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DairyCare COST Action

Scientific Report for Short-term Scientific Mission (STSM)

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STSM Topic:	Metabolomic Study of Amino Acids Supplementation in Dairy Calves
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1. Project description and aim:

This project aims to study the changes on the metabolic composition of semitendinosus muscle from calf groups fed with milk replacer formulas and three supplementations with amino acids: ***A)*** control diet with milk replacer (*Control*); ***B)*** addition of 14.07% proline and18.75% glycine (*Gly+Pro*); ***C)*** addition of 19.19% phenylalanine and 19.79% tyrosine(*Phe+Tyr*); ***D)*** addition of 18.50%lysine, 37.04%methionine and 37.69%Threonine (*Lys+Met+Thr*). Muscle biopsy samples were obtained 8 weeks after the treatment and kept immediately on dry ice. The age of calves on the biopsy day was in the range of 44 – 55 days old with an average of 47 days. The metabolomic study of muscle tissues was carried out using NMR spectroscopy.

2. Description of the work during STSM:

2.1. Sample preparation and data acquisition

Muscle tissue was ground into fine powder using a mortar and pestle in liquid nitrogen. The extraction of the aqueous metabolites was performed using chloroform/methanol method. In brief: 1.2 ml of chloroform/methanol (1:2 v/v) was added and the sample was vortexed for 1 minute. Then an extra 0.4 ml of chloroform was added to compensate the evaporated chloroform and tube was vortexed for 1 more minute. Afterwards 0.4 ml of water was added and the sample was vortexed again for 1 minute. The homogenate was centrifuged at 1935 g for 20 minutes at 4°C. 1000µL of the top (methanol/water) fraction was transferred in a 2.0ml Eppendorf tube and was dried in a vacuum centrifuge/concentrator. To re-suspend the dried sample for NMR analysis, 600µl of phosphate buffer in D_2O (150 mM; pH 6.6 (pD 7.0); with 100 µM of TSP-D4 (deuterated Trimethylsilylpropanoic acid)) was added, followed by 1 minute vortexing and 10 minutes centrifugation at 14,100g at room temperature. The supernatant was transferred into a NMR tube and 1D NOESY spectra were collected in an 800 MHz NMR spectrometerequipped with a room temperature HCN inverse Z-gradient probe. The pulse sequence used was 1D gradient NOESY with water presaturation using the Bruker standard profiling parameters. For select samples, additional homonuclear and heteronuclear spectra were also collected to assist with metabolite identification. These were ^{1}H J-resolved, ^{1}H-^{1}H COSY and ^{1}H-^{1}H TOCSY, ^{1}H DOSY and ^{1}H-^{13}C HSQC spectra.

2.2. Data analysis

Spectra were processed and analyzedwith the TopSpin 3.2 software (Bruker Biospin); compounds were identified and in some cases quantified using the Chenomx software and the identities of difficult cases was verified with the help of J-resolved, COSY/TOCSY and HSQC spectra. Prior to statistical analysis, the NMR data were treated in R, in order to align peaks using SPEAQ. Multivariate statistical analysis on the full spectra was carried out using the MetaboAnalyst 3.0 web interface. For the full spectra, data were

normalized based on their median values and then the intensities were transformed into a logarithmic scale. No additional scaling was performed. PLS analysis of the spectral data was used to identify compounds that varied significantly between groups. These metabolites together with others that are relevant for the project were then quantified using database assisted spectral deconvolution that was performed using the Chenomx suit of programs. The resulting concentration were then further analysed with multivariate and univariate analysis. For the multivariate analysis data normalization was performed based on the mass tissue used for extraction while the variables were scaled according to the Pareto algorithm. Univariate statistics was performed in Excel 2010.

3. Main results obtained during STSM

3.1. Multivariate analysis results

Binned spectral data were used in MetaboAnalyst 3.0 for an initial PCA of all the samples. This was used to identify three outlier samples (two from *B* and one from *C* groups). Upon inspection they were found to have very low concentrations suggesting a problem in the extraction and were removed from the analysis, leaving a total of 29 samples. PCA scores plot of the remaining sample spectra is shown in Figure 1A. No clear separation of the groups is noticeable implying that wide range changes are not prominent between the groups. In order to better identify differences between groups, PLS discriminant analysis was subsequently carried out and some separation could be observed (Figure 1B).

