaches allowed a global vision since proteins as well of metabolites from the citrate cycle, respiratory chain or OXPHOS were identified, helping to understand the physiological adaptation of these individuals.

In conclusion, using "multi-omics" approach to study the supplementations of several AA revealed that the basal MR diet made from cow milk proteins could provide sufficient nutrition for calf's growth. Although no significant performance effects were observed, metabolic adaptations were identified in the molecular analysis. More importantly, as it is the first time we conduct "multi-omics", we successfully established the "multi-omics" approach in dietary supplementation studies in muscle and plasma samples.

7.1.3. Technical improvements throughout the project

Following the previous study in Study 1, we carried out the "-omics" study of Study 2. Compared with previous study, some of the methodologies were improved:

1) The inclusion of plasma sample: In order to have a deeper understanding of the mechanisms of dietary supplementation at the molecular level, we introduced the study of plasma, a biological fluid that not only can reflect the health status of the individuals, but also mirrors the metabolism of other organs. The results especially from LT group confirmed that the skeletal muscle and plasma reflected different metabolisms, but to some extent they share some similar pathways, which can mutually improve the global interpretation of the biological effects.

2) More reliable control and sample handling on metabolomics: As it was discussed before, metabolomics is sensible enough to detect metabolic adaptation within

seconds, faulty and unskilled handling may introduce unwanted observations that are not related to the scientific question being studied (Dunn et al. 2005). Thus for the first time, we recorded every ischemia interval during the muscle biopsy.

3) The development of robust R code: We succeeded in developing homemade R code for all approaches. In the previous study, several commercial statistics programs were required, and some of the downsides are the expensive subscription, cumbrous analytic procedure, bad cross-platform compatibility, low reproducibility and low automation. As the "-omics" is involved with huge amount of data, developing robust, reproductive, automatic and multi-omics-compatible data manipulation and statistical tool will not only ease the work for the researcher, but more importantly, it reduces the chance of technical error and also more capable for the transform of commercialization for the lab.

4) The availability of online-available bioinformatics: Back in the study of Study 1, the bioinformatics were based on very few online programs. Thanks to the fast development by the bioinformatics community, many free online platforms are readily available. In the Study 2, for the first time we used NMRProcFlow (Jacob et al. 2017), a handy tool for the NMR spectrum manipulation; MetaboAnalyst Joint Pathway Analysis, which allows the researcher to integrate the identified metabolites and proteins to explore the pathways and Reactome, similar to MetaboAnalyst Joint Pathway Analysis, but with better pathway visualization and more libraries of pathway data.

7.1.4. Methodological problems and the importance of the biopsy quality

In the study on the effects of supplementation with leucine and threonine (Annex 1), we conclude that the supplementation with these AA affected the metabolism in several ways which in general mirrored the global improvement of performance under the effect of diet. Problems arisen in the Leu group made evident the importance of a proper experimental design and biopsy collection. An important question is whether the biopsy could have affected the conclusions of the LT study. In this case, calves from both treatment groups were well randomized and the effects of the biopsy, if present, would have not influenced our conclusions. Nevertheless, a PCA-based analysis of the influence of treatment and biopsy was performed (Figure 7). PCA results indicate that the biopsy day clearly separates the animals biopsied in the 1st and 2nd day, but since the individuals in the Ctrl and LT were well randomized, both groups were equally affected. This is easily visualized because the effects of biopsy separate groups between right and left in muscle and top and bottom in serum while the effects of treatment separate groups between top and bottom in muscle and left and right in serum. In the case of Leu however (Annex 1: Figure 10), since the whole Leu group is also the 3rd group, the effect by the biopsy is inseparable from the treatment.

In summary, in this study we established reliable, robust and reproducible method for the “-omics” study in dietary supplementation, which also may potentially be applied in other studies such as disease identification and biomarker exploration. Also, this study sets an example of inappropriate sample handling that can give advises for all researches who participate similar research.

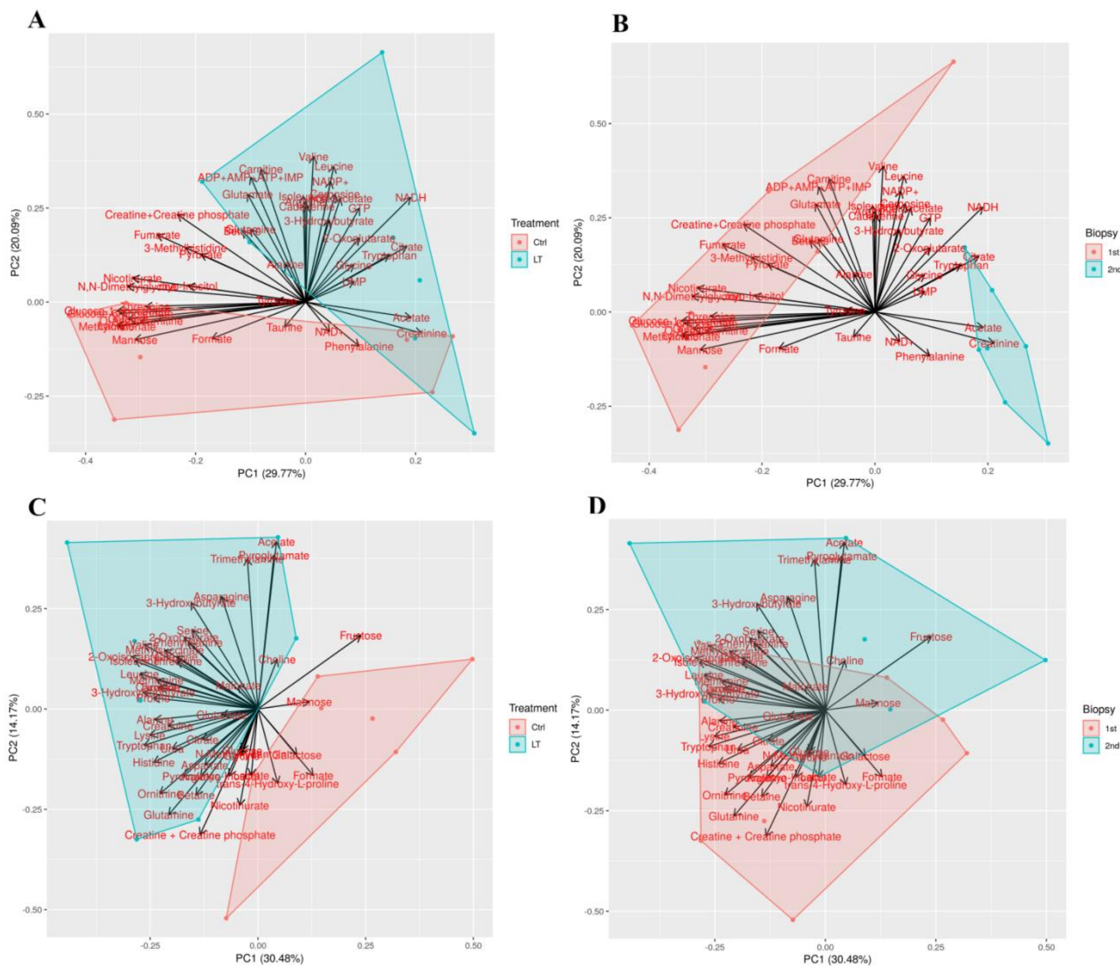

Figure 7. PCA with loadings of muscle (A and B) and serum (C and D) according to treatment (A and C) and biopsy (B and D) of quantified metabolites in Ctrl - LT (1st - 2nd biopsy) comparison.

7.2. The nutritional aspects of AA supplementation

In the present works, we studied several AA, and most of the results were within our expectations. From the perspective of biochemistry, the most obvious results were obtained from LT supplementation in Study 2, where upregulated/increased BCAA degradation, lipid metabolism, threonine degradation, citrate cycle, amino acid biosynthesis and Phospho-S6/S6 ratio were observed. On the other hand, despite the sickness in the leucine-supplemented group, leucine degradation seemed to be unaffected. In MKT supplementation, the mild increase in muscle contraction-associated proteins and serum creatinine may suggest some improvement in muscle function and supplementation

with FY, or with GP did not yield relevant changes. As it was previously described, phenylalanine and tyrosine contribute more to the neurological development and to the author's best knowledge, no study had pointed out their relationship with muscle growth. Lastly, glycine and proline are not EAA for calves, thus the dietary effect is small.

As mentioned above, a plausible explanation of the mild effects of AA supplementation is the use of a milk protein-based milk replacer (MPMR). Although a MPMR has not the same composition than whole milk, its AA profile is similar to whole milk, which meets the majority of AA requirements (Table 3). Indeed, many of the published studies that showed positive effects of AA supplementation used non-animal protein for MR, where methionine, threonine or lysine are commonly known to be limiting (Gorrill, A.D.L. and Nicholson 1969; Kanjanapruthipong 1998; Wang et al. 2010).

Table 3. EAA requirement (g/d) for calf (50 – 58 kg LW) growing at 0.25 kg/d and composition of EAA in whole milk.

AA	EAA requirements	Cow's milk
Methionine	2.1	3.6
Cystine	1.6	1.1
Lysine	7.8	11.7
Threonine	4.9	6.4
Valine	4.8	9.2
Isoleucine	3.4	8.2
Leucine	8.4	13.6
Tyrosine	3.0	7.1
Phenylalanine	5.4	6.8
Histidine	3.0	3.8
Arginine	8.5	5.0
Tryptophan	1.0	2.0

Data collected from Williams AP. Amino acid requirements of the veal calf and beef steer. In: D'Mello JPF, editor. Amino acids in farm animal nutrition. Wallingford, Oxon (UK): CAB International, 1994. p. 329–49. (D'Mello 1994: 329–349)

7.3. The importance of reference interval establishment

Health control through several clinical chemistry analytes is still the most efficient and economical way to investigate the general health of the herd. For this, robust and age-specific RIs are needed. A reliable way of establishing clinical biochemistry RIs is to use data from a large-scale disease-free animal groups with proper statistics (CLSI 2010). Thus, we gathered results from several amino acid supplementation studies. To optimize the number of animals to be included for the RI calculation, a first analysis was performed based on the inspection of distribution histograms of each analyte to prove that there were not apparent differences among treatments. After finding out that nearly all analytes merged from 3 studies followed normal distribution or followed the typical distribution patterns for some certain analytes, we were able to use all the data and succeed in establishing RIs specifically for different ages in young animals (Publication 2). The establishment of age-specific RIs will benefit our understanding of the general status of these animals and provide veterinarians important information for the daily work.

To conclude, RIs are an important tool for veterinarians. The selection of a suitable healthy population defined by specific criteria and with a high number of individuals is a crucial point to elaborate robust RI of wide applicability.

7.4. The Future of "Multi-Omics" in Farm Animal and Veterinary Science

The "-omics" studies have gained a lot of success in farm animal and veterinary science thanks to the technological advances. While the main focus of genomics is the improvement of animal breeding, proteomics and metabolomics, as the "-omics" cascade illustrates (Figure 3), can study the ongoing and recent-happened biological process. Due to the characteristics of metabolomics and proteomics, wide applications have been made from nutritional, productive and pathological problems.

However, most of the studies focus on one "-omics" approach (either metabolomics or proteomics), and as our understanding in biological process and molecular functions go deeper, a sole "-omics" may not provide enough information. Consequently, more integrative "-omics" or "multi-omics" approaches have become popular as a combination of proteomics and metabolomics could not only provide an holistic view from the intact biomolecules, but also can potentially validate and complement one another (Liu et al. 2013).

The "multi-omics" approach has been seldomly used in farm animal and veterinary science and only two reports have used integrative metabolomic and proteomic approaches: 1) the characterization of *longissimus lumborum* muscle quality of Casertana and Large White pigs (D´Alessandro et al. 2011; D'Alessandro and Zolla 2013) and 2) the study of cow milk properties during the dry period (Lu et al. 2013). Furthermore, a more specific term of interactive and integrative metabolomics and proteomics, i.e. foodomics, was introduced in 2009 (Cifuentes 2009) for food analysis using "multi-omics". This will also be beneficial for animal science as one of the scopes is the improvement of food quality and efficiency. In the authors opinion, the future of metabolomics and proteomics in farm animal and veterinary science may be focused on these following parts:

1) A better development of technical standards for each "-omics": As it was reviewed for proteomics (Almeida et al. 2014), despite all "-omics" are closely linked, each one has its own distinct analytical approach. Thus it is important that applications and technical standards can develop, based in deeper expertise of researchers in animal science.

2) The building of animal-specific databases: Despite many platforms such as Uniprot and KEGG offer entries for different organisms, or some animal specific database are built like Bovine Metabolome Database (BMDB) and Livestock Metabolome Database (LMDB), the availability, benchmark and user-friendliness of database for animal and veterinary science are still far low compared with their "human counterpart".

3) A closer lab-to-lab collaboration for better transdisciplinary approaches: It is not necessary and almost impossible for a lab to be able to master all kinds of disciplines/techniques, however, by promoting cooperation with different labs of different scopes, it can better contribute to the future of the "multi-omics". As an example, the author of the thesis not only is benefited the proteomics techniques from the own lab based in Spain, but also was facilitated in lab collaborations with metabolomics lab in ITQB, Portugal, and proteomics lab in Faculty of Veterinary Medicine, University of Zagreb, Croatia.

8. General Conclusions

1) The studies of the effects of amino acid supplementations in milk replacer made from milk protein on unweaned calves using multiple approaches showed good sensitivity and reliability.

2) The supplementation with methionine, lysine and threonine (MKT) in milk replacer caused mild performance improvement of the preruminant calf. Despite the metabolomic analysis in skeletal muscle did not reflect significant changes, Phospho-S6/S6 ratio, serum creatinine and serum insulin were higher in the MKT group. Several muscle proteins (MYH and PDLIM7) related to muscle functionality were found at higher abundance in the proteomic analysis.

3) The supplementations of glycine and proline (GP) or phenylalanine and tyrosine (FY) in milk replacer did not alter the general performance but showed some metabolic adaptations in the metabolomic analysis, which were mainly limited to higher aerobic metabolism possibly due to the extra amino acids. The proteomic analysis of skeletal muscle of the FY group did not yield relevant identifications.

4) The supplementations of leucine and threonine (LT) in milk replacer caused a tendency to increased average daily gain and significantly elevated Phospho-S6/S6 ratio in skeletal muscle.

5) The metabolomic and proteomic analysis of skeletal muscle of calves supplemented with leucine and threonine (LT) revealed increased BCAA metabolism, higher citrate cycle activity and OXPHOS. Antioxidant proteins were found at higher abundance, possibly as a compensatory mechanism.

6) The metabolomic and proteomic analysis of plasma of calves supplemented with leucine and threonine (LT) mainly revealed higher detoxifying proteins (PON1) and apolipoproteins. Identified metabolites indicated increased metabolism of BCAA, butyrate and/or ketone bodies. Leucine was increased in plasma, as well as serine and glycine, probably derived from threonine.

7) The metabolomic and proteomic analysis of plasma and skeletal muscle of calves supplemented with leucine (Leu) revealed that animals had been ill and that there was an experimental flaw in the randomization of muscle biopsies.

8) Supplementation of milk replacer with MKT or LT potentially improves the performance and muscular metabolism of preruminant calves. On the other hand, the basal control milk replacer based on milk protein can possibly cover the majority of the nutritional requirements for growth.

9) Reference intervals for serum clinical chemistry parameters were obtained for unweaned calves at different ages and piglets in the post-weaning period, by using a large healthy population. The main variable was age whereas no major trial- or sex-biased differences were noticed.

10) In calves, ALT, AST, GGT, GPx, SOD, NEFAs, triglycerides, glucose, creatinine, total protein, and urea were greatly elevated at 24 h, some of the elevations were due to the passive transfer from colostrum. In piglets, cholesterol, triglycerides, NEFAs, cortisol and Pig-MAP were increased and TNF-alpha decreased at 0 days post-weaning compared with other times.

9. Annexes

9.1. Annex 1: Metabolome and Proteome Changes in Skeletal Muscle and Blood of Preruminant Calves with Diet Supplemented with Leucine and Threonine

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Abstract

Background

In preruminant calves, both leucine and threonine play important roles in muscle protein biosynthesis. The use of proteomics and metabolomics can improve the understanding of how dietary AA affect growth and metabolism.

Methods and results

Metabolomics, proteomics and clinical chemistry were used to assess the effects of leucine and threonine supplementations added to milk replacer on 14 newborn Holstein male calves: 7 were fed a control diet (Ctrl) and 7 were fed the same Ctrl diet supplemented with 0.3% Leucine and 0.3% Threonine (LT). The experiment lasted 7 weeks, and blood and semitendinosus muscle were collected for analyses. Integrated metabolomics and proteomics showed that branched-chain amino acids (BCAA) degradation and mitochondrial oxidative metabolism (citrate cycle, respiratory chain) are the main activated pathways in muscle. Energy metabolic substrates would be BCAA derivatives and lipid mobilization. The deleterious effects of activated oxidative phosphorylation are balanced by the upregulation of antioxidant proteins. An increase in protein synthesis is indicated by elevated Aminoacyl-tRNA biosynthesis and increased Phospho-S6 to S6 ratio in skeletal muscle.

Conclusion

LT group showed greater BCAA availability and mitochondrial oxidative activity; as the muscle cells undergo greater aerobic metabolism, antioxidant defenses were activated to compensate possible cell damage.

Keywords

Amino acid; calf; leucine; threonine; muscle; plasma; metabolomics; proteomics, serum clinical chemistry

Background

Amino acids (AA) are important for the growth of preruminant calves as they are pivotal for tissue development, endocrine and immune function (Li et al. 2007; Wu 2009). Leucine is an essential AA and one of the branched-chain AA (BCAA) that has been studied in several species due to its function in promoting protein synthesis through the mammalian target of rapamycin (mTOR) pathway in skeletal muscle (Escobar et al. 2005; Yin et al. 2010). Leucine also enhances energy homeostasis through increasing fatty acid oxidation and it provides skeletal muscles with an increased flux of lipids, supplying energy and intermediary substrates to support protein synthesis (Pedroso et al. 2015). Leucine from dietary protein can bypass metabolism in the liver, which results in a rise of plasma leucine levels and the activation of leucine signaling in peripheral tissues in response to a meal (Duan et al. 2016) and, among these tissues/organs, the skeletal muscle is the main site for BCAA oxidation (Bonvini et al. 2018). Threonine is another essential AA that is often considered limiting in milk replacers (MR) for calves (Kanjanapruthipong 1998). It participates in the synthesis of several other important AA such as glycine and serine which are involved in the production of collagen and muscle tissue, and it also plays a central regulatory role in the immune system as it is a major component of intestinal mucins and plasma γ-globulins (Li et al. 1999; Li et al. 2007).

To understand the role of nutritional compounds at the molecular level (Herrero et al. 2012) and to analyze the coordination of multiple systems including the immune, digestive, and endocrine, the identification of large sets of proteins and metabolites is needed. As a result, “-omics” approaches have gained popularity in animal science and nutrition. The combination of proteomics and metabolomics in food and nutrition science, more recently named as “foodomics” (Cifuentes 2009), has been shown to be a more suitable approach compared with other “-omics” like genomics and transcriptomics (Herrero et al. 2012).

The objective of the present work is to determine the effects of MR supplemented with leucine and threonine fed to preruminant calves from 6 days to 7 weeks of age, in skeletal muscle and in plasma using foodomics approaches to obtain the panorama of the effect

of these AA on preweaning calves. Furthermore, the activation of the mTOR pathway was studied in skeletal muscle by determining the phosphorylation of S6 ribosomal protein, a key event in protein synthesis.

Methods

Animal Housing, Diet Management and Dosage Information

Animal management was according to the recommendations of the Animal Care Committee of Institut de Recerca i Tecnologia Agroalimentàries (IRTA) under the approval research protocol FUE-2017-00587321, authorization code 9733.

