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URINE AND FECES METABOLOMICS-BASED ANALYSIS OF CAROB TREATED RATS

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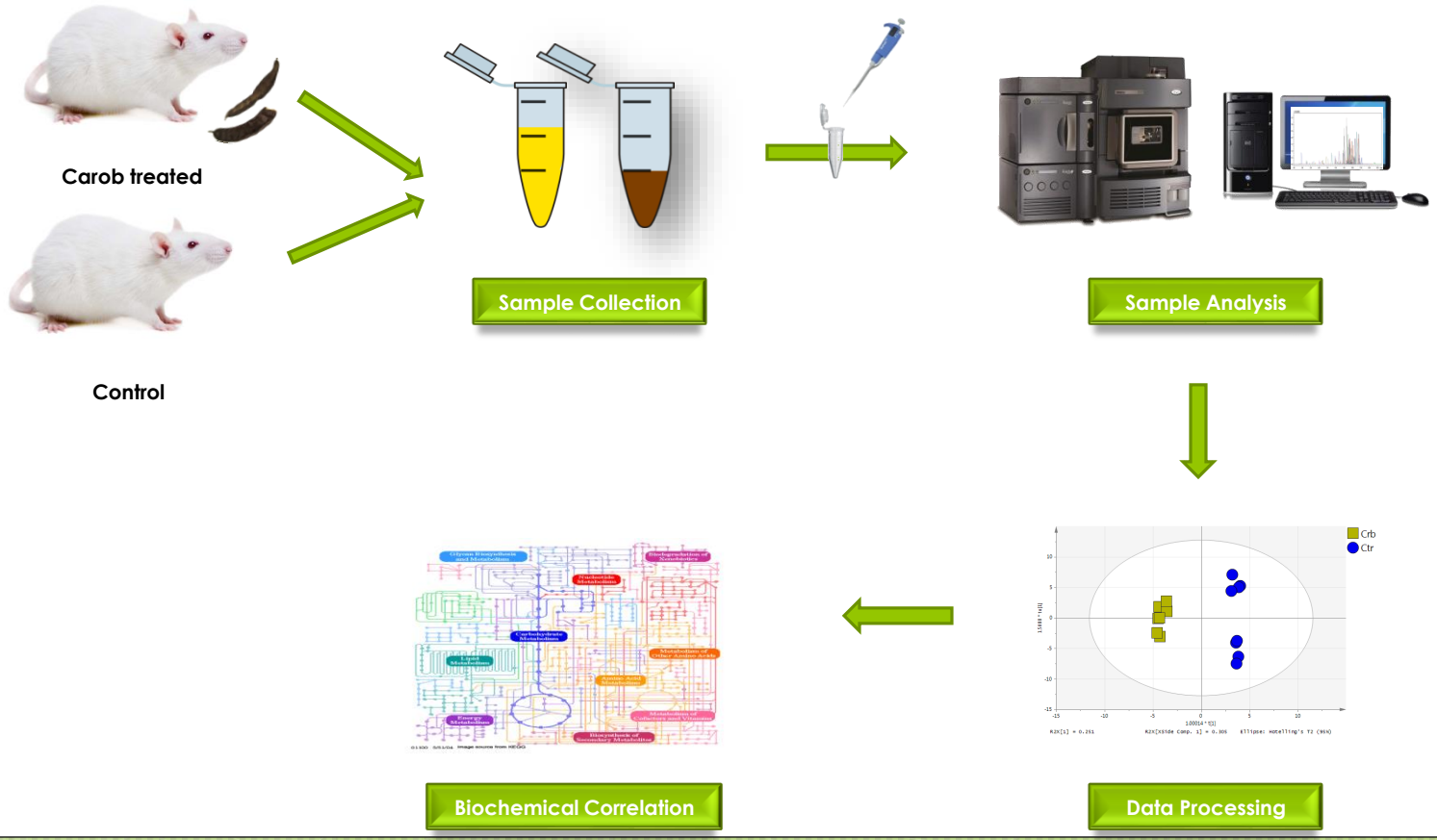
Graphical Abstract



Abstract

Abstract

Ceratonia siliqua L. Fabaceae, commonly known as the carob tree, is native to the eastern Mediterranean countries and its products are widely used in the diet of people living in Mediterranean Europe, Middle East and North Africa. Carobs are considered to be of high nutritional value, as they are virtually fat-free, rich in proteins, antioxidants, vitamins and contain several important minerals. Different types of carob products are available in the local market, such as carob syrup, powder, flour, snack, cream, etc. However, the potential positive health effects of carob-containing products are largely unknown and have not been extensively studied. The aim of this study was to determine significant urine and fecal metabolome alterations in 8 rats treated with carob powder for 15 days as compared to 8 non-treated ones (controls) using liquid chromatography-tandem mass spectrometry (LC-MS/MS) and to underlie specific metabolites that changed according to the treatment.

Urine and fecal samples were collected in five time points during a 15 day period of treatment with carob powder throughout water consumption (10 g powder / L). A targeted HILIC-UPLC-MS/MS method was applied for the determination of 101 polar metabolites (sugars, amino acids, organic acids, amines, etc) in a single run of 40 min in both rat urine and feces. Chromatographic separation was performed on an Aquity BEH amide column (2.1 x 100 mm, i.d. 1.7 μm); the mobile phase was consisted of A: Acetonitrile:H_2O 95:5 v/v (+ 10 mM ammonium formate) and B: H_2O:Acetonitrile 70:30 v/v (+10 mM ammonium formate). The solvents flow rate was set at 0.5 mL/min. Mass spectrometry parameters were optimized for each of the 101 pre-selected analytes.