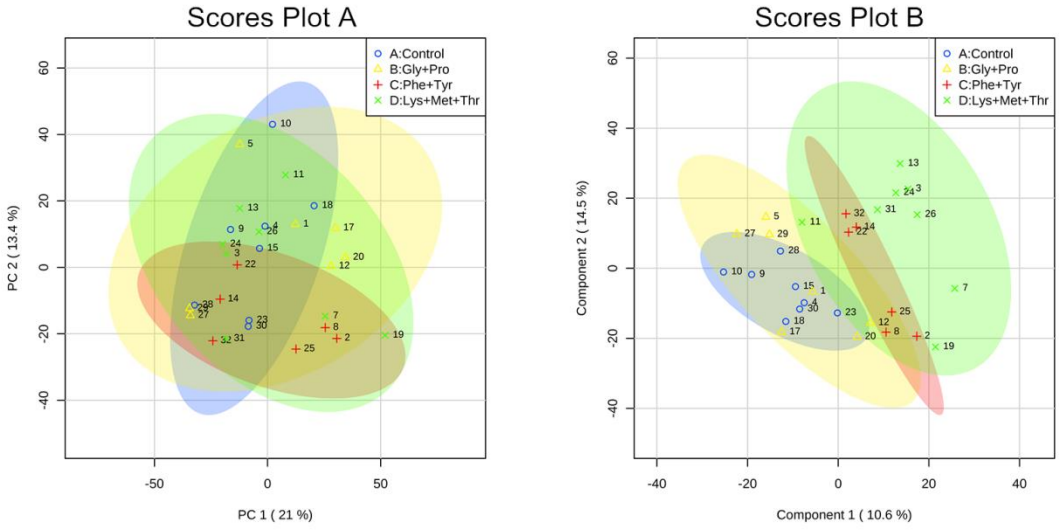

Figure 1.A) PCA and **B**) PLS of full spectral data. The ellipses in the scores plots display 95% confidence regions.

Four PLS-DA plots with $Q^2>0$ are shown in Figure 2, cross validation results of all combinations are shown in Table 1.