Fourteen Holstein male calves (weighing 41.9 ± 2.15 kg of body weight (BW) at age of 5.6 ± 0.67 d) were obtained from Granja Murucuc (Gurb, Barcelona, Spain) and housed individually at IRTA Torre Marimon (Caldes de Montbui, Barcelona, Spain) and fed a milk protein-based MR twice a day. The MR contained 24% crude protein (based in skimmed milk protein and whey protein concentrate) and 20.3% fat, on DM basis. Calves were fed 4 L/d at 15% DM concentration from 1 to 7 days of experiment. Then, MR volume was increased to 6 L/d from 8 to 49 days of study, and it was reduced to one single feeding of 3 L/d until 56 days of study, when animals were weaned. Calves were divided into control (Ctrl, n=7) and Ctrl supplemented with 0.3% leucine and 0.3% threonine (LT, n=7) groups according to the AA profile in the MR formula. Ingredients and chemical composition are shown in Additional file (Table S 1). All AA were provided by Livzon Group (Fuxing Pharmaceutical, Ningxia (China)). All animals had free access to water, and restricted access to a pelleted concentrate starter feed to minimize the impact of concentrate feeding on AA supply. The starter feeding program is shown in Additional file (Table S 2). Chopped barley straw was offered *ad libitum* during this study.

Sample Collection

At 53.6 ± 0.67 days of age and 81.8 ± 3.49 kg of BW, blood was obtained from the jugular vein 4 h after the morning MR feeding, and plasma and serum were obtained by centrifugation at 1,500 g for 10 min and stored in aliquots at −80 °C. Biopsies were obtained from semitendinosus muscle as described in (Yu et al. 2018) and kept at −80 °C.

Prior to assay, frozen muscle tissues were ground into fine powder with liquid nitrogen and at least two ~0.5 g aliquots were made and kept in a 15 ml polypropylene at -80°C.

Serum Clinical Chemistry and Plasma AA

Serum biochemistry analytes were analyzed with the Olympus AU400 analyzer. Methods, reagents and intra-assay CV (%) were previously reported (Yu et al. 2019). Plasma AA were determined by HPLC as described in (Yu et al. 2018) with modifications. An Elite LaCHrom (Hitachi, Tokyo, Japan) equipped with an UV detector (Hitachi L-24200, Tokyo, Japan) with a Novapak C18 column (300mm x 3.9 mm) from Waters were used. The flow rate was 1.0 ml/min, and the column temperature was kept at 42°C. The injection volume was 10 µL and the detection wavelength was set at 254nm. The solvent system consisted of two eluents: (A) 140 mM sodium acetate trihydrate, 12 mM TEA and 1 mg/L EDTA at pH 5.02 and (B) 60% acetonitrile/40% water. The software EZChrom Elite system V3.1.7 (Agilent, Santa Clara, CA, USA) was used for system control and data acquisition.

Proteomic Analysis

Protein Extraction and Quantification

One aliquot of semitendinosus muscle fine powder in a 15 mL tube was sonicated in 3 mL extraction buffer (137 mM NaCl, 50 mM HEPES, 2% SDS, 1% NP-40, 10% glycerol, 2 mM Na_3VO_4, 100 mM NaF and 1% protease inhibitor cocktail (Sigma), pH 7.4) at 40% amplitude following the sequence of 10 s on and 5 s off, three times on ice. The homogenate was centrifuged at 12,000 g for 15 min at 4°C and the supernatant (muscle extract) was kept. Protein concentration was determined by the Bradford method. Samples were kept at -80°C until analyses.

Protein Immunoblot for S6 and Phospho-S6 in Muscle

All antibodies were purchased from Cell Signaling Technology (MS, USA). 15 µg of muscle extract per sample were subjected to 12% SDS-PAGE and transferred to PVDF membranes. Total protein staining (TSP) by Ponceau S was performed for band intensity normalization on membrane after transfer. The membranes were then immunoblotted with primary antibodies (1:2000) against S6 ribosomal protein (#2217) and phospho-S6 ribosomal protein (#4858). The dilution for secondary antibodies were 1:5000 and 1:2000 for S6 and phospho-S6, respectively. Immunoblot bands were processed using Multi Gauge software (Fujifilm, Tokyo, Japan). For normalization, each sample was normalized by its band density divided by its normalization factor f, which was calculated taking into account the mean TPS density of all gels and the mean TPS density of the gel where the sample was placed. Immunoblots were repeated three times with a random distribution of samples between gels.

Sample Preparation for High Resolution LC-MS/MS

Muscle samples were processed using Filter-Aided Sample Preparation (FASP) with some modifications (Wiśniewski et al. 2009). In short, for each sample and internal standard (pooled sample), 35 µg of proteins were diluted with UA buffer (8 M urea in 0.1 M Tris/HCl, pH 8.5) to 200 µL, added to the filter unit (NMWL of 10kDa, Microcon YM-10), subsequently centrifuged at 13,000 g for 15 min at 20 ^{0}C and washed by adding 200 µL of UA buffer followed by centrifugation. Samples were alkylated with 100 µL iodoacetamide (0.05 M IAA in UA buffer) for 20 min in darkness at RT. Additional series of washes with 100 µL of UA buffer (twice) and 100 µl TEAB (0.1 M triethylammonium bicarbonate) (twice) with subsequent centrifugation was conducted. Overnight digestion at 37°C was performed with 50 µL of 0.1 M TEAB containing 2% trypsin (v/v). Tryptic peptides were eluted by centrifugation at 13,000 g for 10 min, and washed with 50 µL of buffer (50% acetonitrile and 50% 0.1 M TEAB, v/v). Collected flowthrough was dried and used for TMT labelling. Before labelling, pellets were dissolved in 50 µL of 0.1 M TEAB.

Plasma-EDTA samples were prepared as described (Horvatić et al. 2019) with some modifications. Briefly, 35 µg of proteins per sample or internal standard were diluted with 100 µL 0.1 M TEAB containing 1% SDS, reduced, alkylated and acetone precipitated overnight. Protein pellets were collected by centrifugation and subsequently dissolved in 50 µL TEAB containing 1 µg trypsin for overnight digestion at 37°C.

Tandem Mass Tag (TMT) Labelling for Muscle and Plasma Proteins

Thermo Scientific™ TMTsixplex™ Isobaric Label Reagent Set (#90061) was used for peptide labelling. All reagents were prepared as reported previously (Martinez-Subiela et al. 2017; Horvatić et al. 2019). In short, for the labelling, 19 µl of reconstituted TMT reagent was added to each tryptic digest and incubated for 1 h, RT. Internal standard was labelled with the TMT *m/z* 126 while other tags were randomized. The reaction was quenched by adding 8 µl of 5% hydroxylamine to each sample and incubating for 15 min. For each sixplex, samples were combined at equal amounts, aliquoted, dried in a vacuum centrifuge and kept in -80°C for future analysis.

High Resolution LC-MS/MS Analyses and Bioinformatics

Mass spectrometric analysis was conducted on an Ultimate 3000 RSLCnano system (Dionex, Germering, Germany) and Q Exactive Plus mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) as reported (Horvatić et al. 2019). In short, after desalting on the trap column, TMT-labelled peptides were separated on the analytical column (PepMap™ RSLC C18, 50 cm x 75 µm) using a 120-min linear gradient of 5 - 45% mobile phase B (0.1% formic acid in 80% ACN) at the flow rate of 300 nL/min. Peptides were introduced into the mass spectrometer using a 10 µm-inner diameter SilicaTip emitter (New Objective, USA) within a nanospray Flex ion source. For DDA analysis Top-8 method was used.

Proteome Discoverer (version 2.0, Thermo Fisher Scientific) was used for subsequent peptide identification and relative quantification. SEQUEST algorithm database search against *Bos taurus* FASTA files (NCBI database, 7/12/2017, 46105 entries) was performed. Proteins with at least two unique peptides and 5% FDR were reported as validly identified. Furthermore, for reporter ion-based protein quantification, internal standards were used to compare the data between the TMT sixplexes. For bioinformatics, NCBI accession number (GI number) was converted to gene symbol by Biological Database Network (https://biodbnet-abcc.ncifcrf.gov/db/db2db.php) and protein function was explored with PANTHER (http://pantherdb.org/).

Validation

PON1 esterase activity was measured in plasma-heparin samples by analyzing the hydrolysis of phenyl acetate (arylesterase activity, ARE), as previously described (Gaita et al. 2010).

Metabolomic Analysis

Muscle and Serum Extractions

The extraction of the aqueous metabolites from muscle powder was performed as described (Yu et al. 2018). For serum, 300 µl of each sample were extracted by methanol protein precipitation method as described (Gowda et al. 2015), with the exception that dried samples were resuspended with 600 µl of NMR buffer.

Data Acquisition, Processing and Analyses

For muscle, data acquisition method and parameters, data processing protocols and analyses were as described elsewhere (Yu et al. 2018). For serum sample, 128 scans and

8 dummy scans were applied. All raw data have been deposited to the EMBL-EBI MetaboLights database (https://www.ebi.ac.uk/metabolights/MTBLS1045).

Spectral Profiling for Chemometrics

All 1r data from either biological sample collected from Bruker were uploaded to NMRProcFlow 1.2 Online spectral processing tool interface (Jacob et al. 2017). Spectra were calibrated referring to the ppm and some peaks were aligned. H_2O peak was zeroed. For bin data, peaks were bucketed following previous knowledge of assignment. Peak intensities for each sample were normalized by its own weight for muscle, no normalization for serum, and centered-scaled for both.

Statistics

All statistical analyses were performed using R (version 3.5.2) under RStudio (version 1.2.1335), "dplyr" (Wickham et al. 2019), "reshape2" (Wickham 2017), "ggpubr" (Kassambara), "ggplot2" (Wickham 2016), "corrplot" (Taiyun et al. 2017) and "openxlsx" (Walker 2019) packages were used for data manipulation, statistics and graphic processes. Performance and intake data were analyzed using a mixed-effects model accounting for the fixed effects of treatment, time of measurement, and their 2-way interaction, and the random effect of calf. Time entered the model as a repeated measure using an autoregressive covariance matrix. Initial BW was used as covariates in the model. For statistical difference (univariate analyses) in the rest of the results, a Wilcoxon test was used, p between 0.05 and 0.1 was inspected, while < 0.05 was considered significant. For MS-proteomics, fold change between two groups was calculated using the function of log2(Mean(LT)/Mean(Ctrl)). Absolute value of fold change ≥ 0.20 and $p < 0.05$ were considered significant. Some proteins/metabolites with p between 0.05 and 0.1 or/and with fold changes between 0.10 and 0.20, but showing significant correlations with representative metabolites, were also included in the analysis.

For univariate analyses in metabolomics, significant chemical shifts from chemometrics were inspected to assist the identification. For multivariate analyses, principle component analyses (PCA) with loadings of both quantified and chemometric data were plotted. The correlation plot in metabolomic results was made and Pearson r was used as distance measurement.

Proteomic-Metabolomic Interactive Analyses

For significant proteins and metabolites, their official gene symbols (proteins) and compound names (metabolites) were analyzed using Reactome (version 69 released on June 12, 2019) (https://reactome.org/) (Fabregat et al. 2018) and Joint Pathway Analysis provided by MetaboAnalyst. *Homo sapiens* was selected as species since *Bos taurus* was not available in both databases. The hypergeometric test, degree centrality and gene-metabolite pathways options were used for enrichment, topology and pathway analyses, respectively, in MetaboAnalyst. For unannotated proteins, literature and Uniprot were referred.

Results

Performance results

No differences were observed in performance data, being the average daily gain (ADG) (kg/d) (mean ± SEM) of Ctrl calves 0.89 ± 0.054 and LT calves 0.96 ± 0.054. The detailed results are shown in Additional file (Table S 3).

Blood biochemistry and AA profile

Analytes with significant differences between groups are shown in Table 4.Decreased GPx and a tendency to increase for triglyceride and cholesterol were observed in the LT group. There was an increase in plasma valine and isoleucine, and a tendency to increase plasma leucine, histidine and phenylalanine concentrations. All analytes were within the reference interval (Yu et al. 2019).

Table 4. Concentrations (mean ± SE) of significantly different serum analytes and plasma AA between Control (Ctrl) and Leucine/Threonine-supplemented (LT) groups.

Analyte	**Ctrl**	**LT**	***p*-value**
Serum Clinical Chemistry			
Cholesterol (mg/dL)	60.29 ± 4.8	84.56 ± 9.48	0.055
Triglyceride (mg/dL)	423.84 ± 38.22	321.89 ± 16.71	0.074
Glutathione Peroxidase (GPx) (U/L)	19.69 ± 3.26	40.26 ± 11.21	0.041
Plasma AA (µM)			
Histidine	71.45 ± 4.53	92.29 ± 9.06	0.055
Valine	212.47 ± 11.46	280.3 ± 34.54	0.021
Isoleucine	94.72 ± 3.52	127.28 ± 13.37	0.007
Leucine	164.74 ± 10.36	220.37 ± 28.84	0.055
Phenylalanine	85.11 ± 3.27	99.95 ± 9.03	0.074

Proteomics in skeletal muscle and plasma

The differentially abundant proteins in muscle and plasma from both groups are shown in Table 5 and Table 6, respectively.

Table 5. Proteins with significantly differential abundances in skeletal muscle between Control (Ctrl) and Leucine/Threonine-supplemented (LT) groups identified after TMT labelling.

Gene symbol (*Bos taurus*)	Protein name	*p*-value	Fold change (LT *vs* Ctrl)
HADHA	Trifunctional enzyme subunit alpha, mitochondrial	0.037	0.37
PRDX1	Peroxiredoxin-1	0.030	0.26
SOD2	Superoxide dismutase 2, mitochondrial	0.043	0.26
SLC25A4	ADP/ATP-carrier protein	0.041	0.21
VCP	Transitional endoplasmic reticulum ATPase	0.041	0.20
COX2	Cytochrome c oxidase	0.030	0.19
COX5A	Cytochrome c oxidase, subunit 5A, mitochondrial	0.030	0.19
NDUFAB1	Chain U, Acyl Carrier Protein, mitochondrial	0.041	0.19
ACO2	Aconitase 2, mitochondrial	0.071	0.11
GYS1	Glycogen synthase 1 protein	0.025	-0.29
RTN2	Reticulon-2	0.010	-0.25

Table 6. Proteins with significantly differential abundances in plasma between Control (Ctrl) and Leucine/Threonine-supplemented (LT) groups after TMT labelling.

Gene symbol (*Bos taurus*)	Protein name	*p*-value	Fold change (LT *vs* Ctrl)
PON1	Paraoxonase 1	0.004	0.41
APOA2	apolipoprotein A-II	0.009	0.35
CRP	C-reactive protein	0.025	0.35
APOC3	Apolipoprotein C-III	0.015	0.33
F2	Prothrombin	0.041	0.2
SERPINA3-5	Serpin A3-5	0.018	-0.44
SERPINA3-6	Serpin A3-6	0.018	-0.44
ITIH4	Inter-alpha-trypsin inhibitor heavy chain H4	0.015	-0.34
SERPINA3-8	Serpin A3-8	0.006	-0.31
FN1	Fibronectin	0.034	-0.24
SERPINA3-7	Serpin A3-7	0.041	-0.2
SERPINA3-2	Serpin A3-2	0.013	-0.19
SERPINA3-1	Serpin A3-1	0.018	-0.19

In muscle, nine proteins were found with higher abundance (HADHA, PRDX1, SOD2, COX2, COX5, NDUFAB1, ACO2, SLC25A4 and VCP) and two at lesser abundance (GYS1 and RTN2) in treated animals in comparison to controls. For increased proteins, GO analysis indicates the positive regulation of ATP biosynthetic process (GO:2001171) and the removal of superoxide radicals (GO:0019430) as the most relevant biological processes and the antioxidant activity (GO:0016209) as the first molecular function. Complete GO analysis is shown in Additional file (Table S 4).

In plasma, five proteins were found with higher abundance (APOA2, APOA3, CRP, F2 and PON1) in treated animals. The GO analysis for biological process showed that three of them (APOA2, APOC3 and PON1) corresponded to regulation of cholesterol transport (GO:0032374) and chylomicron assembly (APOA2, APOC3) (GO:0034378). CRP and F2 are associated to the acute phase response (GO:0006953). The GO analysis for molecular function showed three proteins corresponding to lipoprotein receptor binding (APOA2, APOC3 and PON1) (GO:0070325). Proteins with lower concentration in the LT group included 6 isoforms of the SERPINA3 protein (SERPINA3-1, -2, -5, -6, -7 and -8), ITIH4 and FN1. All eight proteins correspond to the molecular function GO peptidase regulator activity (GO:0061134) and all except FN1 to serine-type endopeptidase inhibitor activity (GO:0004867). Complete GO analysis is shown in Additional file (Table S 5).

For validation of proteomic results, plasma PON1 was determined and found elevated with a statistical tendency ($p = 0.06$) in LT (125.55 U/L ± 9.70) compared with Ctrl (88.16 U/L ± 11.58).

Phosphorylation status of S6 in skeletal muscle

The ratio of Phospho-S6/S6 in muscle was significantly increased in LT compared with Ctrl calves (Fig. 1).

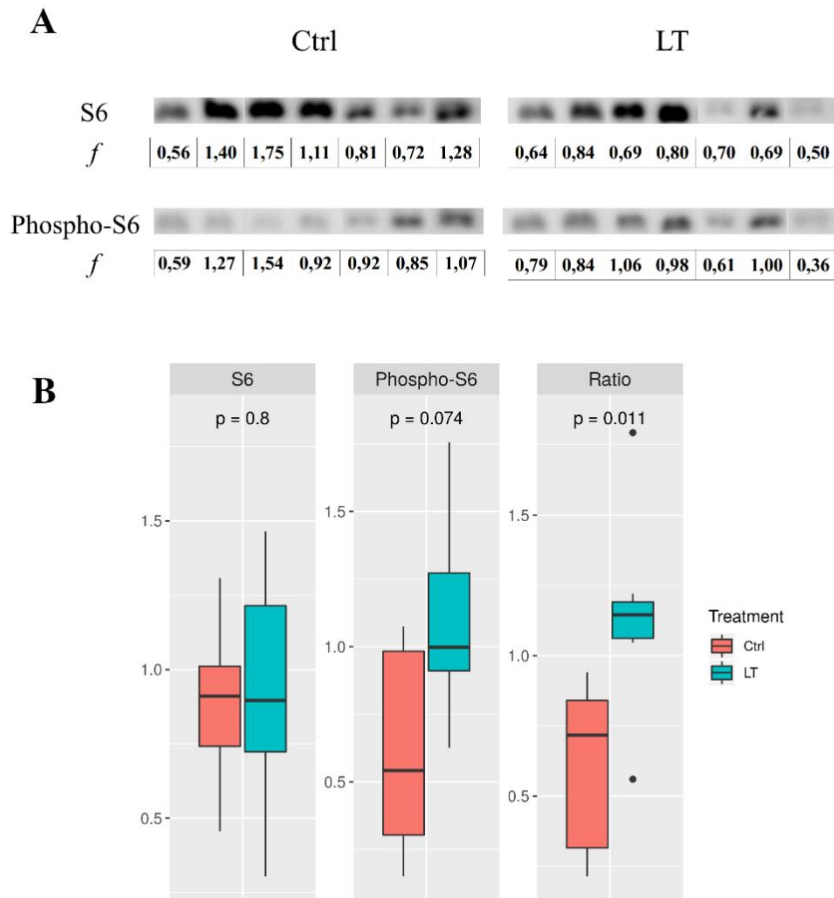


Fig. 1. A. Western blot bands of S6 and Phospho-S6 with their corresponding normalized total protein staining factors (*f*) factors; B. Boxplots of S6, Phospho-S6 and the ratio of Phospho-S6/S6, with p. Mean intensities were scaled to 1.