Approximately 55 urinary and fecal metabolites were identified in both specimens. Data were further processed with multivariate (SIMCA 13) and univariate statistics (ANOVA). The differentiation of treated rats and controls was highlighted using discriminant multivariate models.

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Keywords: targeted metabolomics, carob, rat, urine, feces, LC-MS/MS

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Introduction

Carob tree, *Ceratonia siliqua L.*, (native to the eastern Mediterranean countries) is widely used in the diet of people living in Mediterranean Europe, Middle East and North Africa.

- High nutritional value & fat-free (rich in proteins, antioxidants, vitamins & several important minerals).
- Different types of carob products available in the local market (carob syrup, powder, flour, snack, cream, etc.).

However, the potential positive health effects of carob-containing products are largely unknown and have not been extensively studied.

Effects

- Anticancer
- Antiviral
- Antidiabetes
- Antioxidant
- Digestive
- Antidiarrheal
- Control hyperlipidemia
- Gastroesophageal reflux (in infants)
- Weight loss

http://www.sigmaaldrich.com/life-science/nutrition-research/learning-center/plant-profiler/ceratonia-siliqua.html

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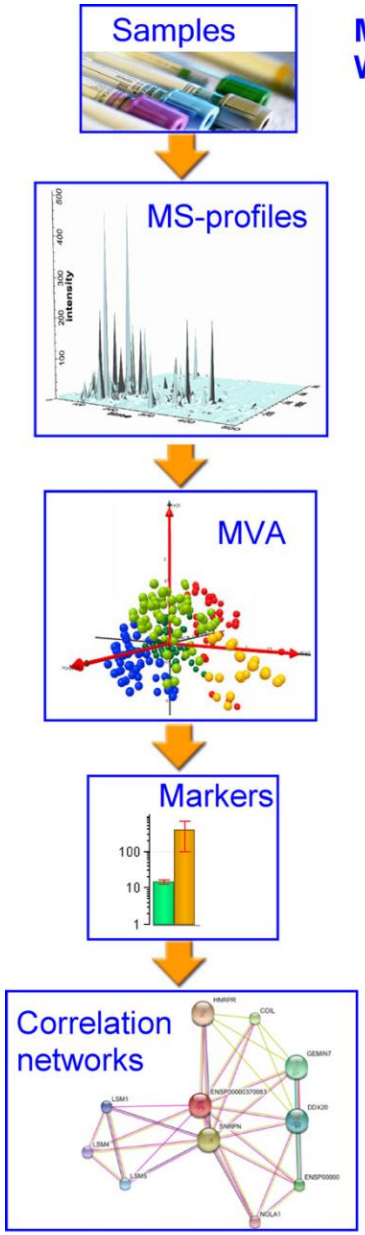
Introduction



Metabolomics Workflow

Metabolomics

- Systematic study of the unique chemical fingerprints that specific cellular processes leave behind
- The study of their small-molecule metabolite profiles

Daviss, Bennett (April 2005). "Growing pains for metabolomics". The Scientist. 19 (8): 25–28.

- Mass spectrometry (MS) dominates in holistic metabolite profiling due to its sensitivity and wingspread availability.
- Liquid chromatography-Mass spectrometry (LC–MS) is currently the most widely used mass spectrometric technology, due to its ability to separate and detect a wide range of molecules.

Theodoridis et al. 2012, Anal. Chim. Acta ,711:7-16

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Literature Review

Matrix	System	Compounds of interest	Column	Ref.
Carob Fruits	HPLC-UV-MS/MS	Polyphenols	Aqua C18 (150 mm x 2 mm, 3μm)	Papagiannopoulos et al. 2004
Carob	LC-MS/MS	Flavonoids	Discovery C-18 column (15 × 4.6 mm, 5 μm)	Vaya et al. 2006
Carob pod	HPLC-PDA, HPLC-MS	Sugars, amino and organic acids, minerals and phenolic compounds	1. Ion-300 column (300 mm x 7.8 mm, 10 μm) 2. Luna Phenyl-Hexyl (250 x 2 mm, 5 μm)	Ayaz et al. 2007
Carob flour	LC-MS/MS	Phenolic Compounds and Alkaloids	HSS T3 (100 mm x 2.1 mm, 1.8 μm)	Ortega et al. 2009
Wild carob seed oil	GC-MS, HPLC	Different lipids	CP-Sil 88 (100 m x 0.25mm, 0.2 μm) Diol phase HPLC column (25 cm×4.6mm)	Matthaus et al. 2011
Carob extracts and mice urine, plasma and cecal	LC-MS/MS, LC-QTOF	Lipids, amino acids, organic acids and phenolic related compounds	C18 Luna 3 n pfp (2) (150 mm x 2 mm)	Jove et al. 2011
Carob leaves	HPLC -MS/MS	Polyphenols	Zorbax Column Synergi 4 μ MAX-RP 80A (150 mm × 4.6 mm)	Aissani et al. 2012
Carob Bean	HPLC-RID	D-pinitol and sugars	CARBOsep Coregel 87P (7.8 × 300 mm)	Turhan 2013
Carob Powder	GC-MS	Volatile compounds	ZB-5ms capillary column (50 m x 0.32mm, 0.25 μm)	Racolța et al. 2014
Carob leaf extracts	HPLC-MS, GC-MS	Phenolic acids	Kinetex C-18 column, (100 x 3 mm) ZB-5MS column (30 m x 0.25 mm, 0.25 μm)	Meziani et al. 2015
Carob pulp	HPLC-DAD-MS	Proteins, phenolic compounds	C18 Alltima (150 mm × 2.1 mm)	Benchikh et al. 2016
Carob pod & Carob syrup	TLC, HPLC-RID	Carbohydrates and Sugars	analytical column (300 mm x 8.0 mm)	Fidan et al. 2016
Carob bean	SPME-GC-MS	Volatile compounds	DB5-MS column (30 m x 0.25 mm, 0.25 μm)	Farag et al. 2017
Carob pod	GC-QTOF, LC-QTOF	Different lipids	1. BPX90 SGE column (30 m x 0.25 mm, 0.25 μm) 2. Phenomenexkinetex C18 (100 mm x 3.0 mm, 2.6 μm)	Nguyen et al. 2017

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Aims

- To determine significant urine and fecal metabolome alterations in rats treated with carob powder using liquid chromatography-tandem mass spectrometry (LC-MS/MS).