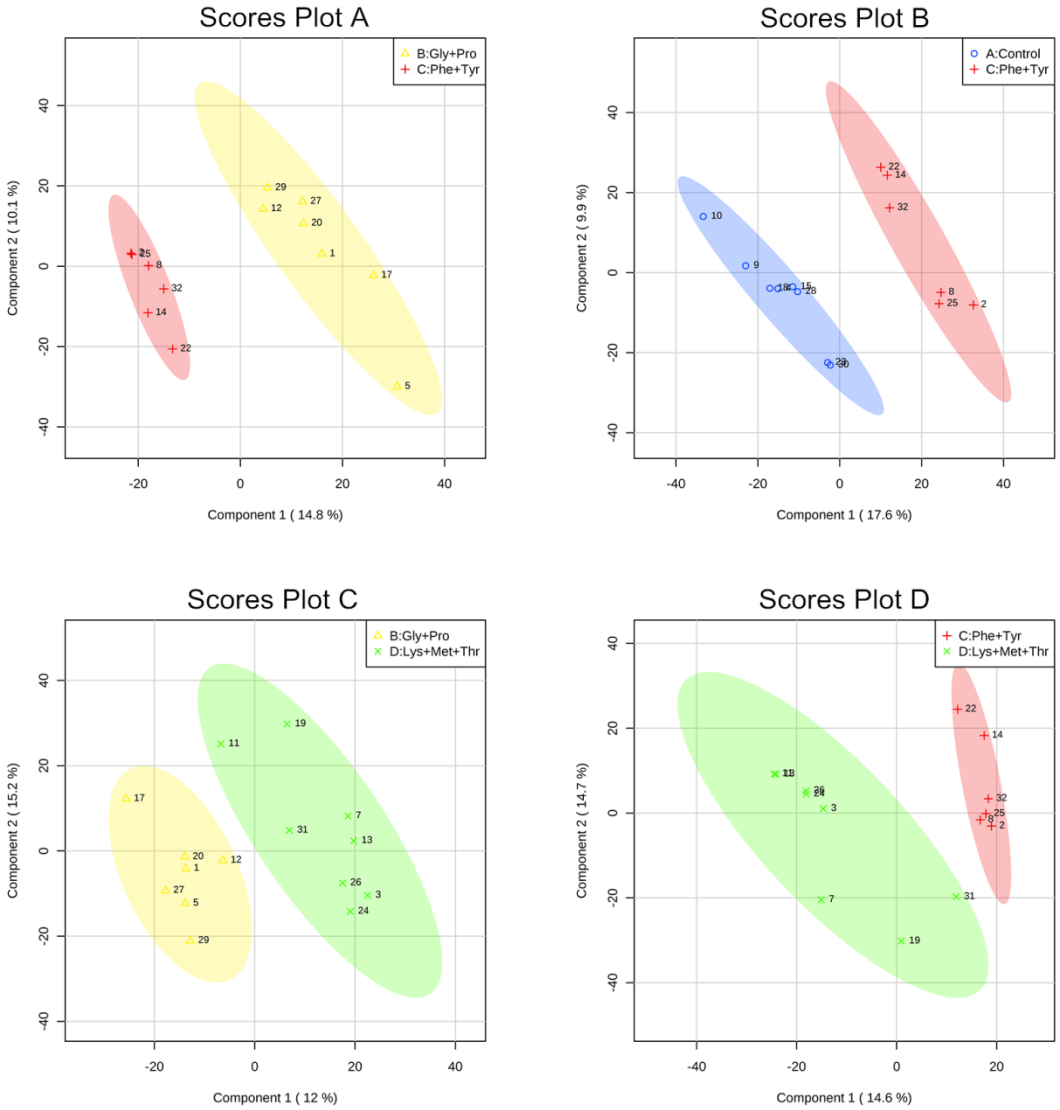

Figure 2.A) PLS scores of *B* vs. *C*. **B**) PLS scores of *A* vs. *C*.**C**) PLS scores of *B* vs. *D*. **D**) PLS scores of *C* vs. *D*.

	Number of Components	R^2	Q^2
A vs. *B*	-	-	-
A vs. *C*	4	0.998	0.385
A vs. *D*	-	-	-
B vs. *C*	2	0.971	0.578
B vs. *D*	2	0.9	0.152
C vs. *D*	2	0.0894	0.121

Table 1. Result from cross validation. Models were not considered and marked as "-" if Q^2<0.

Afterwards, a complete assignment of select spectra was performed and a total amount of 67 compounds were identified using Chenomx software. Representative assignment is shown in the spectrum in Figure 3.