Metabolomics in skeletal muscle and serum

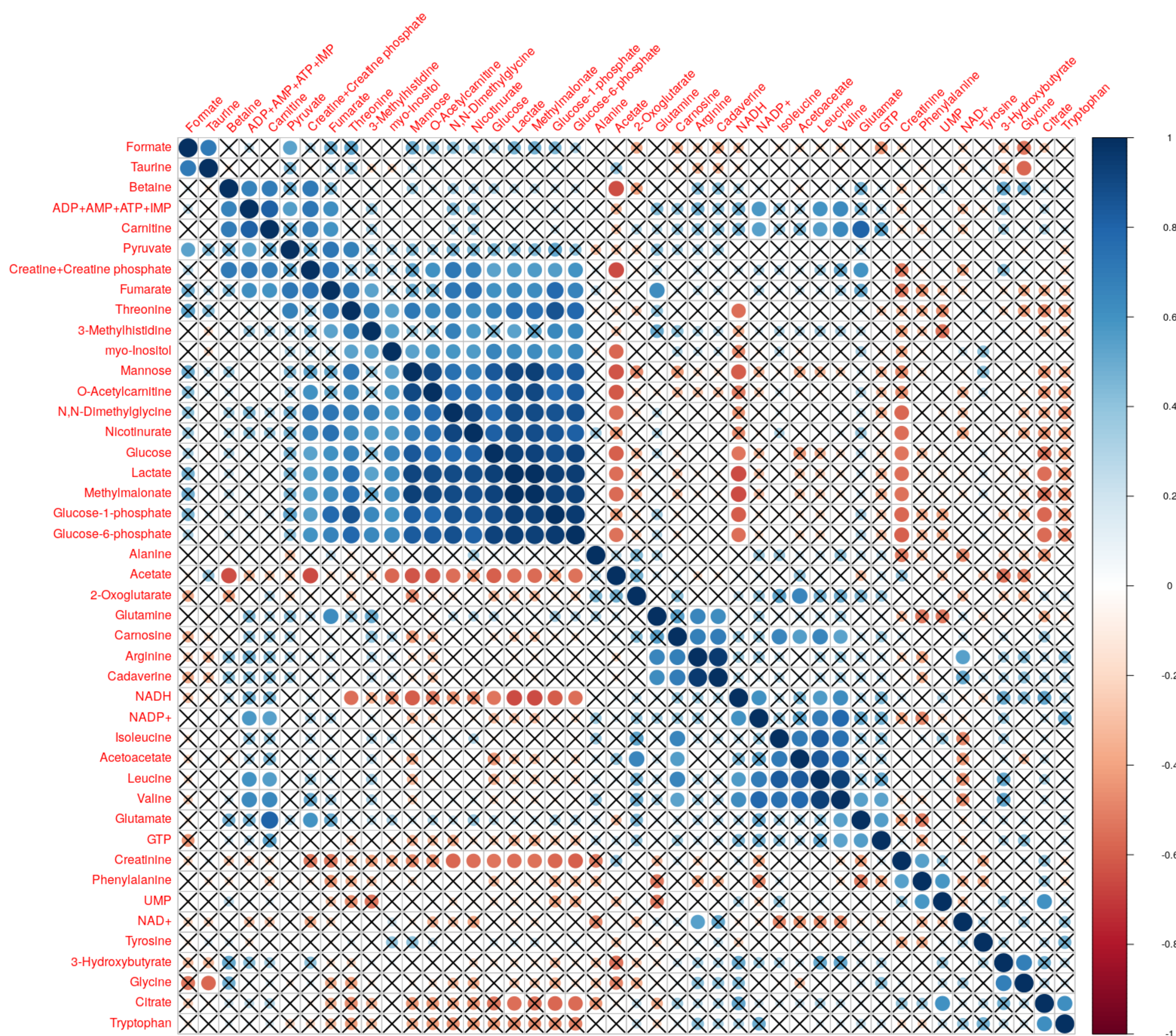
Quantified metabolites of muscle and serum with significant differences are shown in Table 7 and Table 8, respectively. Correlation plots are shown in Fig. 2.

Table 7. Metabolites in skeletal muscle with significantly different concentration between Control (Ctrl) and Leucine/Threonine-supplemented (LT) groups identified by NMR-metabolomics (mean ± SE, µMol/100g muscle).

Metabolite	**Ctrl**	**LT**	***p*-value**
2-Oxoglutarate	0.80 ± 0.07	1.22 ± 0.10	0,021
Acetate	1.08 ± 0.12	1.45 ± 0.13	0,074
Acetoacetate	0.52 ± 0.04	0.67 ± 0.05	0,055
Carnitine	24.71 ± 1.24	27.53 ± 1.28	0,097
GTP	0.50 ± 0.03	0.62 ± 0.02	0,015
Isoleucine	0.43 ± 0.09	0.54 ± 0.05	0,097
Leucine	0.51 ± 0.05	0.70 ± 0.07	0,03
NADH	0.21 ± 0.04	0.35 ± 0.05	0,041
NADP+	0.06 ± 0.00	0.10 ± 0.01	0,03
Valine	0.94 ± 0.06	1.30 ± 0.11	0,03

Table 8. Metabolites in serum with significantly different concentration between LT and Ctrl identified by NMR-metabolomics (mean ± SE, µM).

Metabolite	**Ctrl**	**LT**	***p*-value**
2-Oxobutyrate	6.93 ± 0.71	10.00 ± 1.15	0.072
2-Oxoisocaproate	4.09 ± 0.26	5.66 ± 0.30	0.006
3-Hydroxybutyrate	44.73 ± 4.10	71.01 ± 9.57	0.021
3-Hydroxyisobutyrate	9.34 ± 0.79	11.69 ± 0.57	0.014
Alanine	88.86 ± 3.31	102.76 ± 7.66	0.055
Asparagine	25.24 ± 1.42	30.17 ± 1.37	0.035
Carnitine	5.76 ± 0.25	8.10 ± 0.48	0.002
Citrulline	39.16 ± 3.10	49.77 ± 1.39	0.026
Creatinine	15.86 ± 1.40	19.57 ± 1.15	0.084
Formate	35.59 ± 2.37	30.60 ± 1.46	0.097
Fructose	119.11 ± 10.24	83.50 ± 11.51	0.055
Galactose	24.76 ± 12.07	4.30 ± 0.86	0.096
Histidine	10.87 ± 1.21	16.70 ± 1.88	0.021
Isoleucine	39.70 ± 2.24	50.31 ± 3.32	0.021
Lactate	1223.03 ± 92.05	975.09 ± 126.05	0.097
Leucine	64.06 ± 3.96	81.19 ± 6.02	0.041
Mannose	22.66 ± 1.17	20.01 ± 0.97	0.073
Methylsuccinate	4.46 ± 0.40	5.51 ± 0.32	0.084
Proline	53.79 ± 1.60	64.51 ± 2.40	0.002
Serine	51.36 ± 2.40	68.80 ± 4.81	0.013
trans-4-Hydroxy-L-proline	32.46 ± 2.05	24.17 ± 3.23	0.052
Valine	59.11 ± 3.58	77.06 ± 6.35	0.035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Taurine
Betaine
ADP+AMP+ATP+IMP
Carnitine
Pyruvate
Creatine+Creatine phosphate
Fumarate
Threonine
3-Methylhistidine
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Mannose
O-Acetylcarnitine
N,N-Dimethylglycine
Nicotinurate
Glucose
Lactate
Methylmalonate
Glucose-1-phosphate
Glucose-6-phosphate
Alanine
Acetate
2-Oxoglutarate
Glutamine
Carnosine
Arginine
Cadaverine
NADH
NADP+
Isoleucine
Acetoacetate
Leucine
Valine
Glutamate
GTP
Creatinine
Phenylalanine
UMP
NAD+
Tyrosine
3-Hydroxybutyrate
Glycine
Citrate
Tryptophan
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B

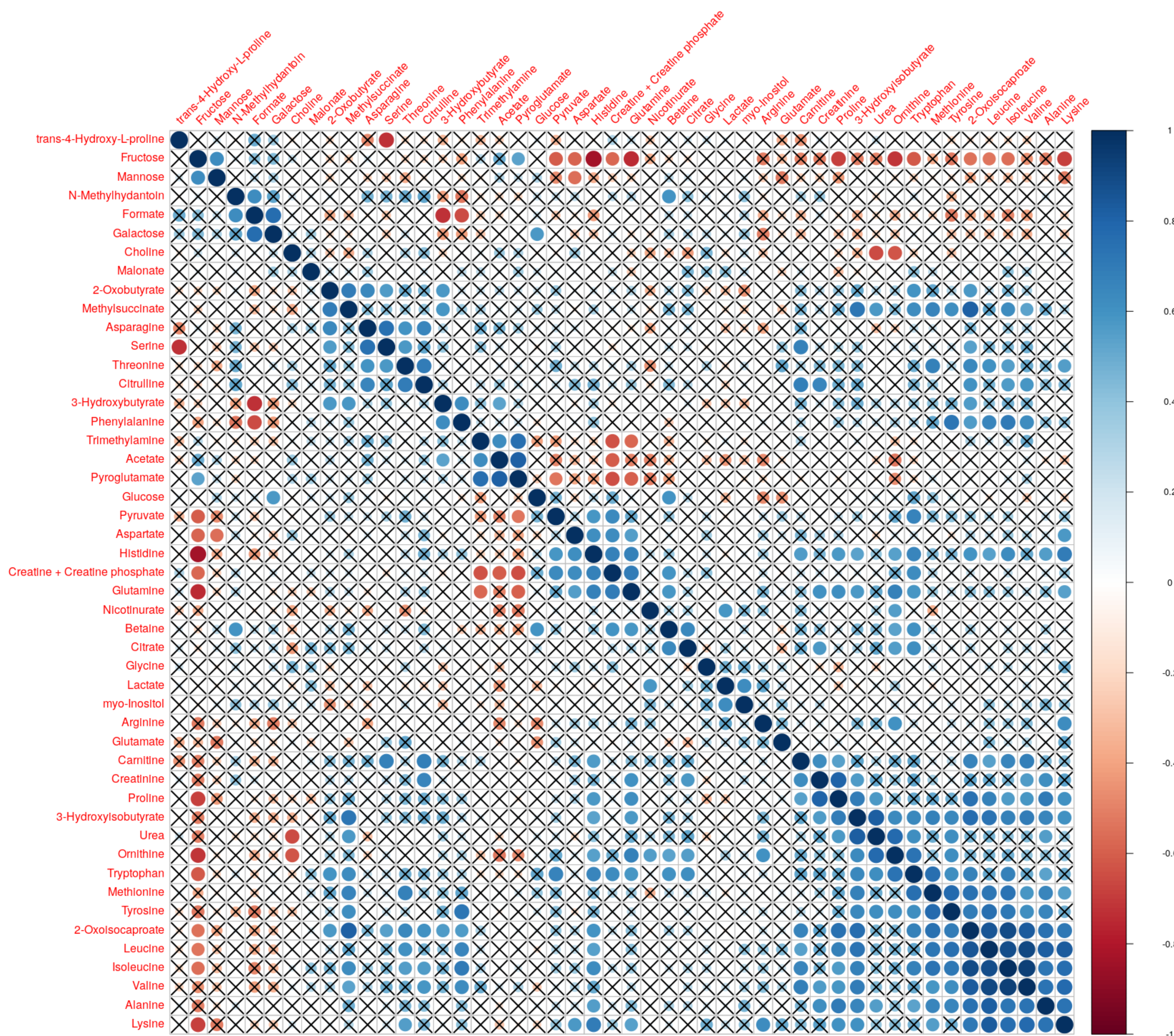

Fig. 2. Correlation plots of quantified metabolites in muscle (A) and serum (B). The bluer and redder colors suggest stronger positive and negative correlation, respectively. The larger size of the circle represents a greater correlation (approaching to 1) and smaller suggests the opposite (approaching to 0). Pearson r correlations were calculated and significant level was set $p < 0.05$, non-significant correlations are depicted with a cross on top of the circle. Compounds are grouped by hierarchical clustering order.

In muscle, two BCAA (leucine and valine), two citrate cycle-related metabolites (2-oxoglutarate and GTP) as well as NADH and $NADP^+$ were significantly elevated in LT. Acetoacetate, carnitine and isoleucine increased (tendency) and showed significant correlation with leucine.

In serum, seven proteinogenic AA (asparagine, histidine, isoleucine, leucine, proline, serine and valine) and two AA derivatives (citrulline and 4-hydroxy-proline) were significantly elevated in LT. Furthermore, catalytic metabolites derived from leucine (2-oxoisocaproate), threonine (2-oxobutyrate) and valine (3-hydroxyisobutyrate) as well as carnitine and 3-hydroxybutyrate, two metabolites related to lipid degradation, were elevated. Correlation plot revealed several positive correlations ($p < 0.05$) between AA and leucine/threonine.

The PCA of quantified metabolites with their corresponding loadings are shown in Fig. 3 (A, B) for muscle and serum, respectively. Verification of consistency with the original data was performed by matching PCA of chemometrics from both biological samples (Additional file Fig. S 1). In muscle, the loadings of several metabolites that are associated with BCAA and energy were tilted towards LT group while several sugars and some AA as well as threonine were tilted towards Ctrl; in serum however, the majority of quantified metabolites were tilted towards the LT group.

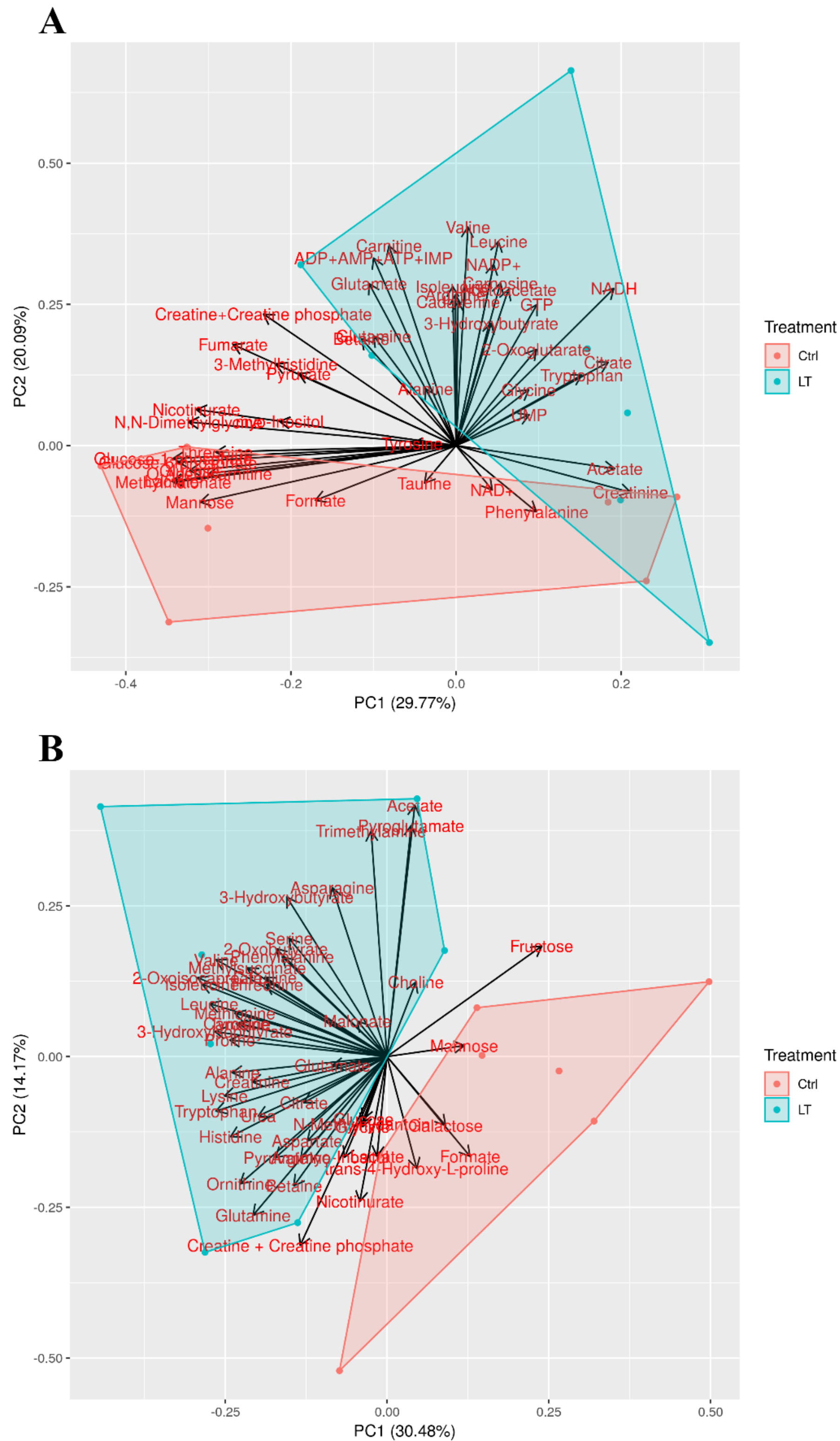

Fig. 3. PCA with loadings of quantified metabolites from muscle (A) and serum (B).

Pathway analysis

The Reactome pathway for proteins analyses yielded several significant pathways, in accordance with the GO ontology. The Reactome analysis for small molecules (KEGG nomenclature) also gave coherent results (Additional file Table S 6).

In muscle, citrate cycle and respiratory electron transport, mitochondrial fatty acid beta-oxidation and detoxification of reactive oxygen species were identified with proteomic data. Amongt the most relevant for metabolites, BCAA catabolism (R-HSA-70895), metabolism of amino acids and derivatives (R-HSA-71291), synthesis of ketone bodies (R-HSA-77111), NADPH regeneration (R-HSA-389542), tRNA modification in the nucleus and cytosol (R-HSA-6782315) and citrate cycle (R-HSA-71403) were observed.

In plasma/serum, chylomicron assembly and remodeling (R-HSA-8963888 and R-HSA-8963901) and lipoprotein assembly and remodeling (R-HSA-8963898 and R-HSA-8963899) were identified with proteomic data and tRNA aminoacylation (R-HSA-379724) and metabolism of amino acids and derivatives (R-HSA-71291) with small compounds.

The joint pathway analyses of upregulated proteins and metabolites in blood and muscle developed with MetaboAnalyst are shown in Table 9. Four pathways (Valine, leucine and isoleucine degradation; butanoate metabolism, Aminoacyl-tRNA metabolism; and Citrate cycle) were found with significant change, coherent with Reactome analysis.

Table 9. Joint pathway analyses of upregulated proteins and metabolites in muscle and blood according to the p values from the pathway enrichment analysis and pathway impact values from the pathway topology analysis. Higher -log(p) means more significant pathway; higher impact means a higher percentage of hit compounds.

Pathway	Muscle (9 proteins and 9 metabolites)								Blood (5 proteins and 16 metabolites)							
	Protein	Metabolite	hit	p	-log(p)	Holm p	FDR	Impact	Protein	Metabolite	hit	p	-log(p)	Holm p	FDR	Impact
Valine, leucine and isoleucine degradation	HADHA	leucine, acetoacetate, valine, isoleucine	5/82	6.69E-05	9.6	0.005	0	0.18	-	leucine, isoleucine, valine, 2-oxoisocaproate, 3-hydroxy isobutyrate	5/82	5.66E-04	7.5	0.04	0.02	0.13
Butanoate metabolism	HADHA	2-oxoglutarate, acetoacetate	3/47	0.003	5.9	0.21	0.07	0.34	-	-	-			-	-	
Aminoacyl-tRNA metabolism	-	leucine, isoleucine, valine	3/87	0.015	4.2	1	0.3	0.04	-	leucine, isoleucine, alanine, valine, serine, proline, histidine, asgaragine	8/87	1.74E-07	15.6	1.39E-05	1.39E-05	0.13
Citrate cycle	ACO2	2-oxoglutarate	2/50	0.039	3.2	1	0.62	0.3	-	-	-			-	-	

Discussion

Efficiency of AA supplementation and its effects on amino acid metabolism

Metabolomics indicated increases in BCAA in serum and muscle, indicating that leucine supplementation reached the blood and the muscle, and that a metabolic connection within BCAA exists. On the other hand, the concentration of threonine in both blood and muscle, despite being supplemented at the same level as leucine, did not present any elevation. This result may be due to the rapid metabolism of threonine to its metabolite serine, since serine was greatly increased and strongly correlated with threonine concentration in serum.