- To underline specific metabolites that are responsible for the differentiations according to the treatment.

In vivo carob study

- 16 male *Wistar* rats
- 2.5-3.5 months of age
- 2 groups, 8 fed rats vs. 8 control ones
- 15 days carob feeding
- 1 week acclimatization period
- Rats were housed in individual cages in standard conditions
- 5 sample collection time points (D0, D1, D5, D10, D15)
- Urine and feces samples were collected
- All samples were analyzed using LC-MS/MS
- Rats body weight and food consumption were measured during the *in vivo* experiment

Fecal samples

- A notably useful specimen to assess the effect of the study factor

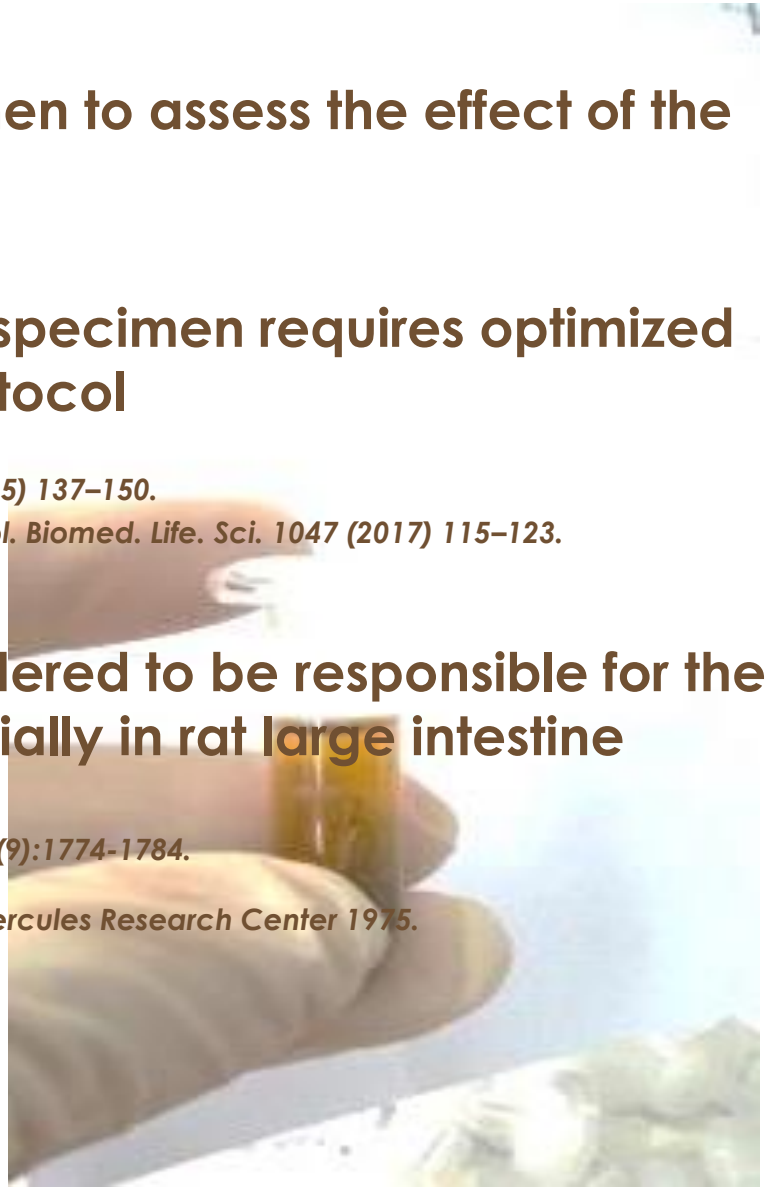
- A particularly complex specimen requires optimized sample preparation protocol

O. Deda, et al, J. Pharm. Biomed. Anal. 113 (2015) 137–150.
O. Deda, et al., J. Chromatogr. B Analyt. Technol. Biomed. Life. Sci. 1047 (2017) 115–123.

- Gut microbiota is considered to be responsible for the carobs metabolism partially in rat large intestine

Harmuth-Hoene and Schelenz, J Nutr 1980;110(9):1774-1784.

Towle and Schranz, Unpublished report from Hercules Research Center 1975.