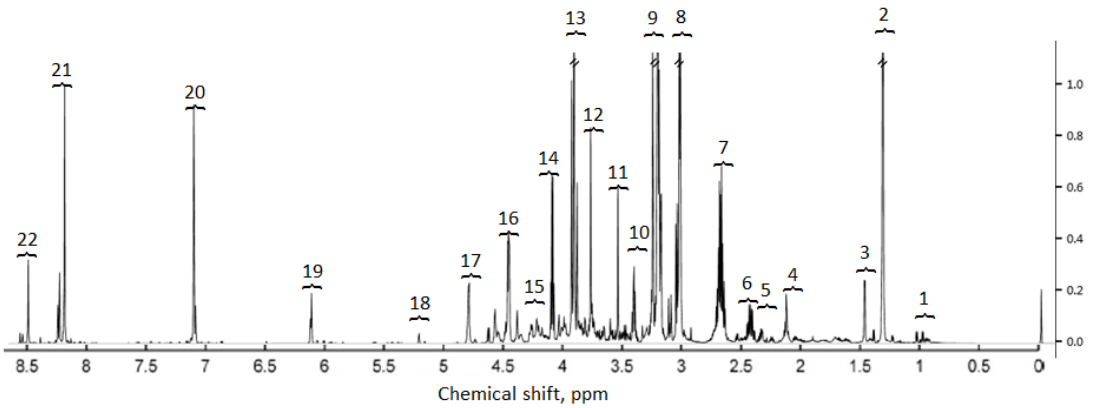

Figure 3. Representative 800 MHz 1H-NMR spectrum of calf muscle aqueous fraction. Assigments: 1: Isoleucine/Leucine/Valine; 2: Lactate; 3: Alanine; 4: Biotin/Carnotine/Glutamine; 5:Glutamate/Pyruvate/Valine; 6: Glutamine/o-Acetylcarnitine; 7: Anserine/Carnosine; 8: Creatine/Creatine phosphate; 9: Anserine/Betaine/Carnosine; 10: Taurine; 11: Glycine; 12: Alanine, Anserine/Glutamine; 13: Betaine/Creatine/Creatine phosphate; 14: Lactate; 15: ADP/AMP/ATP/IMP/NAD+/NADP; 16: Anserine/Carnosine; 17: Unidentified; 18: Glucose/Glucose-6-phosphate; 19: ADP/AMP/ATP/IMP/NAD+/NADP; 20: Anserine/Carnosine/Histidine/Tyrosine; 21: Anserine/Carnosine/Histidine; 22: ADP/AMP/ATP/IMP/NAD+/NADP.

3.2. Univariate analysis results

A selection of compounds to be quantified was made based on the difficulty of fitting and relevance to the study design. This way, 18 compounds were selected: Alanine, Anserine, Carnosine, Creatine, Creatine phosphate, Creatinine, Fumarate, Glutamate, Glycine, Isoleucine, Lactate, Leucine, O-Acetylcarnitine, Phenylalanine, Pyruvate,Taurine, Tyrosine and Valine.After quantification, t.Test was carried out in order to highlight the compounds that cause differences between groups. In Table 2, we only present some compound concentrations in which significant differences were noticed and in Table 3, we present p values of these concentrations comparison, only $p<0.05$ were shown.

	A: Control	*B: Gly+Pro*	*C: Phe+Tyr*	*D: Lys+Met+Thr*
Carnosine	185.236±21.620	185.236±21.620	190.036±37.259	140.547±44.151
O-Acetylcarnitine	4.573±3.900	3.635±1.405	4.364±1.399	2.947±0.904
Phenylalanine	0.478±0.155	0.421±0.096	0.582±0.175	0.396±0.121

Table 2. Concentrations (µMol/100g muscle) of some highlighted compounds.

Groups	Compound	*p-value*
B: Gly+Pro vs. C: *Phe+Tyr*	Carnosine	0.029
B: Gly+Pro vs. *A: Control*	Carnosine	0.044
C: Phe+Tyr vs. *D: Lys+Met+Thr*	Carnosine	0.035
C: Phe+Tyr vs. *D: Lys+Met+Thr*	O-Acetylcarnitine	0.045

C: Phe+Tyr vs. *D: Lys+Met+Thr*	Phenylalanine	0.040

Table 3.*P-values* of some highlighted compounds comparison.

4. Discussion and future work plan

From the unsupervised multivariate analysis (PCA) no clustering of the samples could be observed. The implication of this observation is that differentiation between the sample compositions is not extensive and/or that the intrinsic variability of each sample is more extensive than the differences among groups. Therefore, as an initial conclusion, the effect of formula supplementation does not seem to have a pronounced effect on the metabolites that are present in the muscle.

While global changes are seemingly not dominant, minor changes could still be present. In order to better identify them, supervised analysis was also performed using the PLS and the OPLS methodologies. In this case samples were grouped according to treatment conditions. This information was used to train a model that could predict the probability that a sample could belong to one of the groups used in the training set. The confidence with which such predictions are made can be evaluated from the magnitude of the Q^2 parameter. Values above 0.5 suggest high confidence in the differentiation ability of the model. The methodology also specifies which components of the data are more responsible for the differentiation of the data sets and this is used for the identification of the compounds with the highest variability between the groups.