Unlike most AA that are first metabolized in the liver and turned into ketoacids by transamination, BCAA are not initially metabolized in the liver due to low hepatic activity of branched-chain-amino-acid aminotransferase (BCAT). Thus, the BCAA increase rapidly in the circulation after protein intake and are readily available to extrahepatic tissues (Holeček 2018). Most of the BCAA are initially catabolized in the skeletal muscle, due to its high BCAT activity which can reversibly transfer BCAA amino group to 2-oxoglutarate (α-KG) to form glutamate and the corresponding branched-chain keto acids (BCKA): 2-oxoisocaproate (ketoleucine), 3-methyl-2-oxovalerate (ketoisoleucine) and 2-oxovalerate (ketovaline). The glutamate can further form alanine or glutamine to be released in the bloodstream whereas BCKA are catabolized mainly in the liver (Harper et al. 1984). These three amino acids have parallel pathways for degradation, and the finding of 2-oxocaproate and 3-hydroxyisobutyrate indicates that these AA are indeed catabolized (Newgard 2012). These AA and their metabolites (α-ketoisocaproic acid and β-hydroxy-β-methylbutyrate) have been reported to play important roles in enhancing muscle growth during the neonatal period (Columbus et al. 2015).

Leucine/threonine supplementation and its effects on lipid metabolism

Proteomics showed increases in plasma apolipoproteins APOA2 and APOC3, which are involved in lipid and cholesterol transport (GO:2000910, GO:0060621). PON1, an enzyme involved in detoxification, is bound to HDL in plasma (Aviram et al. 1998). This increase in apolipoproteins was simultaneous to an increase, although not significant, in plasma TGs and cholesterol, as shown by conventional serum biochemical analysis. The increased lipid mobilization may be a mirror of the differences in the ratio protein/total fat in the whole animal, which was greater in LT calves, as observed after CT-imaging performed at the same time than biopsies (Terre M, manuscript in preparation). TGs are transported in the plasma in the form of lipoproteins. The relationship between BCAA and lipid metabolism has been known for years, although the mechanism and role is not yet clear (Newgard 2012). Numerous studies have found that leucine supplementation reduces adiposity in specific conditions (Newgard 2012; Pedroso et al. 2015). An increase in 3-hydroxybutyrate herein, may indicate an increase in ruminal butyrate production, an increase in the production of ketone bodies from the degradation of mobilized fatty acids, or both.

Leucine/threonine supplementation and its effects in skeletal muscle

The proteomic and metabolomic approaches in skeletal muscle highlight other metabolic adaptations. Two main metabolic effects of leucine/threonine supplementation can be observed. On one hand, the GO analysis showed an increase in oxidative metabolism, indicating that a activity of the mitochondrial respiratory chain and oxidative phosphorylation (OXPHO) occurs since components of NADH dehydrogenase (Complex I) (NADH and NDUFAB1) and cytochrome c oxidase (Complex IV) (COX2 and COX5A) (Wallace 1999) are elevated in the LT group. OXPHO would be fueled by a higher rate of the citrate cycle, as suggested by an upregulation of enzymes (ACO2) and metabolites (2-oxoglutarate, GTP, NADH). The ADP/ATP carrier protein SLC25A4, involved in the exchange of cytoplasmic ADP with mitochondrial ATP across the mitochondrial inner membrane, is also upregulated. The citrate cycle would be more active due to the fuel provided by the BCAA, but there is evidence that it can be also fueled by an increase in fatty acid degradation as carnitine, which is involved in the transport of fatty acids to the mitochondria, and HADHA, involved in fatty acid beta-oxidation, are both increased in the LT group. The appearance of acetoacetate and 3-hydroxybutyrate also indicates an

increase in the metabolism of Acetyl CoA. Nutrients (BCAA, butyrate and fats) are metabolized to acetyl-CoA, which subsequently enters the citrate cycle to finally produce ATP as energy for cellular processes (Heiden et al. 2009). Consequently, increased ATP availability leads to increased ATP transfer from mitochondria (upregulation of SLC25A4) as well as transport across membranes in other organelles (upregulation of the ATPase VCP).

On the other hand, the increased abundance of PRDX1 and SOD2 in muscle indicates the activation of superoxide radicals removal (GO:0019430) (Collins et al. 2012). Since in OXPHO is not completely efficient and not every oxygen receives four electrons, a greater amount of superoxide anions and peroxides may be produced in a scenario where OXPHO is activated. The increase in antioxidant defenses may be a compensatory mechanism for the increase in oxygen radicals.

Another potential effect of leucine is its ability to increase protein synthesis through its effect on the mTOR complex 1 (mTORC1) which is known for its central regulation of cell metabolism, growth, proliferation, and survival (Laplante and Sabatini 2009). There is evidence that leucine (Atherton et al. 2010) as well as its metabolite 2-oxoisocaproate (Escobar et al. 2010; Columbus et al. 2015) are able to activate mTOR. Herein, we use the phosphorylation of the ribosomal protein S6, downstream of the mTORC1 signaling pathway, as an indicator of the mTORC1 function, directly related to the upregulation of protein synthesis (Ma and Blenis 2009). In our case, the ratio of phospho-S6/S6 indicated a significant upregulation of mTORC1 in the LT group. Likewise, several AA involved in aminoacyl-tRNA biosynthesis showed significant increases and/or significant positive correlations with leucine, which is another sign of enhanced protein synthesis. Our results suggest that the supplemented leucine stimulated the mTORC1 pathway and protein synthesis, which may have contributed to the observed tendency for the protein/fat ratio increase in the CT-imaging.

Leucine/threonine supplementation and its effects in plasma

The metabolic effects were also visualized in plasma, due to the appearance of two oxidation/citrate cycle biomarkers (carnitine and 2-oxoglutarate), ketone bodies (3-hydroxybutyrate and acetoacetate) and, as mentioned above, BCAA derivatives (2-oxocaproate and 3-hydroxyisobutyrate).

Changes in the plasma proteome indicated that leucine/threonine supplementation seemed to have complex effects in the defense mechanisms of the organism (oxidative stress, native immune system). PON1 is involved in detoxification and binds to HDL, preventing and delaying the oxidation of HDL and LDL and therefore inhibiting the onset and progression of oxidative stress (Antoncic-Svetina et al. 2011) and CRP and ITIH4 are APPs upregulated in acute phase response (Neumaier et al. 2006). On the other hand, a decrease in several serpin 3A isoforms was observed. Serpin A3 belongs to the serpin superfamily of protease inhibitors and it is encoded by a cluster of eight closely related genes in cattle (Pere-Brissaud et al. 2015). The functionality of serpins remain unclear, but they may be involved in blood coagulation, fibrinolysis, cell migration, and hormone transport (Silverman et al. 2001; Gettins 2002). More research is needed to clarify whether these processes can be affected by leucine supplementation.

Conclusion

A schema of the metabolic effects of leucine/threonine supplementation is shown in Fig. 4. Supplementation with leucine and threonine increases oxidative metabolism in muscle through an increase in citrate cycle, the mitochondrial respiratory chain and OXPHOS. The energy substrates are the AA, which are catabolized to ketoacids mainly in the muscle, and not in the liver, and mainly yield acetyl-CoA to enter the citrate cycle. AA catabolism goes along with short fatty acid and/or lipid mobilization, probably from the adipose tissue, which also converts into acetyl-CoA to be oxidized. The generation of oxidative stress in the mitochondria is balanced by the upregulation of antioxidant proteins. Lastly, a complex readjustment of the plasma defense systems also occurs.

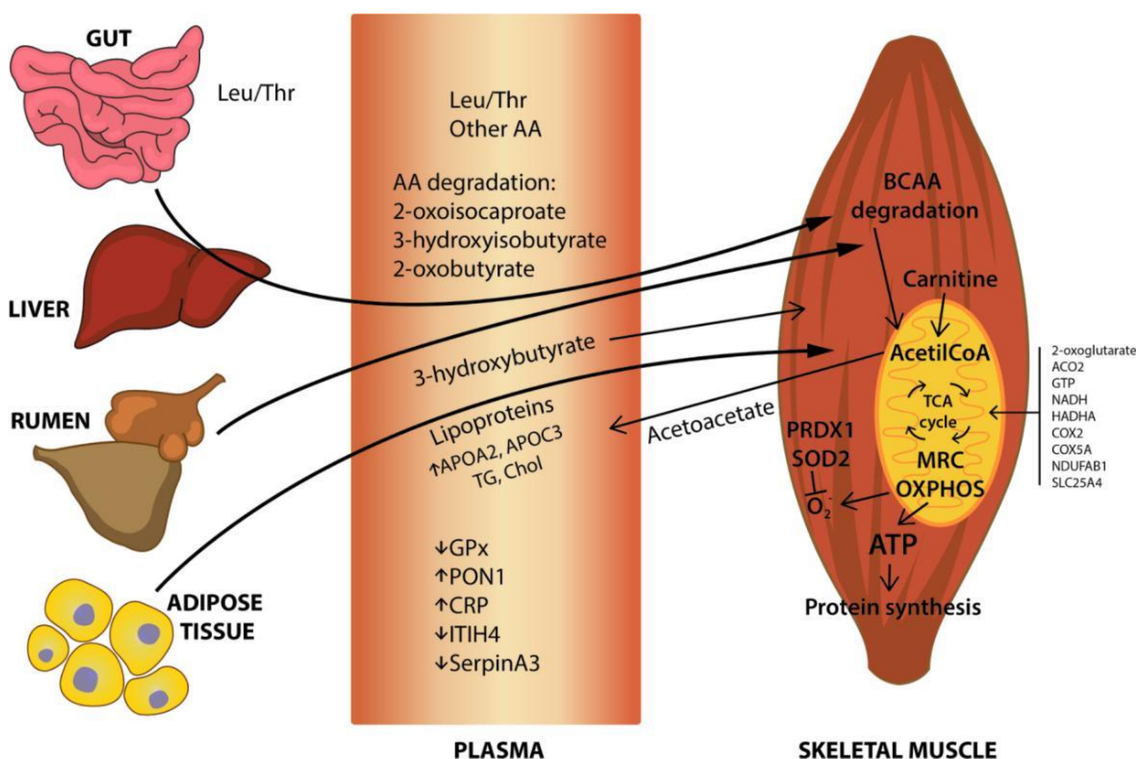

Fig. 4. Schematics of the metabolic effects of leucine/threonine supplementation.

In conclusion, an integrative approach by combining metabolomics, proteomics and conventional clinical chemistry allowed to characterize the metabolic adaptations occurring in these young animals. More studies to analyze the role of the rumen, adipose

tissue and liver, and the metabolomic study of hydrophobic compounds would extend our understanding about the metabolism in dairy calves and help to optimize their nutritional status.

Availability of data and materials

The datasets during and/or analyzed during the current study are available from the corresponding authors on reasonable request.

Abbreviations

AA: amino acid; BCAA: branched-chain amino acid; BCKA: branched-chain keto acids; BW: body weight; CT: computed tomography; DM: dry matter; MR: milk replacer; mTOR: mammalian target of rapamycin; PON1: paraoxonase-1; TSP: total protein staining.

Additional files

Table S 1. Ingredient and chemical composition (% DM) of milk replacer treatments.

	Ctrl	LT	Concentrate	Straw
Ingredients, %			-	-
Skimmed milk powder	39	39	-	-
Whey protein concentrate 35	15	15	-	-
Whey protein concentrate 60	4	3.4	-	-
Fatted whey (50%fat)	39	39	-	-
Premix	3	3	-	-
Leucine	-	0.3	-	-
Threonine	-	0.3		
Chemical composition				
DM	96.6	97.6	89.4	91.5
CP (% DM)	24.1	24.1	17.0	2.4
Fat (% DM)	20.5	23.9	3.8	-
Lactose (% DM)	43.1	43.7	-	-
Ash (% DM)	6.8	6.7	5.0	7.5
ADF (% DM)	-	-	16.8	59.6
NDF (% DM)	-	-	28.8	87.7

Ctrl, control diet; LT, control diet supplemented with 0.3% leucine and 0.3% threonine; CP, crude protein; ADF, acid detergent fiber; NDF, ADF, acid detergent fiber.

Mineral and vitamin composition: Vitamin A 25,000 IU/kg; Vitamin D3 4,500 IU/kg; Vitamin E 300 mg/kg, Vitamin C 300 mg/kg, Vitamin B1 16 mg/kg, Vitamin B2 10 mg/kg, Vitamin B6 10 mg/kg, Vitamin B12 80 mg/T, Vitamin K 5.5 mg/kg, Folic acid 1 mg/kg, Pantothenic acid 23 mg/kg, Niacin 50 mg/kg, Fe 150 mg/kg, Zinc 170 mg/kg, Copper 10 mg/kg, Manganese 40 mg/kg, Iodine 1.3 mg/kg, Selenium 0.4 mg/kg.

Table S 2. Starter concentrate feeding program.

Day	Starter concentrate (g/d)
1 - 7	100
8 - 14	200
15 - 21	300
22 - 28	500
29 - 35	700
36 - 42	1000
43 - 49	1300
50 -	*ad libitum*

Table S 3. Performance and intake of calves fed a milk replacer supplemented with 0.3% leucine plus 0.3% threonine (LT) or a non-supplemented milk replacer.

	Treatments					***p* -values**[1]		
	Ctrl	LT		SEM[2]		T	time	Txtime
Initial age, d	5.7	5.7		0.67		0.88	-	-
Initial BW, kg	43.0	40.8		2.15		0.49	-	-
Final BW, kg	98.8	100		3.82		0.83	< 0.001	0.60
Biopsy BW, kg	82.1	81.5		3.50		0.92	-	-
ADG, g/d	0.89	0.96		0.054		0.48	< 0.001	0.45
Dry matter intake, g/d								
Milk replacer	757	761		8.9		0.77	< 0.001	0.99
Concentrate	735	855		154.7		0.59	< 0.001	0.87
Straw	47	50		5.8		0.73	< 0.001	0.65
Total	1457	1583		155.8		0.57	< 0.001	0.90
Gain to feed	0.66	0.65		0.015		0.62	< 0.001	0.18

Ctrl, control diet; LT, control diet supplemented with 0.3% leucine and 0.3% threonine. p-value < 0.001, value of highly statistical significance.

1 T=effect of leucine and threonine supplementation; time=effect of day of study; Tx time=interaction of Leu and Thr supplementation with day of study.

2 Standard error of the mean.

Table S 4. PANTHER GO-Slim analysis of biological process and molecular functions of the differential proteins in skeletal muscle between Ctrl and LT groups.

	Homo sapiens - REFLIST (20996)	Client Text Box Input (13)	Client Text Box Input (expected)	Client Text Box Input (over/under)	Client Text Box Input (fold Enrichment)	Client Text Box Input (raw P-value)	Client Text Box Input (FDR)
GO biological process complete							
positive regulation of ATP biosynthetic process (GO:2001171)	11	2	.01	+	> 100	2.75E-05	3.95E-02
removal of superoxide radicals (GO:0019430)	13	2	.01	+	> 100	3.69E-05	4.49E-02
regulation of ATP biosynthetic process (GO:2001169)	14	2	.01	+	> 100	4.22E-05	4.77E-02
cellular response to superoxide (GO:0071451)	15	2	.01	+	> 100	4.78E-05	4.73E-02
cellular response to oxygen radical (GO:0071450)	15	2	.01	+	> 100	4.78E-05	4.45E-02
response to superoxide (GO:0000303)	18	2	.01	+	> 100	6.67E-05	5.28E-02
response to oxygen radical (GO:0000305)	19	2	.01	+	> 100	7.37E-05	5.07E-02
mitochondrial electron transport, cytochrome c to oxygen (GO:0006123)	20	2	.01	+	> 100	8.11E-05	4.75E-02
aerobic electron transport chain (GO:0019646)	20	2	.01	+	> 100	8.11E-05	4.58E-02
positive regulation of purine nucleotide biosynthetic process (GO:1900373)	21	2	.01	+	> 100	8.88E-05	4.84E-02
positive regulation of nucleotide biosynthetic process (GO:0030810)	21	2	.01	+	> 100	8.88E-05	4.68E-02
aerobic respiration (GO:0009060)	80	3	.05	+	60.57	1.65E-05	3.73E-02
cellular oxidant detoxification (GO:0098869)	91	3	.06	+	53.24	2.40E-05	4.75E-02
mitochondrial ATP synthesis coupled electron transport (GO:0042775)	91	3	.06	+	53.24	2.40E-05	4.22E-02
ATP synthesis coupled electron transport (GO:0042773)	92	3	.06	+	52.67	2.48E-05	3.92E-02
cellular detoxification (GO:1990748)	100	3	.06	+	48.45	3.16E-05	4.16E-02
respiratory electron transport chain (GO:0022904)	112	3	.07	+	43.26	4.39E-05	4.63E-02
oxidative phosphorylation (GO:0006119)	117	3	.07	+	41.41	4.98E-05	4.38E-02
detoxification (GO:0098754)	120	3	.07	+	40.38	5.37E-05	4.47E-02
cellular respiration (GO:0045333)	168	4	.10	+	38.45	2.93E-06	1.16E-02
positive regulation of small molecule metabolic process (GO:0062013)	135	3	.08	+	35.89	7.56E-05	4.98E-02
energy derivation by oxidation of organic compounds (GO:0015980)	238	5	.15	+	33.93	2.37E-07	1.25E-03
ATP metabolic process (GO:0046034)	203	4	.13	+	31.82	6.11E-06	1.93E-02
generation of precursor metabolites and energy (GO:0006091)	426	6	.26	+	22.75	1.11E-07	8.77E-04
response to inorganic substance (GO:0010035)	533	5	.33	+	15.15	1.17E-05	3.09E-02
oxidation-reduction process (GO:0055114)	960	9	.59	+	15.14	5.51E-10	8.71E-06
GO molecular function complete							
antioxidant activity (GO:0016209)	87	3	.05	+	55.69	2.11E-05	4.90E-02
oxidoreductase activity (GO:0016491)	764	7	.47	+	14.80	1.23E-07	5.74E-04

Table S 5. PANTHER GO-Slim analysis of biological process, cellular component and molecular functions of the differential proteins in serum between Ctrl and LT groups.