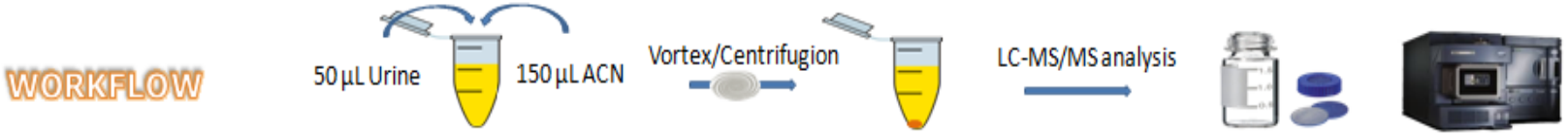


Preparation of carob drinking solution

- 10 g carob powder diluted in warm water (10 ppm)
- Flasks of rats were filled with 750 ml water
- Let them be cooled and place them back to cages
- Preparation of fresh solutions and refill every 2 days

Sample preparation

Urine samples



Fecal samples

- Extraction with 1-propanol: water solution, in a ratio of 1:4 fecal sample weight to extraction solvent
- Vortex-mixing
- Sonication for 10 min
- Ultra-centrifugation (20.000 rpm, 4°C, 30 min)
- Filtration through syringe filters PTFE 0.22 µm

Optimized sample preparation protocol based on: *O. Deda, H. G. Gika, G. Theodoridis, Methods Mol Biol., 2017; In press*

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LC-MS Analysis

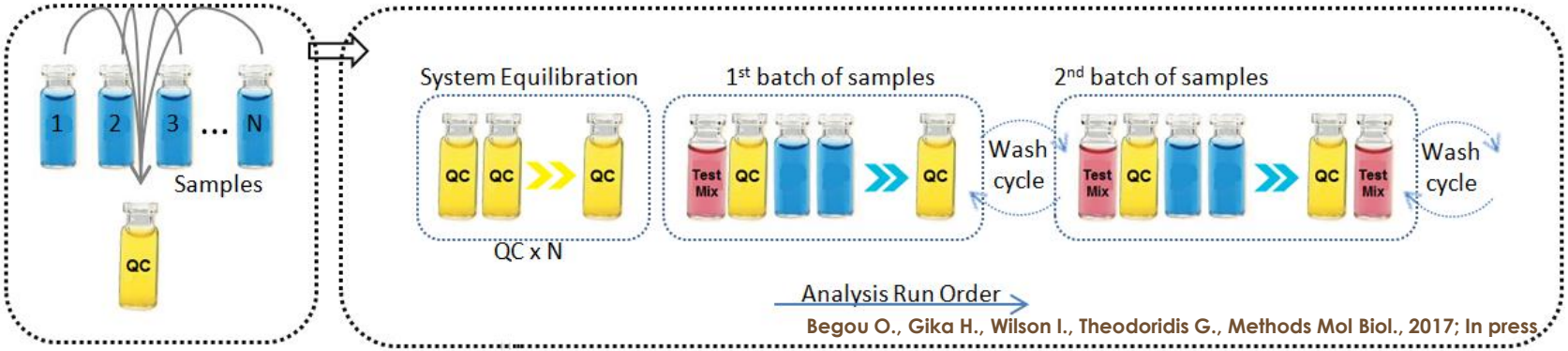
UHPLC-MS/MS Conditions

- Column: Acquity BEH Amide (150×2.1mm i.d., 1.7 µm).
- Mobile Phase: A: ACN: H_2O 95:5 v/v, 10mM $HCOONH_4$, B: ACN: H_2O 30:70 v/v, 10mM $HCOONH_4$
- Flow rate : 0.50 mL/min.
- Instrument: AcquityH UPLC class, Xevo TQD.

The mass spectrometry parameters were optimized for each of the 100 pre-selected analytes (amino-acids, organic acids, sugars, nucleosides, amines and other molecules).

Linear Gradient

Time (min)	%A	%B
0.00	100.0	0.0
4.00	100.0	0.0
25.00	60.0	40.0
30.00	15.0	85.0
30.01	100.0	0.0
40.00	100.0	0.0

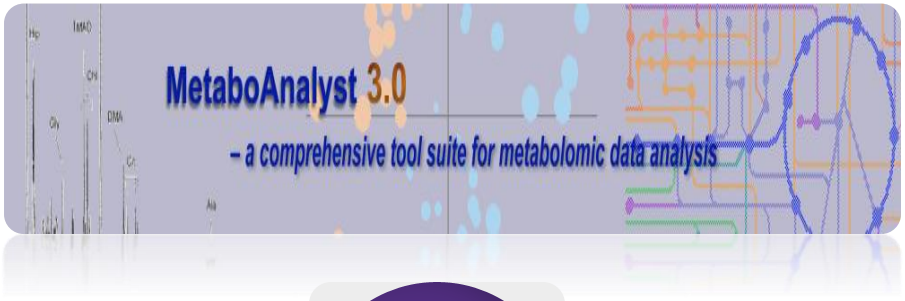


Begou O., Gika H., Wilson I., Theodoridis G., Methods Mol Biol., 2017; In press

HILIC/MS-MS analysis
QCs samples & standard mixes to evaluate stability & repeatability

Data handling

Software



- MassLynx (Waters, UK)
- TargetLynx (Waters, UK)
- SIMCA 13.0 (Umetrics, Sweden)
- MS Excel (Microsoft, USA)
- MetaboAnalyst 3.0 (Xia et al., 2015)

Statistical analysis

- Multivariate statistics (PCA, PLS-DA, OPLS-DA), VIP
- Univariate statistics (t-test, fold change)
- Normalization: Log transformation
- Scaling: Univariate (UV) & Auto
- RSD% of QCs to evaluate stability of the system