PLS-DA was performed on each set of groups. This way we were able to find a good separation between *B:Gly+Pro* and *C:Phe+Tyr* and a borderline separation between *A:Control* and *C:Phe+Tyr*. This implies that the group *C:Phe+Tyr,* is the one with the biggest extend of differences from the rest.

In order to better evaluate the reasons for the differentiation between groups, univariate analysis was also performed. From this analysis it was shown that carnosine plays an important role in differentiating several group comparisons. Since carnosine is described as a biomarker of muscle growth/muscle dystrophy, we may use carnosine to predict the calf growth and/or efficiency of amino acid supplementation.

The differences in the muscle metabolome composition are not as evident as we expected, especially when comparing the treatment groups with the control. One possible reason for this result is that the extend of amino acid supplementation is relatively small (nutrition facts of groups were shown in Figure 4), thus it may not be sufficiently to change whole metabolome profile. Another possible reason could be that the effects of the supplementation maybe more pronounced in the amino acid composition of the muscle proteins rather than the free metabolites. This could be studied in the future with the analysis of the muscle protein hydrolysates.

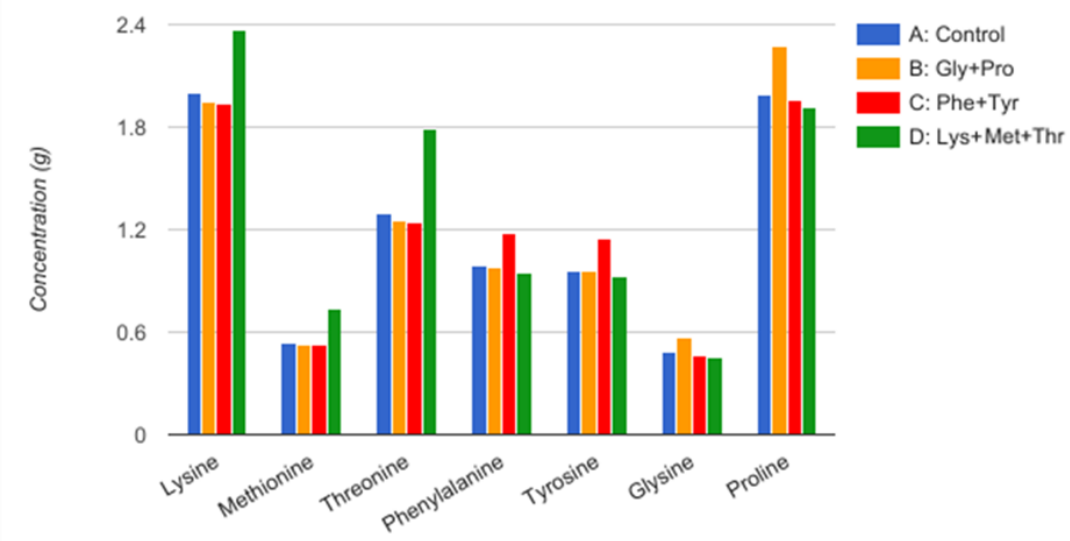

Figure 4. Differences in the concentration of supplemented amino acids (g) in the milk replacers in the four experimental groups

Another possible reason could be the insufficiency of the extraction method and may cause internal difference since 3 samples were detected as outlier due to low concentration problem. However, due to previous extraction trial with some test samples with each sample 4 replicas and the reproducibility shows a good result (data not shown), probably a more reliable extraction validation is needed.

Finally, due to the inherently low sensitivity of NMR, it is also possible that more extensive changes could be present at the level of the least abundant metabolites that were below the detection limit of the method. However, these would correspond to a very small portion of the total metabolites (>5%). Another possibility is that differences exist in the lipidic portion of the metabolome that was not studied here.

We are currently analyzing the variable importance in projection (VIP) of the whole spectra, the regions with difference will be marked and assigned by comparing to previous compounds assignment. With a complete compound set, we can go deeper into the study by doing the pathway study using MetaboAnalyst 3.0. Also, this project presented here is complementary to a wider project related to animal production, biochemical analysis and skeletal muscle proteomics, which involves multidisciplinary research. By the end of the project, we hope we can provide a full explanation.