	Homo sapiens - REFLIST (20996)	Client Text Box Input (9)	Client Text Box Input (expected)	Client Text Box Input (over/under)	Client Text Box Input (fold Enrichment)	Client Text Box Input (raw P-value)	Client Text Box Input (FDR)
GO biological process complete							
negative regulation of sterol import (GO:2000910)	2	2	.00	+	> 100	9.79E-07	2.58E-03
negative regulation of cholesterol import (GO:0060621)	2	2	.00	+	> 100	9.79E-07	2.21E-03
negative regulation of very-low-density lipoprotein particle remodeling (GO:0010903)	3	2	.00	+	> 100	1.63E-06	2.58E-03
regulation of sterol import (GO:2000909)	4	2	.00	+	> 100	2.45E-06	3.52E-03
regulation of cholesterol import (GO:0060620)	4	2	.00	+	> 100	2.45E-06	3.22E-03
regulation of very-low-density lipoprotein particle remodeling (GO:0010901)	5	2	.00	+	> 100	3.42E-06	4.16E-03
chylomicron remodeling (GO:0034371)	9	2	.00	+	> 100	8.96E-06	1.01E-02
chylomicron assembly (GO:0034378)	10	2	.00	+	> 100	1.07E-05	1.13E-02
phospholipid efflux (GO:0033700)	12	2	.01	+	> 100	1.48E-05	1.38E-02
negative regulation of cholesterol transport (GO:0032375)	12	2	.01	+	> 100	1.48E-05	1.30E-02
negative regulation of sterol transport (GO:0032372)	12	2	.01	+	> 100	1.48E-05	1.23E-02
triglyceride-rich lipoprotein particle remodeling (GO:0034370)	13	2	.01	+	> 100	1.71E-05	1.35E-02
negative regulation of lipase activity (GO:0060192)	17	2	.01	+	> 100	2.78E-05	2.09E-02
high-density lipoprotein particle remodeling (GO:0034375)	17	2	.01	+	> 100	2.78E-05	2.00E-02
reverse cholesterol transport (GO:0043691)	18	2	.01	+	> 100	3.09E-05	2.04E-02
acute-phase response (GO:0006953)	41	4	.02	+	> 100	2.30E-09	1.82E-05
negative regulation of lipid catabolic process (GO:0050995)	22	2	.01	+	> 100	4.48E-05	2.84E-02
cholesterol efflux (GO:0033344)	23	2	.01	+	> 100	4.87E-05	2.96E-02
plasma lipoprotein particle assembly (GO:0034377)	26	2	.01	+	> 100	6.13E-05	3.59E-02
negative regulation of lipid transport (GO:0032369)	28	2	.01	+	> 100	7.05E-05	3.85E-02
negative regulation of lipid localization (GO:1905953)	43	3	.02	+	> 100	8.18E-07	2.59E-03
plasma lipoprotein particle remodeling (GO:0034369)	29	2	.01	+	> 100	7.54E-05	3.97E-02
protein-lipid complex remodeling (GO:0034368)	29	2	.01	+	> 100	7.54E-05	3.85E-02
acute inflammatory response (GO:0002526)	73	5	.03	+	> 100	7.72E-11	1.22E-06
protein-lipid complex assembly (GO:0065005)	30	2	.01	+	> 100	8.04E-05	3.97E-02
protein-containing complex remodeling (GO:0034367)	30	2	.01	+	> 100	8.04E-05	3.85E-02
regulation of cholesterol transport (GO:0032374)	46	3	.02	+	> 100	9.92E-07	1.96E-03
regulation of sterol transport (GO:0032371)	46	3	.02	+	> 100	9.92E-07	1.74E-03
acylglycerol catabolic process (GO:0046464)	33	2	.01	+	> 100	9.64E-05	4.48E-02
neutral lipid catabolic process (GO:0046461)	33	2	.01	+	> 100	9.64E-05	4.36E-02
regulation of lipid localization (GO:1905952)	138	4	.06	+	67.62	2.46E-07	1.30E-03
regulation of lipid transport (GO:0032368)	108	3	.05	+	64.80	1.18E-05	1.17E-02
regulation of cytokine secretion (GO:0050707)	195	3	.08	+	35.89	6.65E-05	3.75E-02
inflammatory response (GO:0006954)	485	5	.21	+	24.05	7.89E-07	3.12E-03
defense response (GO:0006952)	1320	5	.57	+	8.84	1.01E-04	4.43E-02
regulation of transport (GO:0051049)	1829	6	.78	+	7.65	2.94E-05	2.02E-02
GO cellular component complete							
spherical high-density lipoprotein particle (GO:0034366)	8	3	.00	+	> 100	1.28E-05	
chylomicron (GO:0042627)	14	2	.01	+	> 100	2.78E-02	
high-density lipoprotein particle (GO:0034364)	27	3	.01	+	> 100	3.13E-04	
lipoprotein particle (GO:1990777)	39	3	.02	+	> 100	8.83E-04	
plasma lipoprotein particle (GO:0034358)	39	3	.02	+	> 100	8.83E-04	
protein-lipid complex (GO:0032994)	41	3	.02	+	> 100	1.02E-03	
blood microparticle (GO:0072562)	143	5	.06	+	81.57	2.85E-06	
collagen-containing extracellular matrix (GO:0062023)	406	4	.17	+	22.98	2.38E-02	
extracellular exosome (GO:0070062)	2097	7	.90	+	7.79	4.29E-03	
extracellular vesicle (GO:1903561)	2118	7	.91	+	7.71	4.59E-03	
extracellular organelle (GO:0043230)	2120	7	.91	+	7.70	4.62E-03	
extracellular space (GO:0005615)	3337	8	1.43	+	5.59	4.53E-03	
extracellular region part (GO:0044421)	3535	8	1.52	+	5.28	7.10E-03	
extracellular region (GO:0005576)	4341	8	1.86	+	4.30	3.52E-02	
GO molecular function complete							
high-density lipoprotein particle receptor binding (GO:0070653)	3	2	.00	+	> 100	4.50E-03	
lipoprotein particle receptor binding (GO:0070325)	26	3	.01	+	> 100	5.45E-04	

Table S 6. Reactome identified pathways from protein and metabolite data in skeletal muscle and plasma-serum.

Extra large table, for viewing please refer to the link below:

https://drive.google.com/drive/folders/1yybSBWQTCQRf6VSFc6jpbKGme8vkieit?usp=sharing

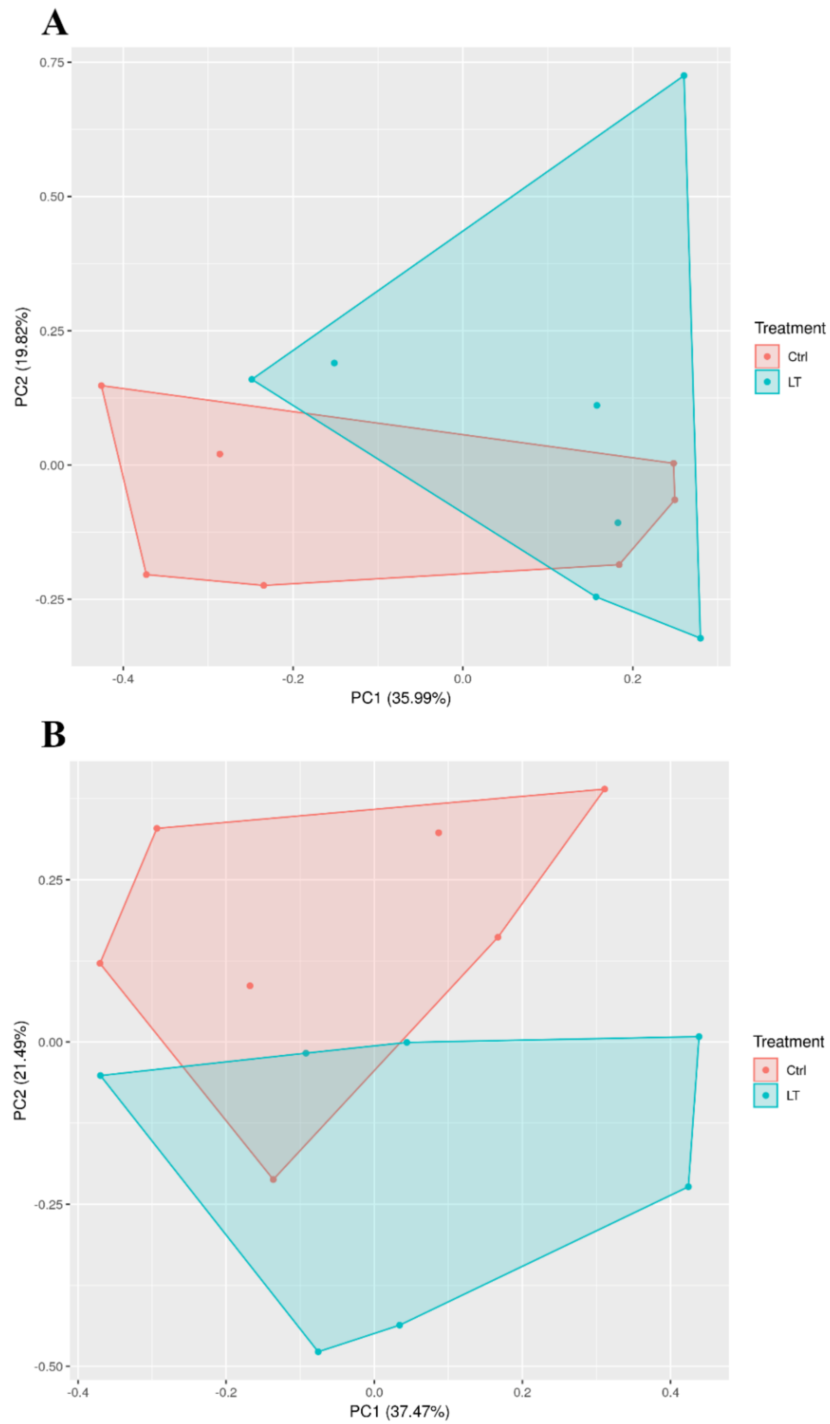

Fig. S 1. **A**. PCA of muscle chemometrics; **B**. PCA of serum chemometrics. Ctrl, control diet; LT, control diet supplemented with 0.3% leucine and 0.3% threonine.

Author Contributions

K.Y. performed the experimental sessions in metabolomics and proteomics, data analysis, bioinformatics, statistics and manuscript writing. M.M. (manuscript, metabolomics and data analysis), A.H. (manuscript, proteomics and data analysis), M.T. (experimental design, manuscript, sampling, data analysis and statistics), A. Bach (experimental design, manuscript revision), J.K. (manuscript and proteomics), N.Y., E.R. and R.P (analytical work), N.G. (data analysis, bioinformatics and statistics), A.A. and P.D. E. (manuscript revision), A.Bassols (experimental design, analytical work supervision, manuscript writing and revision). All authors read and approved the final manuscript.

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Conflict of Interest

The authors declare no conflict of interest.

9.2. Annex 2: Metabolome and Proteome Changes in Skeletal Muscle and Blood of Preruminant Calves with Diet Supplemented with Leucine

Unpublished complementary work from the same study reported in Annex 1.

Objective

The original objective of the present work was to determine the effects of MR diet supplemented with leucine (Leu) fed to preruminant calves 6 days to 7 weeks in skeletal muscle biopsies and in plasma by using metabolomics and proteomics approaches to investigate the effect of supplemented leucine on preweaning calves. However, as some technical/experimental impingements were found, we modified the original objective to take into account these events, and to consider the technical faults to point out adequate precautions.

Introduction

This study is part of the Study 2 and it was performed in parallel with the experiment described in Annex 1 (Leucine/Threonine supplementation). After the “multi-omics” analysis, we were informed from the farm that most of the animals in the Leucine-supplemented group (Leu) presented serious digestive and respiratory symptoms and the productive parameters were not as expected. Despite unidentified etiology, these animals presented symptoms at necropsy similar to *Clostridium*, *E. Coli* and *Cryptosporidium* infection while some others showed pneumonia (data not shown). Moreover, a technical fault happened in the biopsy as, among the three biopsies that were performed, animals in the control group (Ctrl) and the group supplemented with leucine and threonine (LT) were well randomized between biopsy days, whereas the third biopsy day was not adequately randomized since this batch only contained individuals from the Leu group (shown in Figure 8). Due to the biased biopsy and sickness reasons from Leu group, this group was excluded from the manuscript reported in Annex 1.

Since the “multi-omics” assay was performed before the announcement from the farm, it was interesting to show and interpret the results and, also by discussing about this incident, be able to emphasize about the precautions that should be taken into account.

ID	03	04	05	06	01	02	07	08	10	13	09	11	12	14	15	16	17	18	19	20
Treatment	Ctrl	Ctrl	Ctrl	Ctrl	LT	LT	LT	Ctrl	Ctrl	Ctrl	LT	LT	LT	LT	Leu	Leu	Leu	Leu	Leu	Leu
Biopsy	1st	1st	1st	1st	1st	1st	1st	2nd	2nd	2nd	2nd	2nd	2nd	2nd	3rd	3rd	3rd	3rd	3rd	3rd

Figure 8. Scheme of treatment and biopsy programs in Study 2. Ctrl, control diet; LT, control diet supplemented with 0.3% leucine and 0.3 % threonine; Leu, control diet supplemented with 0.3% leucine. Date (dd/mm/yyyy) of biopsies: 1st, 14/06/2018; 2nd, 21/06/2018; 3rd, 26/07/2018.

Methods

Animal house and diet management, sample collection, serum clinical chemistry, proteomic analysis, metabolomic analysis, statistics and proteomic-metabolomic interactive analyses were as described in Annex 1. Ingredients and chemical composition of milk replacers are shown in Table 10.

Table 10. Ingredients and chemical composition (% DM) of milk replacer treatments.

	Ctrl	Leu	Concentrate	Straw
Ingredients, %			-	-
Skimmed milk powder	39	39	-	-
Whey protein concentrate 35	15	15	-	-
Whey protein concentrate 60	4	3.4	-	-
Fatted whey (50%fat)	39	39	-	-
Premix	3	3	-	-
Leucine	-	0.3	-	-
Threonine	-	-		
Chemical composition, % DM				
DM	96.6	97.1	89.4	91.5
CP	24.1	24	17.0	2.4
Fat	20.5	20.9	3.8	-
Lactose	43.1	42.2	-	-
Ash	6.8	6.8	5.0	7.5
ADF	-	-	16.8	59.6
NDF	-	-	28.8	87.7

Ctrl, control diet; Leu, control diet supplemented with 0.3% leucine.

[1] Mineral and vitamin composition: Vitamin A 25,000 IU/kg; Vitamin D_3 4,500 IU/kg; Vitamin E 300 mg/kg, Vitamin C 300 mg/kg, Vitamin B_1 16 mg/kg, Vitamin B_2 10 mg/kg, Vitamin B_6 10 mg/kg, Vitamin B_{12} 80 mg/T, Vitamin K 5.5 mg/kg, Folic acid 1 mg/kg, Pantothenic acid 23 mg/kg, Niacin 50 mg/kg, Fe 150 mg/kg, Zinc 170 mg/kg, Copper 10 mg/kg, Manganese 40 mg/kg, Iodine 1.3 mg/kg, Selenium 0.4 mg/kg.

Results

Productive results

The productive results are shown in Table 11. In comparison with Ctrl and LT groups, worse ADG and Gain to feed were observed by the farm staff for the Leu group due to the sickness.

Table 11. Performance and intake data.

Treatments						*p* [1]		
	Ctrl	LT	Leu	SEM[2]		T	time	Txtime
Initial age, d	5.7	5.7	5.3	0.76		0.94	-	-
Initial BW, kg	43.0	40.8	39.7	2.19		0.58	-	-
Final BW, kg	98.8	100	79.3	2.22		0.01	<0.001	<0.001
Biopsy BW, kg	82.1	81.5	68.1	3.75		0.03	-	-
ADG, g/d	0.89	0.94	0.61	0.049		<0.001	<0.001	0.003
Dry matter intake, g/d								
Milk replacer	757	761	659	10.1		0.28	<0.001	0.29
Concentrate	735	855	556	129.8		0.22	<0.001	0.06
Straw	47	50	41	7.8		0.73	<0.001	0.65
Total	1457	1583	1,256	125.9		0.14	<0.001	0.1
Gain to feed	0.66	0.65	0.54	0.021		<0.001	<0.001	<0.001

Ctrl, control diet; Leu, control diet supplemented with 0.3% leucine. p <0.001, value of highly statistical significance.

[1] T = effect of leucine and threonine supplementation; time = effect of day of study; Tx time = interaction of Leu and Thr supplementation with day of study.

[2] Standard error of the mean.

Plasma biochemistry and AA profile

Serum biochemistry results are shown in Table 12. AST and SOD decreased, and tendencies to decrease were observed for cholesterol and total protein; glucose and GPx were increased. In all cases, values for all animals were within the reference interval.

Table 12. Mean concentrations (mean ± SD) of serum biochemical analytes.

Analyte	Unit	Ctrl	Leu	*p*	Reference Interval[1]
ALT	U/L	10.93 ± 1.57	8.28 ± 3.21		5.2 - 15.3
AST	U/L	41.43 ± 5.91	30.17 ± 8.84	< 0.05	19.0 - 63.3
Cholesterol	mg/dL	60.29 ± 12.7	40.38 ± 16.79	< 0.1	25.4 - 118.3
Creatinine	mg/dL	0.77 ± 0.14	0.64 ± 0.1		0.47 - 0.99
GGT	U/L	18.43 ± 4.04	15.17 ± 2.23		11.0 - 27.6
Glucose	mg/dL	79.3 ± 12.52	94.82 ± 11.83	< 0.1	63.4 - 123.0
GPx	U/L	423.84 ± 101.13	536.95 ± 122.92	< 0.1	288.7 - 711.0
Haptoglobin	mg/mL	0.13 ± 0.07	0.21 ± 0.13		0.08 - 0.38
NEFAs	mmol/L	0.2 ± 0.03	0.2 ± 0.06		0.06 - 0.35
SOD	U/mL	0.43 ± 0.17	0.18 ± 0.1	< 0.05	0.03 - 0.57
TGs	mg/dL	19.69 ± 8.61	26.92 ± 6.28		6.7 - 58.5
Total protein	g/dL	5.51 ± 0.51	4.93 ± 0.46	< 0.1	3.87 - 6.13
Urea	mg/dL	10.53 ± 2.79	12.75 ± 4.29		6.8 - 18.3

Ctrl, control diet; Leu, control diet supplemented with 0.3% leucine; ALT, Alanine Aminotransferase; AST, Aspartate Aminotransferase; GGT, Gamma-Glutamyl Transferase; GPx, Glutathione Peroxidase; NEFAs, Non-Esterified Fatty Acids; SOD, Superoxide Dismutase; TGs, Triglycerides; TP, Total Proteins. *p*-value was calculated using Wilcoxon test and < 0.1 and < 0.05 correspond to statistical tendency and significance, respectively.

[1] Reference interval for preweaning calves at 7 weeks of old (Yu et al. 2019).

Proteomics in skeletal muscle and plasma

The differentially abundant proteins in muscle and plasma from both groups are shown in Table 13 and Table 14, respectively.

In muscle, seven proteins were found at higher abundance (HADHA, LDHB, PPIA, EIF5A2, PRDX1 and LOC524236) and lower abundance proteins were HBA, HSPB6, FHL3, UQCRFS1, SYNM, LGALS1, FHL1, NEB, PGM1, RTN2 and PGAM1.

For increased proteins, HADH, LDHB and PRDX1 are involved in catalytic activity (GO:0140096); EIF5A2 and LOC524236 are involved in nuclear acid binding (GO:0003676) whereas EIF5A2 is in ribosome binding (GO:0043022) and LOC524236 in DNA binding (GO:0003677) and protein heterodimerization activity (GO:0046982) in molecular function. PPIA is involved in peptidyl-amino acid modification (GO:0018193) in biological process.

In downregulated proteins, two filament binding proteins (NEB and SYNM) are separately involved in actin filament binding (GO:0051015) and intermediate filament binding (GO:0019215), respectively and two Four and a half LIM domains proteins (FHL1 and FHL3) are involved in metal ion binding (GO:0046872) in molecular function. As for the biological process, HSPB6 is involved both in muscle and nervous system process (GO:0003012 and GO:0050877, respectively) as well as protein homodimerization activity (GO:0046982); LGALS1 is in T cell costimulation (GP:0031295); three proteins (PGM1, PGAM1 and RTN2) are involved in sugar-related metabolism/catabolism: PGM1 is in glucose metabolic (GO:0006006), glycogen biosynthetic (GO:0005978) and galactose biosynthetic (GO:0019388) processes; PGAM1 is in regulation of glycolytic process (GO:0006110) and RTN2 is in regulation of glucose import (GO:0046324) and UQCRFS1 is in mitochondrial electron transport, ubiquinol to cytochrome c (GO:0006122).

Table 13. Proteins with significantly differential abundances between Leu and Ctrl in skeletal muscle.

Accession NCBI	**Gene symbol (*bos taurus*)**	**Protein name**	**p**	**Fold change**
296486764	HADH	hydroxyacyl-coenzyme A dehydrogenase, mitochondrial	0.021	0.32
59858383	LDHB	lactate dehydrogenase B	0.043	0.2
741923293	PPIA	peptidyl-prolyl cis-trans isomerase A	0.015	0.15
300797376	EIF5A2	eukaryotic translation initiation factor 5A-2	0.025	0.15
28189631	PRDX1	similar to peroxiredoxin 1, partial	0.029	0.13
296490775	LOC524236	histone cluster 1, H2ae-like	0.01	0.12
1057719460	HSPB6	heat shock protein beta-6, partial	0.025	-0.21
73586807	FHL3	Four and a half LIM domains 3	0.01	-0.19
157833692	UQCRFS1	Chain A, Rieske Iron-sulfur Protein	0.034	-0.19
296475605	SYNM	synemin, intermediate filament protein isoform 1	0.041	-0.19
28189797	LGALS1	similar to galactose-binding lectin, partial	0.047	-0.17
1387298754	FHL1	four and a half LIM domains protein 1-like isoform X2	0.047	-0.16
289444	NEB	nebulin, partial	0.017	-0.14
115305028	PGM1	Phosphoglucomutase 1	0.029	-0.14
32880227	RTN2	reticulon-2 isoform C	0.014	-0.13
296472661	PGAM1	phosphoglycerate mutase 1	0.047	-0.13

Ctrl, control diet; Leu, control diet supplemented with 0.3% leucine. *p* was calculated using Wilcoxon test and fold change using the function of log2(Mean(Leu)/Mean(Ctrl)).