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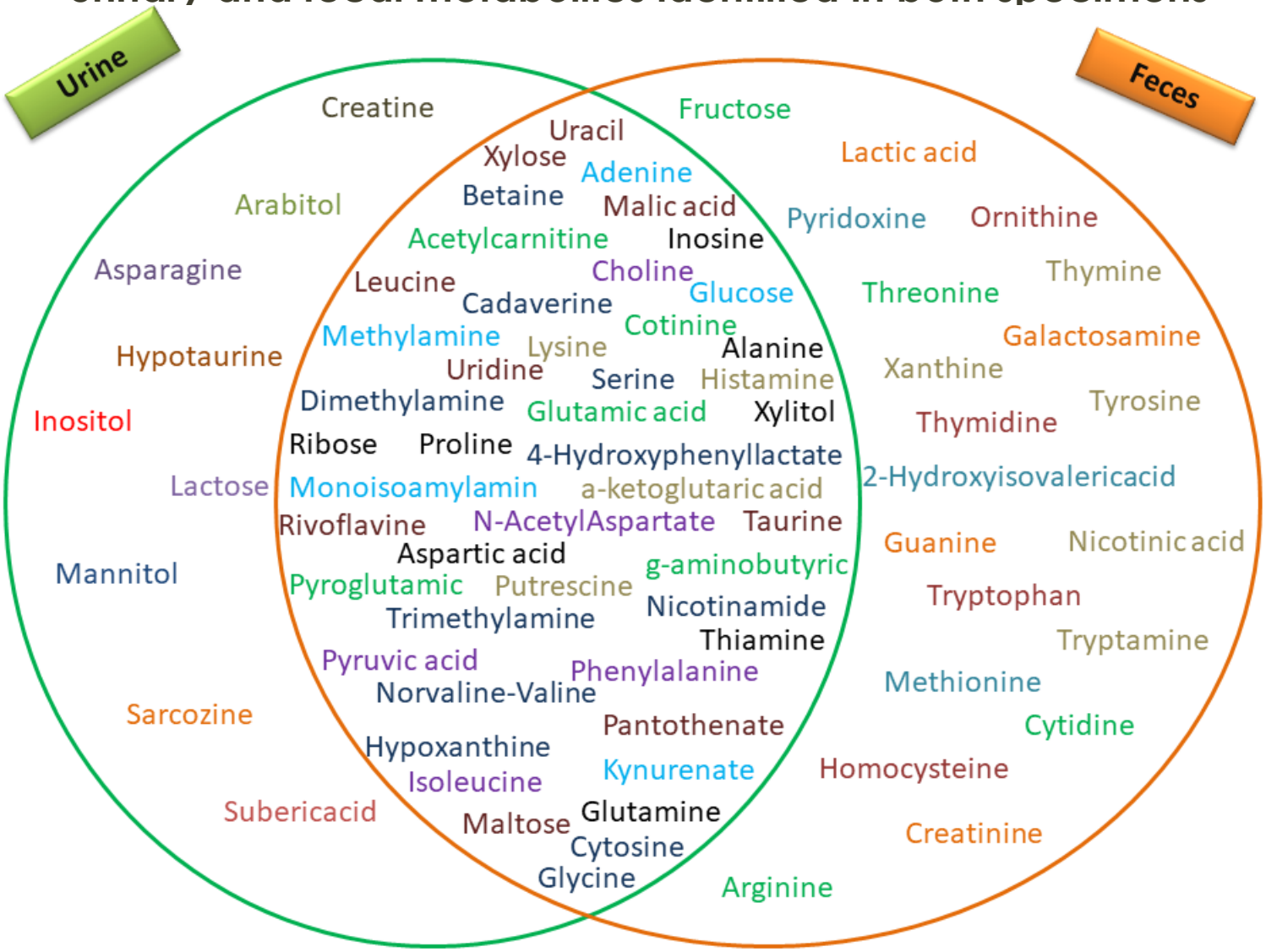
Results

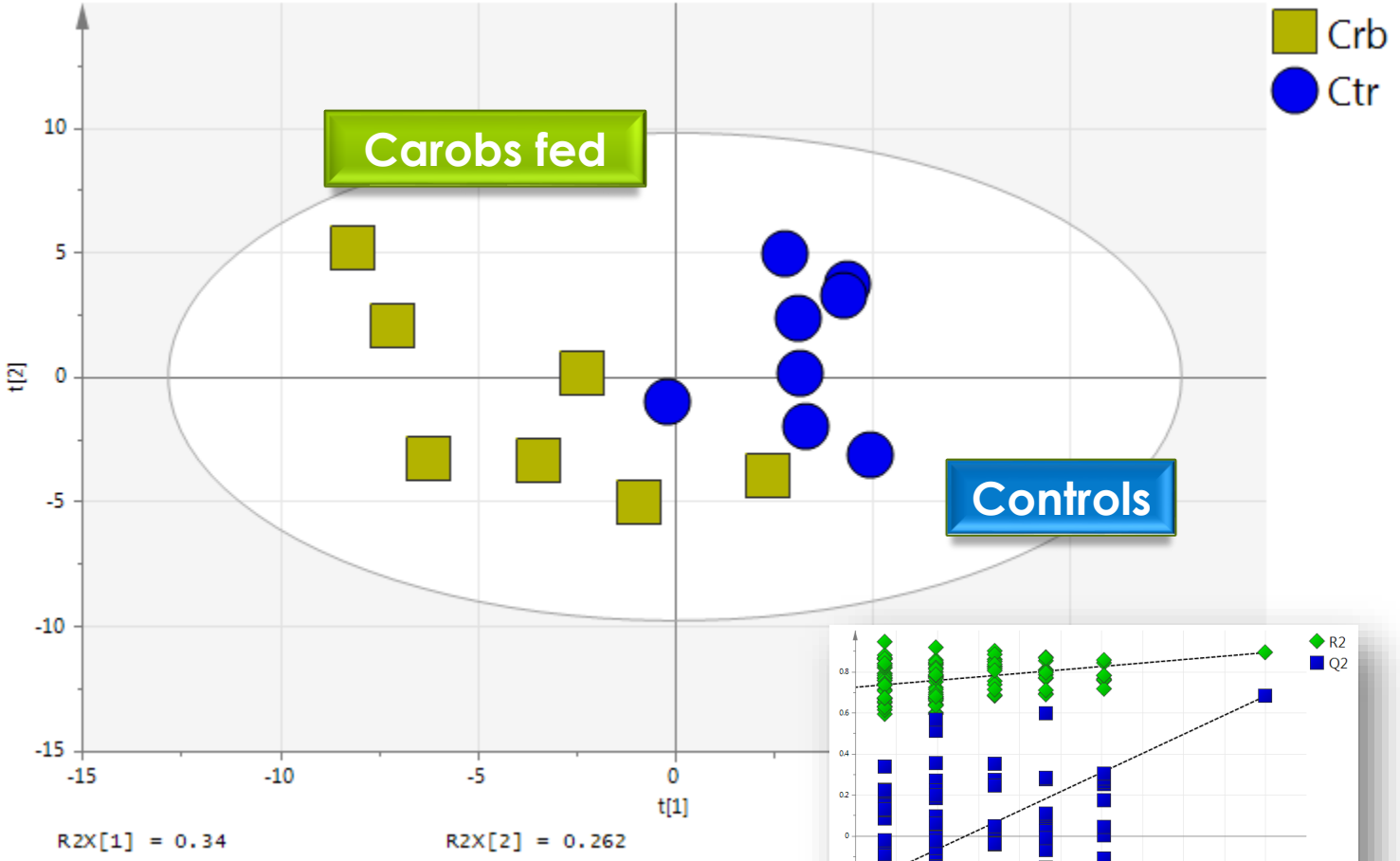
Urinary and fecal metabolites identified in both specimens



Results

Preliminary results

Fecal samples



PLS-DA scores plot of fecal samples (Day 1)

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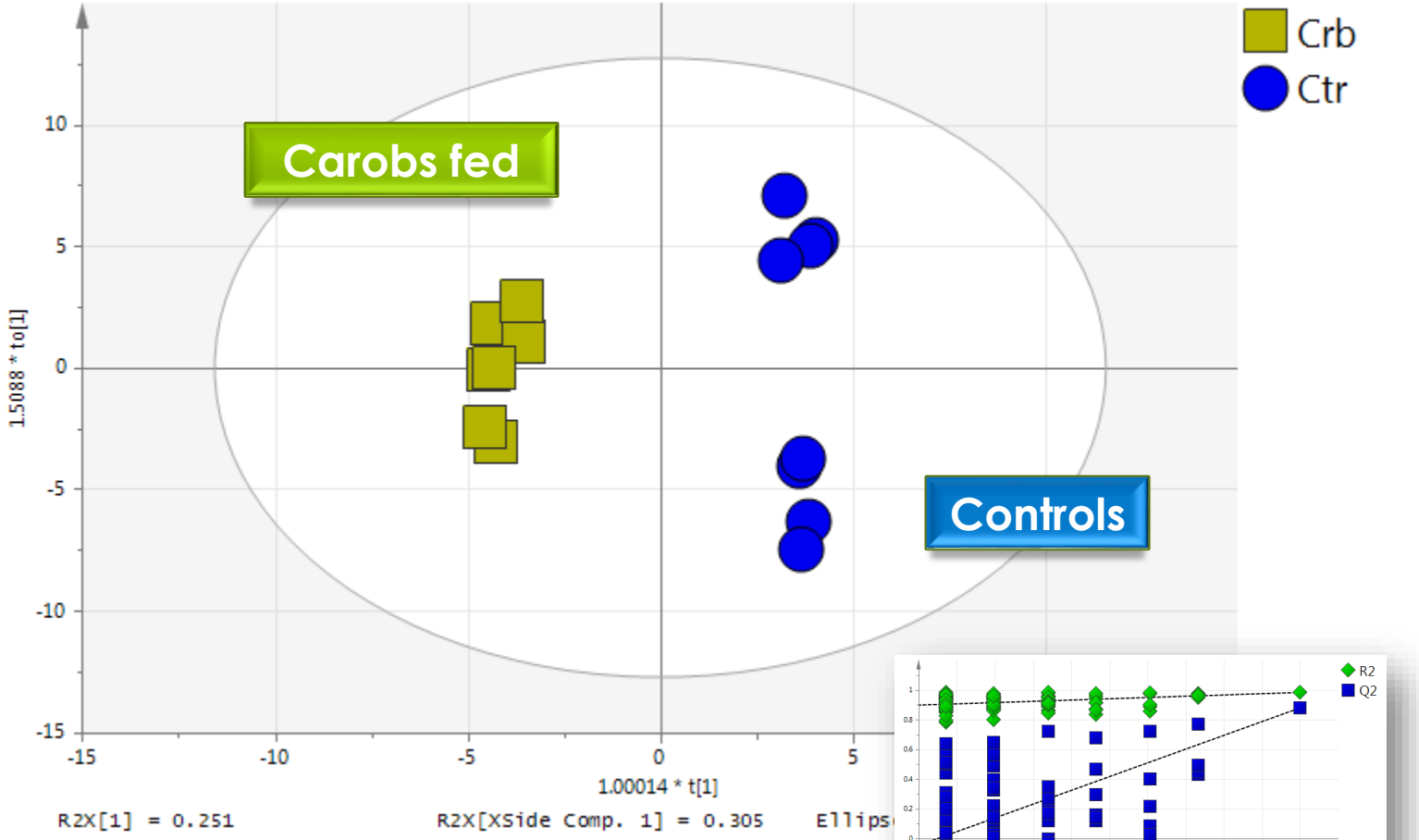
Results