In plasma, SAA3, SAA1, SAA2, ORM1, FGG, GPX3, FGB, LOC515150, SERPINA3-7, C9, F2, AGT, PLG, ITIH3, F5, PROS1 and C5 were found elevated. Less abundant proteins were IGG1C, HBA, PLA2G2D5, PLA2G2D3, PLA2G2D1, PLA2G2D4, C4A, C2, LOC525947, ALB, C1QC, APOH, CFI and CFB.

In the GO analysis of molecular function, the elevated plasma protein, PLG, F2, SERPINA3-7 and AGT are involved in peptidase activity (GO:0008233) whereas SERPINA3-7, AGT, ITIH3 and C5 are in peptidase inhibitor activity (GO:0002020). In terms of biological process, three serum amyloid A proteins (SAA1, SAA2 and SAA3), ORM1 and F2 are in acute phase response (GO:0006953); C5 and C9 are involved in complement activation, alternative (GO:0006957) and classical pathways (GO:0006958); PROS1, F5, FGB and FGG are involved in coagulation-related processes: GO:0030168 and GO:0007596; GPX3 responses to oxidative stress (GO:0006979) and finally, since there was not information on LOC515150 in databases, NCBI BLAST showed that this protein shows 100% identity to apolipoprotein R [*Bos taurus*].

For downregulated proteins, four Phospholipase A2 proteins (PLA2G2D1, PLA2G2D3, PLA2G2D4 and PLA2G2D5) are involved in lipid catabolic process (GO:0016042) in biological process. Five proteins from complement system (C1QC, C2, C4A, CFB, CFI and IGG1C) are involved in complement activation, alternative (GO:0006957) and classical pathways (GO:0006958) or innate immune response (GO:0045087) in biological process. LOC525947 is a serotransferrin-like protein and is involved in enzyme inhibitory activity (GO:0004857). APOH is in heparin binding (GO:0008201) in molecular function. ALB has several molecular binding functions to fatty acid (GO:0005504), metal ion (GO:0046872), toxic substance (GO:0015643)) and it is a negative acute phase protein (Conner et al. 1988).

Table 14. Proteins with significantly differential abundances between Leu and Ctrl in plasma.

Accession NCBI	**Gene symbol (*bos taurus*)**	**Protein name**	***p***	**Fold change**
620597439	SAA3	serum amyloid A3	0.012	0.73
296471870	SAA1	serum amyloid A protein-like	0.008	0.71
245184	SAA2	serum amyloid A, SAA	0.005	0.7
159895416	ORM1	alpha-1 acid glycoprotein	0.038	0.57
296478819	FGG	Fibrinogen gamma-B chain precursor	0.038	0.47
449500	GPX3	selernium-dependent glutathione peroxidase	0.005	0.37
151555876	FGB	FGB protein, partial	0.026	0.37
296479410	LOC515150	complement component 4 binding protein, alpha chain-like	0.027	0.31
38683423	SERPINA3-7	endopin 2B	0.045	0.27
75947612	C9	Complement component 9	0.038	0.24
4139478	F2	Chain L, Thrombin Inhibitor from Triatoma Pallidipennis	0.038	0.23
74354323	AGT	Angiotensinogen (serpin peptidase inhibitor, clade A, member 8)	0.022	0.2
163552	PLG	plasminogen, partial	0.038	0.2
156120445	ITIH3	inter-alpha-trypsin inhibitor heavy chain H3 precursor	0.032	0.17
163040	F5	factor V	0.038	0.16
163698	PROS1	vitamin K-dependent protein S precursor	0.044	0.16
82704085	C5	complement component C5a, partial	0.012	0.15
7547266	IGG1C	IgG1 heavy chain constant region, partial	0.038	-1.1
12084212	HBA	Chain A, Carbonmonoxy Liganded Bovine Hemoglobin Ph 5.0	0.027	-0.91
296489961	PLA2G2D5	calcium-dependent phospholipase A2 PLA2G2D5	0.037	-0.29
296489959	PLA2G2D3	calcium-dependent phospholipase A2 PLA2G2D3	0.037	-0.29
296489958	PLA2G2D1	calcium-dependent phospholipase A2 PLA2G2D1	0.037	-0.29
54606405	PLA2G2D4	putative calcium-dependent phospholipase A2	0.037	-0.29
262050656	C4A	complement C4 precursor	0.032	-0.27
1387238402	C2	complement C2 isoform X1	0.045	-0.25
1387190927	LOC525947	inhibitor of carbonic anhydrase isoform X1	0.038	-0.23
229552	ALB	albumin	0.045	-0.21
1387228013	C1QC	complement C1q subcomponent subunit C isoform X1	0.006	-0.19
982959007	APOH	beta-2-glycoprotein 1 isoform X1	0.045	-0.19
84000165	CFI	complement factor I precursor	0.005	-0.17
66866253	CFB	complement factor B, partial	0.027	-0.13

Ctrl, control diet; Leu, control diet supplemented with 0.3% leucine. *p* was calculated using Wilcoxon test and fold change using the function of log2(Mean(Leu)/Mean(Ctrl)).

Metabolomics in skeletal muscle and serum

For metabolomics, quantified metabolites of muscle and serum are shown in Table 15 and Table 16 respectively. The correlation plots with symbolic *p* values are shown in Figure 9.

In muscle, increases in the Leu group were found in glutamate, leucine, NADH, NADP+ and UMP; three sugars (glucose, glucose-6-phosphate and mannose), 3-methylhistidine, betaine, glutamine, lactate and methylmalonate were found decreased. The correlation plot reveals that isoleucine is the only elevated metabolite with statistical tendency that have significant positive correlations with leucine.

In serum, acetate, aspartate, carnitine, formate, galactose and leucine were found increased and creatine + creatine phosphate, lactate, methylsuccinate and tryptophan were decreased in Leu group. Correlation plot revealed positive correlations ($p < 0.05$) between six AA (asparagine, isoleucine, leucine, lysine, methionine and serine) and threonine.

Table 15. Mean metabolite concentrations (mean ± SD) in skeletal muscle (µmol/100g muscle).

Metabolite	KEGG ID	Ctrl	Leu	*p*
2-Oxoglutarate	C00026	0.80 ± 0.20	1.17 ± 0.57	
3-Hydroxybutyrate	C01089	0.27 ± 0.04	0.30 ± 0.08	
3-Methylhistidine	C01152	11.69 ± 1.63	8.25 ± 1.87	< 0.01
Acetate	C00033	1.08 ± 0.32	1.48 ± 0.29	< 0.1
Acetoacetate	C00164	0.52 ± 0.11	2.71 ± 2.53	
ADP+AMP+ATP+IMP	C00008/C00020/C00002/C00130	33.15 ± 3.28	31.78 ± 4.71	
Alanine	C00041	7.23 ± 2.15	5.22 ± 1.14	
Arginine	C00062	2.80 ± 0.63	2.90 ± 1.35	
Betaine	C00719	24.03 ± 6.27	17.15 ± 1.76	< 0.05
Cadaverine	C01672	0.52 ± 0.12	0.75 ± 0.30	
Carnitine	C00318	24.71 ± 3.28	22.01 ± 3.01	
Carnosine	C00386	83.46 ± 23.57	100.56 ± 10.53	< 0.1
Citrate	C00158	1.25 ± 0.63	1.56 ± 0.42	
Creatine + Creatine phosphate	C00300/C02305	276.39 ± 45.49	237.98 ± 32.81	
Creatinine	C00791	10.99 ± 11.72	19.34 ± 5.71	
Formate	C00058	0.52 ± 0.17	0.42 ± 0.05	
Fumarate	C00122	0.08 ± 0.04	0.04 ± 0.01	
Glucose	C00031	9.75 ± 6.98	1.49 ± 0.39	< 0.05
Glucose-1-phosphate	C00103	2.74 ± 2.34	0.47 ± 0.32	
Glucose-6-phosphate	C00668	8.64 ± 7.19	0.74 ± 0.59	< 0.05
Glutamate	C00025	4.20 ± 1.48	6.21 ± 1.15	< 0.05
Glutamine	C00064	8.95 ± 1.91	5.76 ± 1.63	< 0.05
Glycine	C00037	26.58 ± 8.75	34.47 ± 13.82	
GTP	C00044	0.50 ± 0.07	0.57 ± 0.09	
Isoleucine	C00407	0.43 ± 0.23	0.64 ± 0.12	< 0.1
Lactate	C00186	307.11 ± 235.50	41.62 ± 12.14	< 0.05
Leucine	C00123	0.51 ± 0.13	0.98 ± 0.22	< 0.01

Mannose	C00159	3.29 ± 2.65	0.38 ± 0.17	< 0.05
Methylmalonate	C02170	2.19 ± 1.63	0.35 ± 0.10	< 0.05
myo-Inositol	C00137	3.25 ± 0.60	2.88 ± 0.37	
NAD+	C00003	1.38 ± 0.26	1.50 ± 0.36	
NADH	C00004	0.21 ± 0.11	0.36 ± 0.07	< 0.05
NADP+	C00006	0.06 ± 0.01	0.11 ± 0.02	< 0.01
Nicotinurate	C05380	1.95 ± 0.59	1.35 ± 0.22	< 0.1
N,N-Dimethylglycine	C01026	0.75 ± 0.23	0.55 ± 0.12	
O-Acetylcarnitine	C02571	2.49 ± 1.21	4.10 ± 4.85	
Phenylalanine	C00079	0.30 ± 0.09	0.37 ± 0.11	
Pyruvate	C00022	0.67 ± 0.20	0.49 ± 0.23	
Taurine	C00245	9.40 ± 2.51	8.75 ± 2.04	
Threonine	C00188	3.65 ± 1.58	2.26 ± 0.99	< 0.1
Tryptophan	C00078	0.17 ± 0.02	0.16 ± 0.01	
Tyrosine	C00082	0.36 ± 0.05	0.41 ± 0.09	
UMP	C00105	0.27 ± 0.05	0.35 ± 0.05	< 0.05
Valine	C00183	0.94 ± 0.15	1.07 ± 0.18	

Ctrl, control diet; Leu, control diet supplemented with 0.3% leucine. *p*-value was calculated using Wilcoxon test.

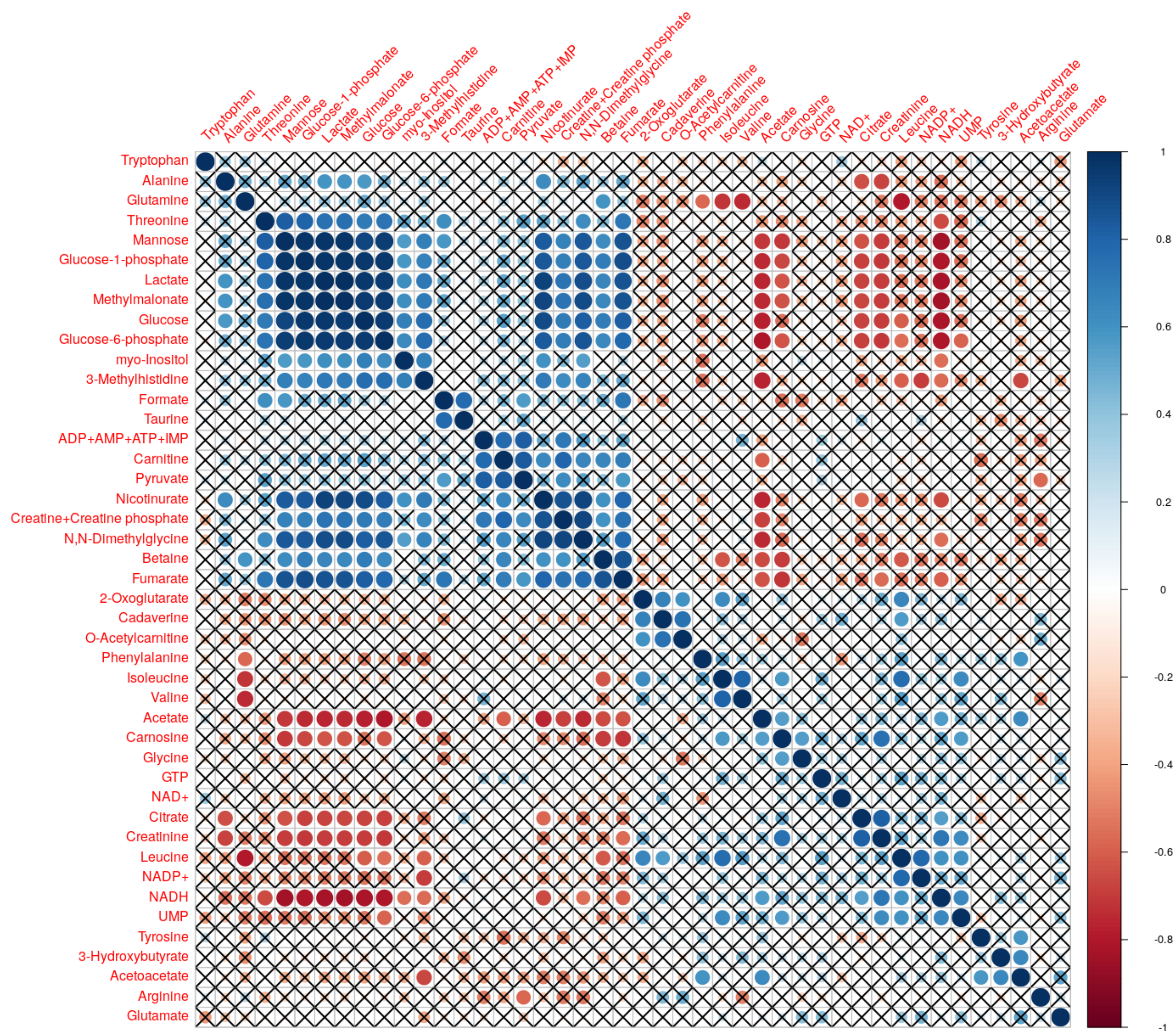
Table 16. Mean metabolite concentrations (mean ± SD) in serum (µM).

Metabolite	KEGG ID	Ctrl	Leu	*p*
2-Oxobutyrate	C00109	6.93 ± 1.88	8.20 ± 1.77	
2-Oxoisocaproate	C00233	4.09 ± 0.70	3.68 ± 1.24	
3-Hydroxybutyrate	C01089	44.73 ± 10.86	42.67 ± 9.90	
3-Hydroxyisobutyrate	C06001	9.34 ± 2.10	10.98 ± 2.19	
Acetate	C00033	142.66 ± 36.44	214.77 ± 47.76	< 0.05
Alanine	C00041	88.86 ± 8.77	78.47 ± 19.42	
Arginine	C00062	79.74 ± 17.47	90.58 ± 11.96	
Asparagine	C00152	25.24 ± 3.75	25.67 ± 5.96	
Aspartate	C00049	7.26 ± 1.95	11.72 ± 3.29	< 0.01
Betaine	C00719	111.79 ± 21.22	91.87 ± 9.82	
Carnitine	C00318	5.76 ± 0.66	10.10 ± 1.85	< 0.01
Choline	C00114	16.06 ± 7.42	11.93 ± 1.50	< 0.05
Citrate	C00158	60.74 ± 13.38	49.65 ± 8.76	
Citrulline	C00327	39.16 ± 8.19	48.90 ± 9.24	< 0.1
Creatine + Creatine phosphate	C00300/C02305	64.99 ± 12.85	42.63 ± 14.75	< 0.05
Creatinine	C00791	15.86 ± 3.71	19.52 ± 4.78	
Formate	C00058	35.59 ± 6.26	47.10 ± 12.23	< 0.05
Fructose	C02336	119.11 ± 27.09	151.45 ± 32.37	
Galactose	C00984	24.76 ± 31.93	275.08 ± 191.37	< 0.01
Glucose	C00031	851.30 ± 235.00	1041.67 ± 116.65	< 0.1
Glutamate	C00025	62.57 ± 4.26	59.13 ± 9.28	
Glutamine	C00064	52.77 ± 14.41	48.17 ± 12.01	
Glycine	C00037	159.83 ± 25.68	140.97 ± 35.55	
Histidine	C00135	10.87 ± 3.21	9.70 ± 2.38	
Isoleucine	C00407	39.70 ± 5.93	42.62 ± 9.98	

Lactate	C00186	1223.03 ± 243.55	597.38 ± 30.98	< 0.01
Leucine	C00123	64.06 ± 10.47	87.52 ± 14.47	< 0.05
Lysine	C00047	38.30 ± 10.55	39.15 ± 13.15	
Malonate	C00383	38.27 ± 8.61	48.15 ± 9.26	< 0.1
Mannose	C00159	22.66 ± 3.10	26.97 ± 3.35	< 0.1
Methionine	C00073	15.36 ± 2.70	16.08 ± 5.35	
Methylsuccinate	C08645	4.46 ± 1.06	2.68 ± 0.45	< 0.01
myo-Inositol	C00137	24.83 ± 4.33	26.90 ± 4.73	
Nicotinurate	C05380	3.36 ± 1.64	2.78 ± 0.97	
N-Methylhydantoin	C02565	3.70 ± 1.34	5.63 ± 2.25	< 0.1
Ornithine	C00077	14.46 ± 8.03	12.78 ± 4.71	
Phenylalanine	C00079	21.17 ± 2.70	24.35 ± 6.01	
Proline	C00148	53.79 ± 4.23	55.48 ± 13.20	
Pyroglutamate	C01879	74.13 ± 25.80	78.87 ± 14.53	
Pyruvate	C00022	9.86 ± 3.14	7.05 ± 2.36	
Serine	C00065	51.36 ± 6.34	42.42 ± 10.88	
Threonine	C00188	66.43 ± 13.23	77.88 ± 14.83	
trans-4-Hydroxy-L-proline	C01157	32.46 ± 5.41	33.43 ± 15.05	
Trimethylamine	C00565	4.34 ± 2.92	4.00 ± 2.02	
Tryptophan	C00078	15.41 ± 2.53	11.65 ± 1.86	< 0.05
Tyrosine	C00082	20.64 ± 4.51	21.42 ± 5.72	
Urea	C00086	119.61 ± 31.60	115.55 ± 30.55	
Valine	C00183	59.11 ± 9.46	63.38 ± 15.50	

Ctrl, control diet; Leu, control diet supplemented with 0.3% leucine. *p*-value was calculated using Wilcoxon test.