Preliminary results

Fecal samples



OPLS-DA scores plot of fecal samples (Day 15)

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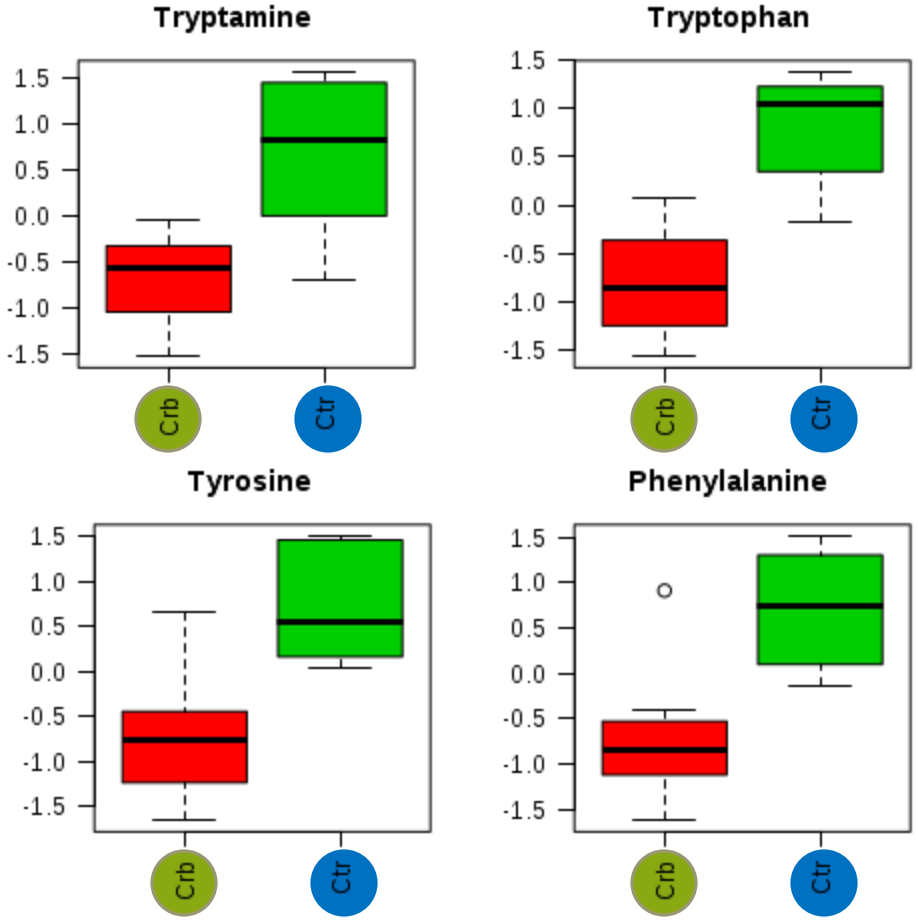
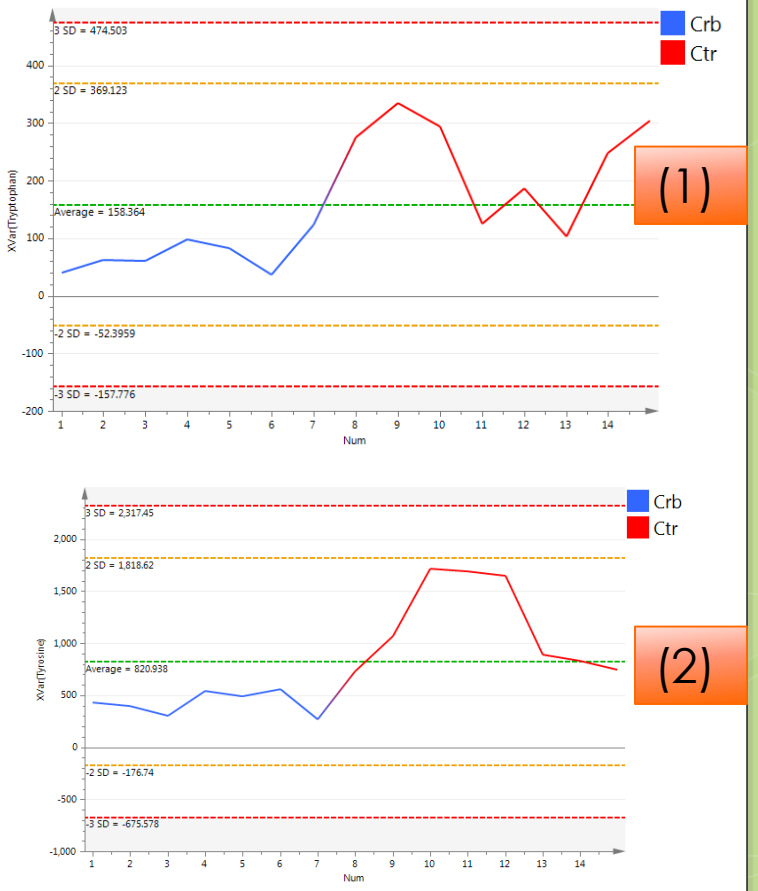
Results

Preliminary results

Fecal samples



Box plots of differentiated compounds in day 15 derived by t-test and VIP values



Examples of Hotelling's line of Tryptophan (1) & Tyrosine (2)

Results

Preliminary results
Fecal samples

- Differentiation between the 2 groups in the day 1 was observed.
- Multivariate statistical analysis managed to separate fecal samples in the day 15.
- Both Multivariate and Univariate statistical analysis demonstrate specific compounds altered in rats fed with carob powder for 15 days (tryptamine, tryptophan, tyrosine, phenylalanine).