A



B

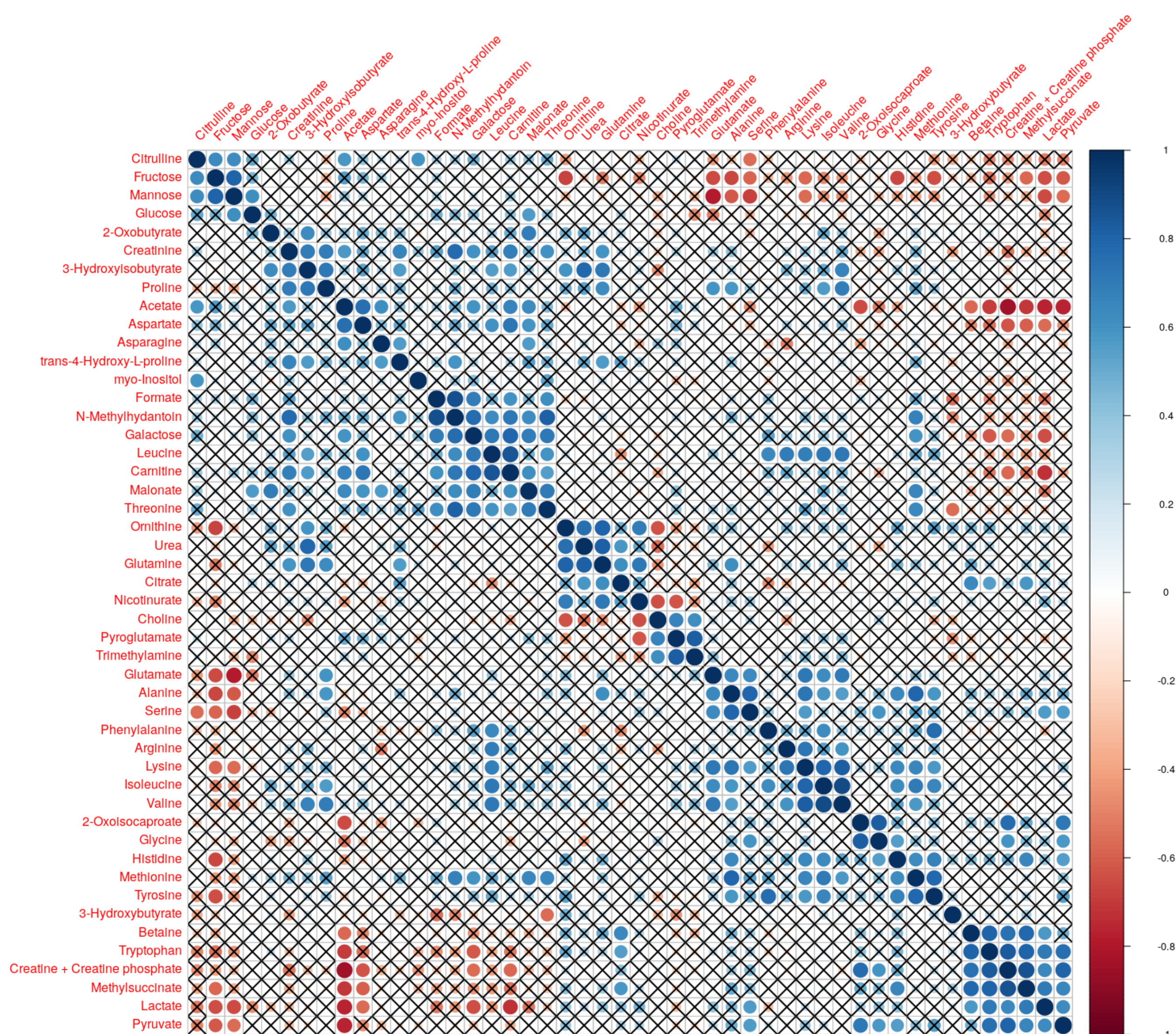


Figure 9. Correlation plots of quantified metabolites in muscle (**A**) and serum (**B**). Blue and red colors indicate stronger positive and negative correlation, respectively. The bigger size of the circle represents the higher absolute correlation (approaching to 1) and smaller suggest the opposite (approaching to 0). The Pearson r correlations were calculated and significant level was set $p < 0.05$, for non-significant correlation, a cross is marked on top of the circle. Compounds are grouped by hierarchical clustering order.

Since important differences were observed between Ctrl and Leu groups in glycolytic metabolites as serum lactate or muscle glucose, an interference from the biopsy day was suspected. As stated above, individuals in the Leu group were not properly randomized between biopsy days (all individuals in Leu group were sampled in the 3rd biopsy day, whereas those in the Ctrl were randomly assigned to 1st and 2nd biopsy days). Thus, a deeper study of the influence of the biopsy day was performed.

The PCAs of quantified metabolites with their corresponding loadings based on the effect of treatment are shown in Figure 10 (A, C) for muscle and serum, respectively. In order to investigate the biopsy effect, PCA with the same data set but based on the effect of biopsy for muscle and serum are shown in Figure 10 (B, D).

For the effect of treatment, verification of consistency with the original data was performed by matching PCA analyses of chemometrics from both biological samples (Figure 11).

In both muscle and serum, Leu group (3rd biopsy) were found to be separated from Ctrl (1st and 2nd biopsies). From the general aspect of loadings in both muscle and serum, it can be observed that several AA were tilted towards to the Leu group. However, several muscle metabolites such as lactate, several sugars (glucose, glucose-6-phosphate and mannose), formate, threonine and methylmalonate were tilted to the opposite direction of Leu group; in serum, four sugars (glucose, mannose, fructose and galactose) were found to be tilted towards Leu.

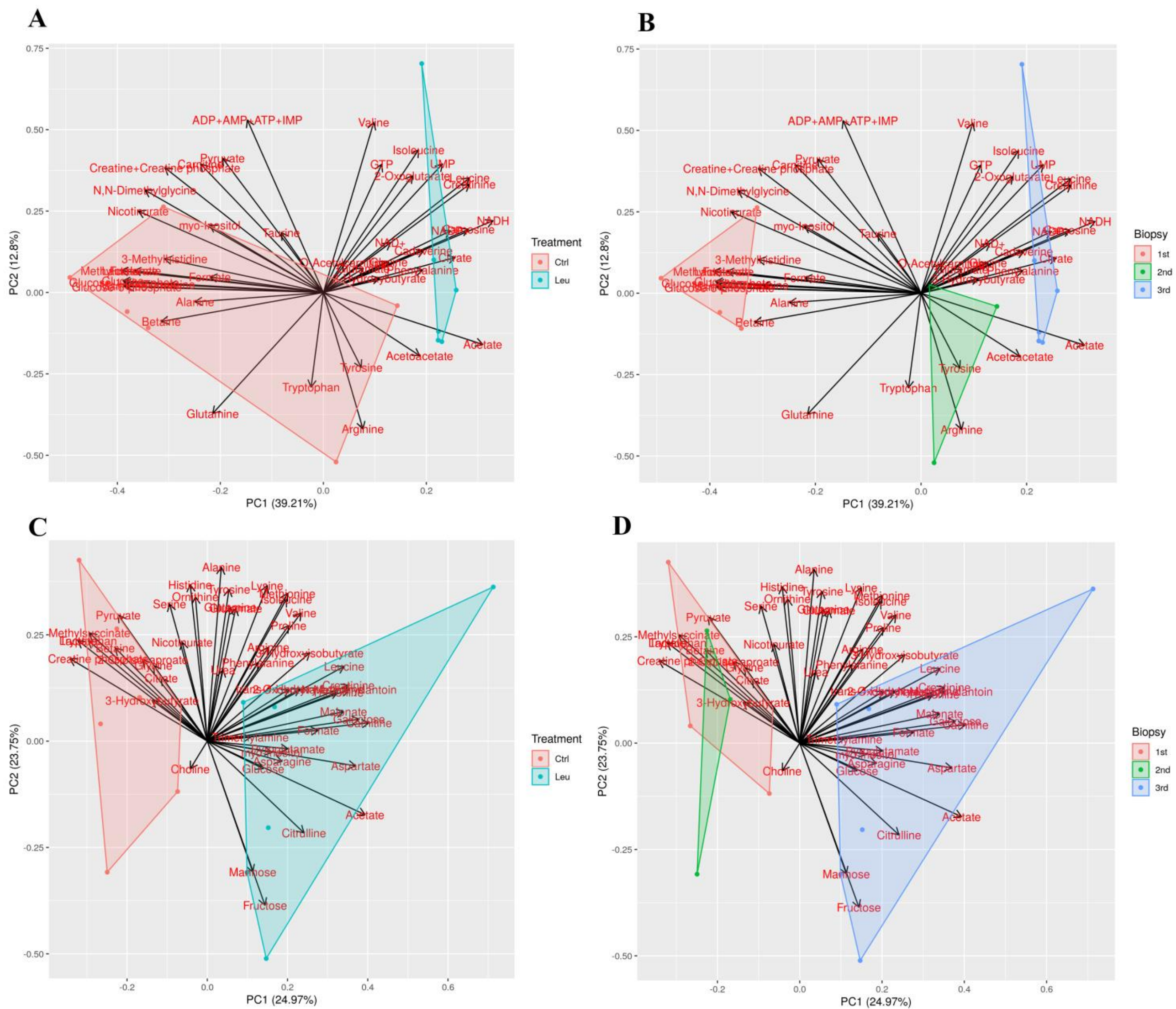


Figure 10. PCA with loadings of muscle (**A** and **B**) and serum (**C** and **D**) according to treatment (**A** and **C**) and biopsy (**B** and **D**) of quantified metabolites in Ctrl - Leu (1st - 2nd - 3rd biopsy) comparison.

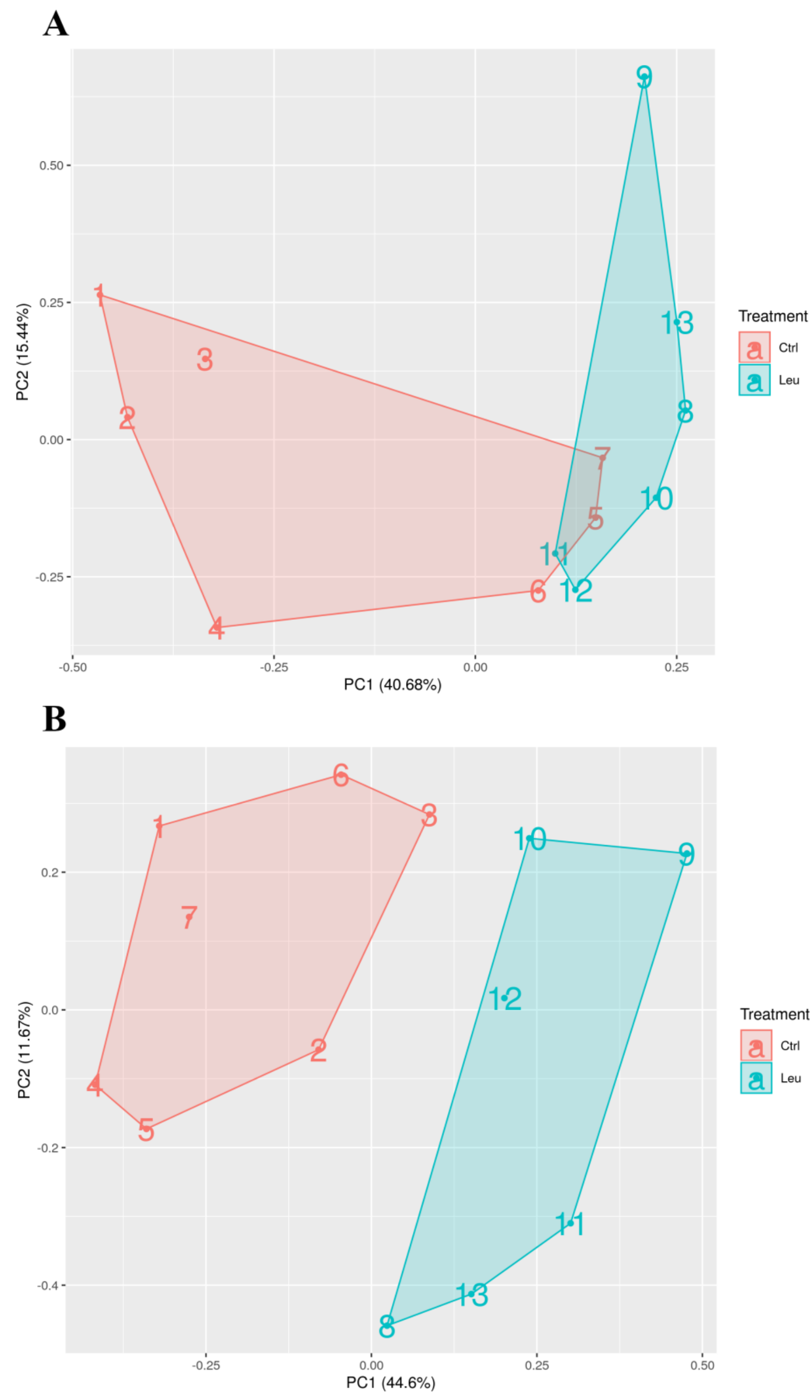


Figure 11. PCA with sample identifiers of **A**. muscle chemometrics (495 bins); **B**. serum chemometrics (216 bins). Ctrl, control diet; Leu, control diet supplemented with 0.3% leucine.

Pathway analysis

The Reactome pathway analysis for proteins yielded several significant pathways, in accordance with the GO ontology. The Reactome analysis for small molecules (KEGG nomenclature) also gave coherent results.

Ctrl – Leu pathways

In skeletal muscle, the Reactome pathway analysis indicated that Beta oxidation (R-HSA-77346, R-HSA-77286, R-HSA-77289, etc.) and Branched-chain amino acid catabolism (R-HSA-70895) were the main upregulated pathways. Glycogen synthesis (R-HSA-3322077) and Metabolism of carbohydrates (R-HSA-71387) were the main downregulated pathways.

In serum, the Reactome pathway analysis identified innate immune system (R-HSA-168249), platelet degranulation (R-HSA-114608) and hemostasis (R-HSA-109582) as the most important upregulated pathways. Regulation of Complement cascade (R-HSA-977606) and innate immune system (R-HSA-168249) for proteins and Creatine metabolism (R-HSA-71288) for metabolites were the most downregulated pathways.

In the joint pathway analysis from MetaboAnalyst, upregulated and downregulated pathways are shown in Table 17 and Table 18. In the upregulated pathways, butanoate and glutathione metabolisms, valine, leucine and isoleucine degradation were found in the muscle and aminoacyl-tRNA biosynthesis was found in blood; for the downregulated pathways, galactose and starch and saccharose metabolism, glycolysis/gluconeogenesis and pentose phosphate pathways were found in muscle.

Table 17. Joint pathway analyses of upregulated proteins and metabolites in muscle and blood Ctrl – Leu comparison according to the p from the pathway enrichment analysis and pathway impact values from the pathway topology analysis. The higher the -log(p) is, the more significance is the pathway; the higher the impact is, the higher percentage of hit compounds are involved in overall compounds.

Pathway	**Muscle (6 proteins * and 5 metabolites)**							
	Protein	Metabolite	hit	p	-log(p)	Holm p	FDR	Impact
Butanoate metabolism	HADHA	Glutamate	2/47	0,015	4,2	1	0,73	0,16
Glutathione metabolism	-	NADP, Glutamate	2/75	0,038	3,3	1	0,73	0,15
D-Glutamine and D-glutamate metabolism	-	Glutamate	1/9	0,038	3,3	1	0,73	0,43
	Blood (17 proteins § and 6 metabolites)							
Aminoacyl-tRNA biosynthesis	-	Leucine; Aspartate	2/87	0,034	3,4	1	1	0,03

* LOC524236 was converted to HIST1H2AC for homo sapiens, which shows 97% identities and 97% positives and 0% gaps.

§ LOC515150 was converted to C4BPA for homo sapiens, which shows 54% identities and 74% positives and 0% gaps.

Table 18. Joint pathway analyses of downregulated proteins and metabolites in muscle in Ctrl – Leu comparison according to the p from the pathway enrichment analysis and pathway impact values from the pathway topology analysis. The higher the -log(p) is, the more significance is the pathway; the higher the impact is, the higher percentage of hit compounds are involved in overall compounds.

Pathway	**Muscle (11 proteins and 8 metabolites)**							
	Protein	Metabolite	hit	p	-log(p)	Holm p	FDR	Impact
Galactose metabolism	PGM1	Glucose, Glucose-6-phosphate, Mannose	4/55	3.90E-04	7.8	0.031	0.031	0.35
Glycolysis / Gluconeogenesis	PGM1, GPAM1	Glucose-6-phosphate, lactate	4/91	0.003	5.9	0.21	0.1	0.34
Starch and sucrose metabolism	PGM1	Glucose, Glucose-6-phosphate	3/78	0.015	4.2	1	0.38	0.26
Amino sugar and nucleotide sugar metabolism	PGM1	Glucose-6-phosphate, Mannose	3/84	0.019	4	1	0.98	0.29
Pentose phosphate pathway	PGM1	Glucose-6-phosphate	2/48	0.044	3.1	1	0.66	0.2

Discussion

Being aware of the problems raised in the Leu experiment (ill animals and not randomized biopsy day), the effects due to Leu supplementation, to the disease appearance and to the biopsy will be separately analyzed in the present Discussion. The results obtained in the comparison between Ctrl and LT groups previously described in Annex 1 (with no inherent methodological issues and all the analytical work made in parallel with the L group) will be taken into account for the discussion.

Changes observed in proteins will be mostly due to long term effects (Leu treatment or pathogens), whereas metabolites, especially those related with glycolysis, will be mostly affected by the biopsy.

Efficiency of leucine supplementation, its catabolism and metabolic effects in the Leu group

Leucine was found significantly elevated both in muscle and serum, and the quantification proved by the original chemometrics files (partially shown in Figure 12).

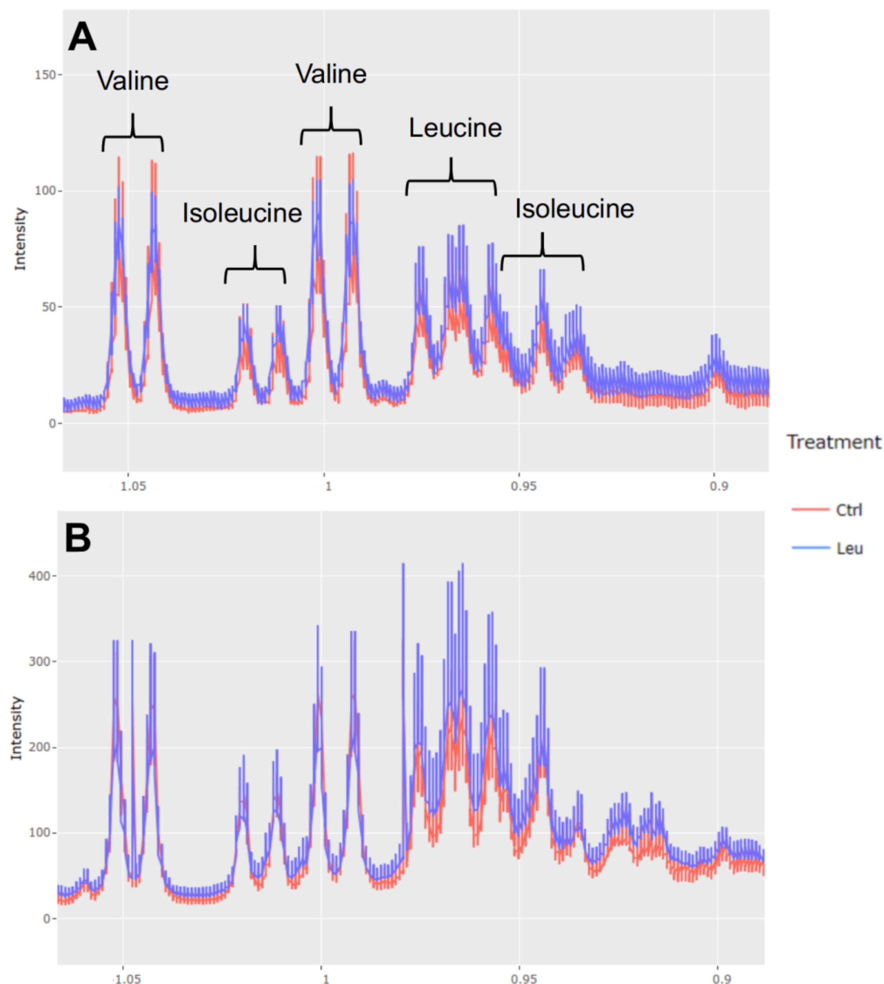


Figure 12. Reconstructed mean spectral densities of each treatment from chemometrics data from muscle (**A**) and serum (**B**). Only chemical shifts of valine, leucine and isoleucine (δ 0.9 – 1.1) were shown. Ctrl, control diet; Leu, control diet supplemented with 0.3% leucine.

Both Reactome and MetaboAnalyst showed upregulated BCAA catabolism in the muscle, suggesting the general availability of leucine as well as its ongoing catabolism after leucine supplementation.

The increase in the Beta oxidation pathway and the concentration of serum carnitine, known to stimulate fatty acid oxidation (Bremer 1983), are in accordance with the higher rate of Acetyl CoA oxidation observed in the Leu/Thr compared with Ctrl (Annex 1). Similarly, butanoate and glutathione metabolisms, valine, leucine and isoleucine degradation were found upregulated in the muscle and aminoacyl-tRNA biosynthesis was found in blood.

Disease-associated biomarkers in the plasma of the Leu group

As it was previously mentioned, animals in this group suffered digestive and/or respiratory diseases. At the time of biopsy, the individuals were apparently recovered (visible symptoms had disappeared around 22 days of age), but they were smaller than the rest of animals. The specific pathogen was not identified but this is a frequent situation in commercial farms. The most relevant proteomic changes observed in plasma are characteristic of a general infectious or inflammatory condition: Immune-related biological processes such as acute phase response (GO:0006953), complement system (GO:0006957 and GO:0006958) and oxidative stress (GO:0006979) were found elevated. Likewise, immune and coagulation pathways were found altered in Reactome. It is interesting to note that these inflammatory markers are still increased even when clinical symptoms have disappeared.

Acute phase proteins as SAA1, SAA2 and SAA3 were elevated as well as fibrinogen, a well-known inflammatory marker in large animals. Albumin, a negative acute phase protein, is decreased in the Leu group (Petersen et al. 2004; Cray et al. 2009). The complement system, as part of the innate immune system, underlies one of the main effector mechanisms of antibody-mediated immunity and involves more than 30 proteins in the blood (Walport 2001). Since several complement proteins are cleaved and activated during the activation of the system, the general mechanism involves the upregulation as well as downregulation of different complement components. Finally, the oxidative stress marker GPx was found elevated in the proteomic analysis and in the conventional biochemical analysis (which serves as validation of this marker) and Phospholipase A2 is also involved in inflammation (Rosenson and Stafforini 2012).

Biopsy/temperature-induced bias in the muscle of Leu group

Obvious separations of PCA profiles between Ctrl and Leu groups (quantified: Figure 10 and chemometrics: Figure 11) based on treatment and biopsy effect were noticed in both muscle and serum samples.

In muscle of Ctrl - Leu, several sugars (glucose, glucose-6-phosphate and mannose), alanine and lactate were found showing loading effects towards the 1st biopsy, and on the opposite direction in the 3rd biopsy (accordingly, these metabolites were decreased in Leu according to Table 15). The differences in glucose and glucose 6-P were probably associated to changes in the glycolytic flux due to differences in the duration of the biopsy procedure. During biopsy, as the tissue is removed from the body, due to the supply cut of blood that contains oxygen, ischemia happens and cells become dependent on anaerobic glycolysis for the ATP supply, which leads to the accumulation of lactate, protons and NAD+ (Baines 2009; Kalogeris et al. 2012). Thus, the higher sugars as well as upregulated sugar metabolism, alanine and lactate concentrations in the first biopsies are due to a prolonged biopsy time due to lack of experience. Our hypothesis was proved as we recorded the post-biopsy ischemia intervals (time between tissue is cut from the body and properly snap-frozen), resulting mean intervals of 64 ± 4.8 s for the 1st biopsy; 51 ± 5.7 s for the 2nd biopsy and 35 ± 2.9 s for the 3rd biopsy. That means that the decrease in sugar metabolism in the Leu group (Table 18) was probably due to an increased glycolytic rate in the Ctrl group (biopsies 1^{rst} and 2nd) compared to the Leu group (3rd biopsy).

While in muscle the three biopsies were clearly separated, in serum the 1st and 2nd biopsies were overlapping and the 3rd biopsy was isolated from the previous two. Yet, the ischemia effect caused by the biopsy should not interfere in the serum as blood was equally taken 4 h after the morning feeding and before the biopsy. However, as the season/temperature may also affect the metabolism, we queried the temperature information of Monells from http://www.meteo.cat/observacions/xema, and the registered weather (temperature / humidity) of 1st, 2nd and 3rd biopsy days were 17 °C / 82%, 19.1 °C / 77% and 24,4 °C / 57%, respectively. Interestingly, glucose, mannose, fructose and galactose were elevated in Leu and are responsible for the separation between Ctrl – Leu. It was previously reported that the blood glucose is elevated in higher temperature seasons in several species (Suarez and Barrett-Connor 1982; Antunović et al. 2002; Sakatani et al. 2012). This suggests that a relationship may exist between the higher temperature in the 3rd biopsy day and differences in metabolites.

Conclusion

In the Leu group and in comparison with the Ctrl group, animals were affected by complex metabolic processes. On one hand, supplemented leucine was available in muscle and blood, and an increase of leucine catabolism was noticed. On the other hand, disease-associated biomarkers that participate in innate immune and coagulation pathways were predominant identified in plasma by proteomic approaches, and pathogen-unidentified diseases were confirmed by veterinarians. Lastly, at least biopsy, temperature and diet effects caused the Leu group be separated from Ctrl. Since the Leu group samples were obtained the same biopsy day rather than properly randomized with Ctrl, it is extremely difficult to discover/separate these effects, thus making it impossible to further discuss the sole dietary effect of the Leu group. This illustrates the precautions that must be taken for animal and sample handling to achieve a correct interpretation of the -omics results.

9.1. Annex 3: Skeletal muscle proteomics analysis reveals changes associated with dietary amino acid supplementation in unweaned calves

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This work is complementary to the results presented in Publication 1 ("Skeletal muscle metabolomics and blood biochemistry analysis reveal metabolic changes associated with dietary amino acid supplementation in dairy calves". Yu K, Matzapetakis M, Valent D, Saco Y, De Almeida AM, Terré M, Bassols A. Sci Rep. 2018;8(1):13850. doi: 10.1038/s41598-018-32241-4).

Introduction

As the muscle tissue is the fastest growing tissue in young animals (Drackley 2008), amino acids (AA) are indispensable resource for the protein accumulation in the muscle and the lack of certain AA, especially limiting AA, may cause growth delay and even higher mortality rate which can threat the animal welfare as well as the farm production. Among the AA for preruminant calves, methionine (M), lysine (K) and threonine (T) are the most studied not only because these are important proteinogenic AA, but also many studies considered them limiting especially in MR from soybean proteins (Pelaez and Walker 1979; Jenkins and Emmons 1983; Kanjanapruthipong 1998; Hill et al. 2008). While some evidences suggest that supplementing these AA regardless of the MR type showed better performances (Hill et al. 2008; Yu et al. 2018), other studies reported that no improvement were observed in MR supplemented with methionine (Castro et al. 2016) or threonine and isoleucine (Morrison et al. 2017). Another two AA: phenylalanine (F) and tyrosine (Y) are less studied in farm animal, however, these two AA are important as, besides participating in the role as constituents of protein, they are crucial for the neurological system as tyrosine is the precursor for catecholaminergic neurotransmitters (dopamine, norepinephrine and epinephrine) (Fernstrom and Fernstrom 2007) and phenylalanine, an essential AA, can synthesize tyrosine. Studies have shown that serum catecholamine concentration is correlated with availability of tyrosine, and subsequently can affect behavior in dogs (Gazzano et al. 2018) and rats (Yeghiayan et al. 2001), suggesting that phenylalanine and tyrosine may contribute to the improvement of neurological function.

Mass spectrometry (MS)-based proteomics is considered nowadays the principal method to investigate biological process and to discover biomarkers in farm animal science and veterinary medicine (Almeida et al. 2014; Bilić et al. 2018; Horvatić et al. 2019). We previously presented a metabolomic study of supplementations of methionine, lysine and threonine (MKT) and phenylalanine and tyrosine (FY) in milk replacer (MR) for preruminant calves (Yu et al. 2018), in which we found that, despite no major differences were found between groups, a selection of reduced quantified metabolites suggested that FY and MKT groups had different metabolic profiles compared with Ctrl. Thus in this

study, we conduct a proteomic approach, aiming to elucidate a clearer profile of the effects of these dietary AA in the young animal.

Following this previous study, the objective of the present work was to determine the effects of MR diets supplemented with 1) methionine, lysine and threonine and 2) phenylalanine and tyrosine fed to preruminant calves 3 days to 7 weeks old in skeletal muscle biopsies by using proteomics to fully understand the effects of these AA's on preweaning animals. Furthermore, the activation of the mTORC1 pathway, a key pathway in protein synthesis, was studied in skeletal muscle by determining the phosphorylation state of the S6 ribosomal protein.

Material and Methods

Animal and housing, diet treatments and performance recordings, blood and muscle sample collection are the same as the ones from Publication 1 except that the GP group was excluded. The proteomic analysis and protein immunoblot for S6 and Phospho-S6 in skeletal muscle biopsies follow the methods described in Annex 1.

Serum Leptin, Glucagon and Insulin

Serum leptin was detected using radioimmunoassay (RIA) from Multi-Species Leptin RIA kit (Cat. #XL-85K, EMD Millipore, MO, USA), glucagon and insulin were detected using commercial ELISA kits (Mercodia Glucagon ELISA and Mercodia Bovine Insulin ELISA, Uppsala, Sweden). Leptin and glucagon assays are compatible with bovine as declared by the manufacturers. Intra-assay coefficients of variation (CV) were: insulin (4.1%), leptin (3.4%) and glucagon (6.7%).

Statistics

All statistical analyses were performed using homemade code in R (version 3.5.2) under RStudio graphic interface (Open Source License, version 1.2.1335), R build-in tools as well as “dplyr” (Wickham et al. 2019) and “outliers” (Lukasz Komsta 2011) packages were used for statistical analyses and “ggplot2” package (Wickham 2016) was used for graphic processes. The interquartile fence is set between $[Q1-3*IQR, Q3+3*IQR]$ and values outside the fence are considered outliers and are removed. For statistical difference comparisons, pair-wise wilcoxon non-parametric test was used and p-value <0.05 is considered significant. For muscle proteome, wilcoxon p-value <0.05 and absolute value of fold change that is ≥ 0.10 were considered significant where fold change between two groups was calculated using the function of log2(Mean(Treatment)/Mean(Ctrl)) where Treatment is either FY or MKT.

Results

Hormonal status in AA-supplemented calves

The levels with statistical *p*-values of serum insulin, glucagon, leptin, insulin/glucagon and leptin/glucagon are shown in Figure 13. Insulin was found elevated with statistical tendency ($p = 0.093$) in the MKT group compared with Ctrl.

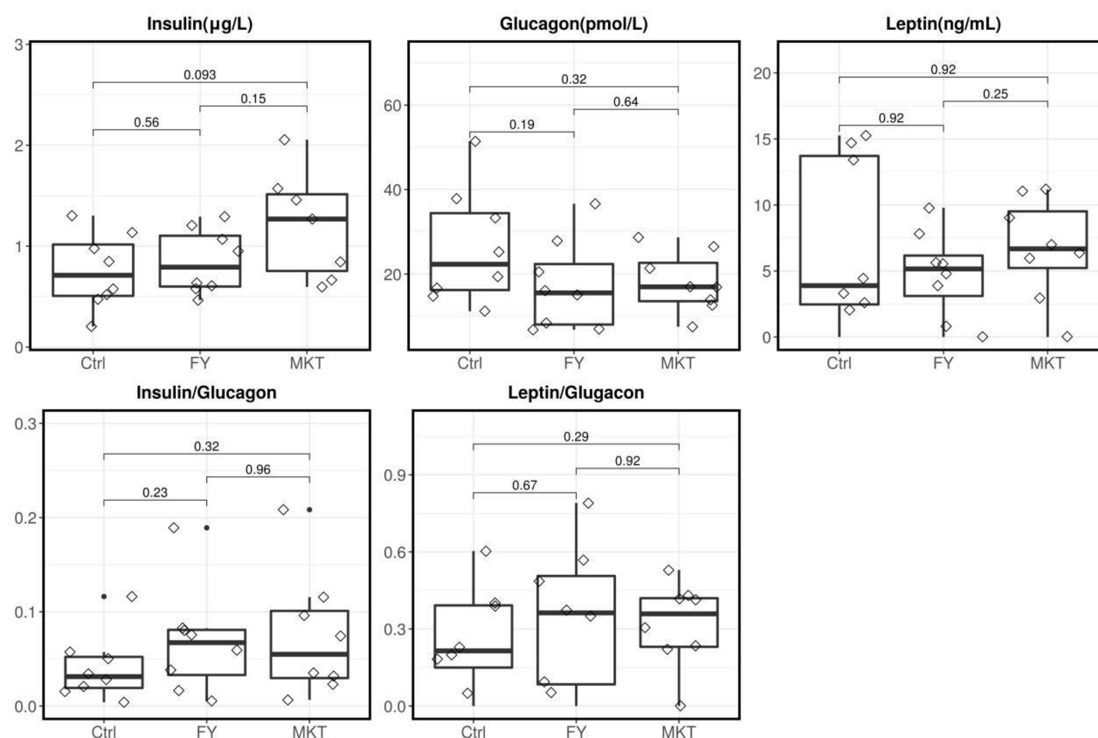

Figure 13. Boxplots with *p*-values of pair-wise Wilcoxon non-parametric comparison of insulin, glucagon and leptin, insulin/glucagon and leptin/glucagon.

Phospho-S6/S6 ratio state

The phosphorylation status of the S6 protein was determined in skeletal muscle biopsies by western blot. The boxplot of Phospho-S6/S6 ratio is shown in Figure 14. The Phospho-S6/S6 ratio was found decreased in the FY group compared with Ctrl ($p = 0.083$) and MKT ($p = 0.014$). No statistical difference was found between Ctrl and MKT.

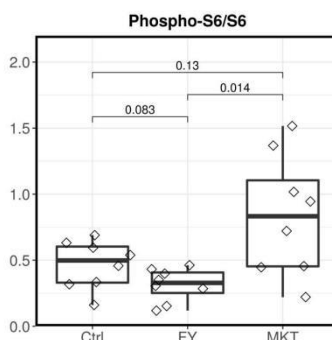

Figure 14. Boxplots with *p*-values of pair-wise Wilcoxon non-parametric comparison of Phospho-S6/S6 ratios.

Proteomic approach to the characterization of the effects of AA supplementation on skeletal muscle

After TMT-labeling and MS analysis, 1153 quantifiable proteins were identified. Differentially abundant proteins between Ctrl - MKT and Ctrl - FY groups are shown in Table 19 and Table 20, respectively. Three groups of proteins: serpin A3 (SERPINA3-1 and SERPINA3-2), myosin (MYH6 and MYH7) and enigma protein (PDLIM7) were found elevated in MKT group compared with Ctrl. In terms of molecular function in GO analysis, MYH6 and MYH7 are in actin filament binding (GO:0051015) and ATP binding (GO:0005524) while PDLIM7 is in actin binding (GO:0003779); SERPINA3-1 and SERPINA3-2 are responsible for serine-type endopeptidase inhibitor activity (GO:0004867). In biological process, MYH6 is responsible for muscle filament sliding (GO:0030049), PDLIM7 for muscle structure development (GO:0061061) and SERPINA3(-1 and -2) are in negative regulation of endopeptidase activity (GO:0010951). In the comparison between Ctrl and FY, only SERPINA3-7 was found decreased in the FY.

Table 19. Proteins with significantly differential abundances on MKT/Ctrl.

Accession NCBI	Gene Symbol (bos taurus)	Protein name	p-value	Fold change
86438018	SERPINA3-1	Serpin A3-1	0,02	0,24
121531626	SERPINA3-2	Serpin A3-2	0,02	0,24
41386711	MYH7	Myosin-7	0,04	0,12
1387195856	MYH6	Myosin-6	0,04	0,11
120419518	PDLIM7	Enigma protein	0,04	0,11

Ctrl, control diet; MKT, control diet supplemented with 0.62% lysine plus 0.22% methionine plus 0.61% threonine. *p*-value was calculated using Wilcoxon test and FC (fold change) using the function of log2(Mean(MKT)/Mean(Ctrl)).

Table 20. Proteins with significantly differential abundances between on FY/Ctrl.

Accession NCBI	Gene Symbol (bos taurus)	Protein name	p-value	Fold change
121531634	SERPINA3-7	Serpin A3-7	0,02	-0,17

Ctrl, control diet; FY, control diet supplemented with 0.2% phenylalanine plus 0.2% tyrosine. *p*-value was calculated using Wilcoxon test and FC (fold change) using the function of log2(Mean(FY)/Mean(Ctrl)).

Discussion and Conclusion

The previous analysis performed in the same groups of animals (Yu et al. 2018) did not show significant differences between groups, although a statistical tendency for higher ADG was observed in MKT group compared with Ctrl ($p = 0.096$) and FY groups ($p = 0.097$). Furthermore, the metabolomic analysis indicated that indeed some metabolic changes occurred in skeletal muscle, especially in the MKT and FY groups. As the growth in young animal mainly occurs in the skeletal muscle (Drackley 2008), we used a proteomic approach to further analyze the undergoing dietary effects.

Among the several hormones that were analyzed in serum, only insulin was found elevated with statistical tendency in the MKT group. Insulin is the primary anabolic hormone (Hay 2007; Hay 2008), and elevated plasma insulin has been associated with higher protein synthesis in skeletal muscle (Fujita et al. 2006). This result shows consistency with the growth performance of MKT. Moreover, the mean Phospho-S6/S6 ratio in skeletal muscle extracts in the MKT group was the highest among the three groups despite no statistical difference was found between MKT and Ctrl due to the high variability in MKT. Thus several potential indications of enhanced muscle tissue growth were observed. To better understand the functionality, we characterized the proteome in skeletal muscle biopsies of MKT and FY calves.

The upregulated proteins in the muscle of the MKT group can be categorized into three groups: myosin heavy chain (MYH), PDLIM7 and serpin A3, where MYH and PDLIM7 interact with actin filaments (Niederländer et al. 2004). Myosin is one of the major proteins in contractile fibers, and many studies have highlighted its importance in muscular function. For example, it has been described *in vivo* the positive regulation of mTOR/p70^{S6K} and MYH in rat myocytes (Boluyt et al. 2004), while another *in vitro* study describes a positive relationship between depletion of MYH and atrophy in skeletal muscle (Clarke et al. 2007). For PDLIM7, a 2DE-based proteomic study identified this protein as upregulated in muscle of zebrafish (*Dario renio*) fed with a high-lysine diet (de Vareilles et al. 2012). In terms of PDLIM7 functionality, the PDZ domain binds to actin filaments whereas the LIM domains bind to protein kinases (Guy et al. 1999). As it

participates in the muscle fiber structure, it is assumed that PDLIM7 is positively related with the skeletal muscle development. Related to serpin A3, a relationship with muscle growth or dietary supplementation has not been described, to our best knowledge. Serpin A3 belongs to the serpin superfamily of protease inhibitors and it is encoded by a cluster of eight closely related genes in cattle (Pere-Brissaud et al. 2015). The functionality of serpins remain unclear, but they may be involved in blood coagulation, fibrinolysis, cell migration and hormone transport (Silverman et al. 2001; Gettins 2002). Yet, more research is needed to clarify its relationship with the supplemented AA, if any.

To conclude, the tendency to higher insulin, non-significant higher mTOR level, and upregulated MYH and PDLIM7 may suggest a better muscular function leading to a general improved performance due to MKT supplementation. However, as the all the changes are marginal, it may indicate that the basal diet (Ctrl) already meets most of the requirements for an optimal muscular growth in comparison with the supplemented diet.

In the FY group, the Phospho-S6/S6 ratio was significantly lower compared with MKT. This result may be in consonance with the previous results as FY showed non-significant lowest ADG. In the proteomic analysis, the only change was the downregulation of SERPINA3-7. Altogether, it can be concluded that the basal Ctrl diet already meets most of the optimal requirements for calf growth, and that the additional phenylalanine and tyrosine may have a negligible effect.

In conclusion, the proteomic approach revealed few proteome adaptations. In the case of MKT, evidences indicate that AA supplementation can help muscle development, whereas FY does not alter the muscular proteome.

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