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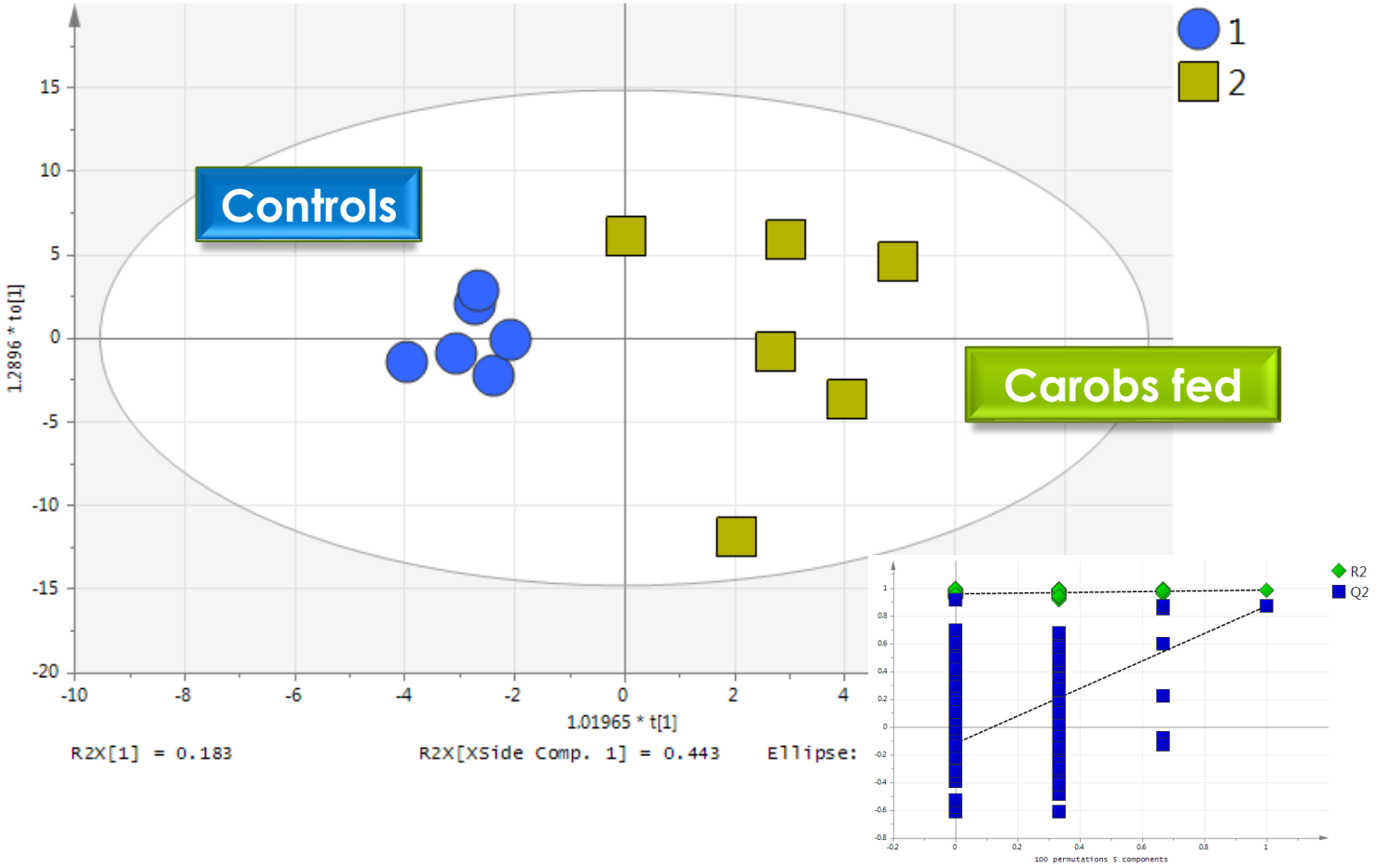
Results

Preliminary results
Urine samples



OPLS-DA scores plot of urine samples (Day 1)

Results

Preliminary results
Urine samples

- Mild differentiation between the 2 groups in the day 1.
- Multivariate statistical analysis did not manage to separate urine samples, statistical significantly, in the day 15.
- Univariate statistical analysis demonstrates specific compounds altered in rats fed with carob powder for 15 days (glucose, inositol, thiamine, alanine).

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Limitations

Preliminary results
Urine samples

- Lower number of urine samples (unable to collect from some rats at the specific time point).
- Matrix effect may affect the obtained results.
- Normalization could be applied in raw data from urine samples in order to overcome matrix effect.

Conclusions

- Statistically significant differentiations, according to food consumption, were observed between weeks for both fed and control groups.
- The metabolomics based analysis manage to separate the analyzed samples according to the treatment.
- Carob treated rats showed different metabolic profiles comparing to the controls allowing their discrimination by LC-MS/MS-urine and fecal profiling analysis.
- Carobs consumption may affect the fecal metabolome in greater scale than urine metabolome.

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Conclusions

- Based on our preliminary results tryptamine, was found to be affected in both days 1 and 15 of sample collection.
- Affected metabolic pathways derived from fecal sample analysis: aminoacyl-tRNA biosynthesis, phenylalanine tyrosine and tryptophan biosynthesis.
- Jove et al., 2011 observed that cecal metabolome was affected more than urine and plasma metabolome in mice fed with carobs.
- Based on our preliminary results, and the only relevant metabolomics-based published study (Jove et al., 2011), as well as older studies (Harmuth-Hoene and Schelenz, 1980), it could be considered that carobs greatly affect gut microbiota.

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Images

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Acknowledgments

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