



## Urine and fecal samples targeted metabolomics of carobs treated rats

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### ABSTRACT

*Ceratonia siliqua*, known as the carob, is considered to be of high nutritional value and of great economic significance due to its unique composition. The beneficial effects of carob against cancer, metabolic syndrome, diabetes, diarrhea, hyperlipidemia and gastro esophageal reflux disease are only a few of its therapeutic actions. Metabolomics-based analysis provides an ultimate tool, for the deciphering of nutritional intervention derived metabolic alterations. In the present study, 16 male Wistar rats were treated with carob powder for a 15-day period. Fecal and urine samples were collected at 5 time points (0, 1, 5, 10 and 15 days). By the applied HILIC-MS/MS method, 63 and 67 hydrophilic metabolites were detected in the fecal and urine samples, respectively, including amino acids, organic acids, sugars, vitamins and other endogenous compounds. A clear group separation based on fecal metabolome was observed after 1 day and 15 days treatment, while only a mild differentiation at day 1 was observed based on urine metabolome. Twenty-one fecal metabolites were responsible for the separation including amino acids and their derivatives, vitamins and organic acids. However, only 7 metabolites were altered in rat urine samples. Metabolic alterations in fecal samples could be attributed to physiological and biochemical adaptations derived from the nutritional intervention. Fecal targeted metabolomics were proven to be suitable for uplifting and highlighting such alterations.

### 1. Introduction

Carob tree (*Ceratonia siliqua* L. *Fabaceae*) and its different products such as carob powder, syrup, cream, etc., are widely used in the everyday diet mostly of Eastern Mediterranean people. Carob is considered to be of high nutritional value, as it is proven an excellent source of polyphenols, fibers, flavonoids, sugars and amino acids, minerals, and it is also fat-free [1–8]. Almost all of carob products, such as carob pod, seed, leaves, syrup, powder and flour have been analyzed using different analytical platforms as liquid and gas chromatography combined with either a UV/DAD detector [4,9] or a mass spectrometer [1,10]. Its unique composition makes it of great economic and nutritious importance [11]. A plethora of studies have also reported the great beneficial effect of carob consumption regarding health benefits.

Cancer, metabolic syndrome, diabetes, diarrhea and hyperlipidemia are few of the subjects tested [12–16]. However, despite the fact that there are numerous of studies showing the beneficial effect of carob consumption, only one study in the literature is focusing on the holistic analysis of rat urine, plasma and cecal metabolome after a three week treatment with carob, hazelnut and almond [17].

The last years, carob is also considered to be an excellent source of animal food as it is a high-energy source, due to its containment in small molecular weight carbohydrates [18,19]. Carob pod or pulp have been used in different animals diet as rabbits [18], pigs [19,20], fish [21], lambs [22–24] and poultry [25] and its effects in body weight, growth performance, immune status, nutrients absorption, meat quality and cecal characteristics were evaluated. The studies showed that in certain amount of carob per body weight, carob products can be an

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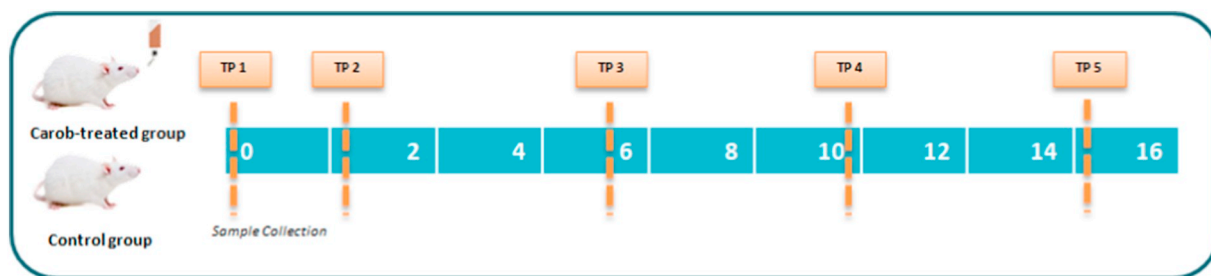


Fig. 1. The two groups, the experimental duration and the urine and fecal samples collection are illustrated.

excellent food source without undesirable effects. For instance, in pigs' diet carob is considered to be an excellent mean for fattening and meat quality improvement [19,20], while in lambs does not affect their milk production lowering the milk production costs [24]. However, when carob is used in larger amounts, its rich content in tannins leads to significant depression in growth rate, with tannins acting as inhibitors, and also to lower digestibility of proteins and nutrient intake [18,24].

Over the past two decades, scientific community has experienced a great growth in different fields due to the advance of *omics* technologies (genomics, transcriptomics, proteomics, and metabolomics), with more and more publications related to the *omics* topic taking place. Metabolomics or Metabonomics, a rapidly evolving multidiscipline, are related to the holistic simultaneous profiling of small endogenous compounds in different matrices, such as biofluids, tissues, food etc. However, due to the demanding requirements of different fields, beside the mentioned *omics* technologies, many *omics* subdisciplines such as lipidomics, foodomics, metallomics, etc. have raised [26]. Foodomics, a metabolomics' subcategory mostly focused on the Food and Nutrition domain, have been recently used as an excellent tool for providing nutritional advices in population groups with specific dietary requirements [27], in order to improve their nutrition or nutritional habits [26]. Food metabolomics studies focus on finding differences in the profile of small metabolites in the different species and varieties [28], but also in the metabolome after a specific food consumption. These kinds of studies have been favored by the extremely great development of different analytical platforms, such as NMR spectroscopy and mass spectrometry (MS) combined with gas and liquid chromatography (GC/LC-MS), that enables the simultaneous analysis of a large number of metabolites present in complex matrices [28]. LC-MS seems to outweigh against the other platforms for both targeted and untargeted profiling [29]. However, none of the methodologies described thus far is able to provide a whole coverage of the metabolome in a single run [30].

In the present study, the affected metabolic pathways after a 15-day period of carob treatment are examined through the use of an HILIC-MS/MS based metabolomics study in rat urine and fecal samples.

## 2. Experimental

### 2.1. Chemicals and reagents

All solvents, acetonitrile, methanol, 2-propanol, ammonium formate, formic acid, (Sigma Aldrich, St. Louis, USA and Merck, Darmstadt, Germany) used were of LC/MS analytical grade. Water (18.2 M $\Omega$  cm) was purified in Milli-Q purification system (Merck Darmstadt, Germany).

### 2.2. In vivo study

Urine and feces samples were collected from 16 male Wistar rats, weighting 364.3 g  $\pm$  33.1 g that were between 2.5 and 3.5 months of age for a period of 15 days. Each animal was housed in individual cage, under a regulated 12 h light/12 h dark cycle, controlled temperature

(22–25 °C) and humidity conditions (50%). The implementation of the animal experiment took place in the Laboratory of Animal Physiology Facility, Veterinary Medicine School of Aristotle University of Thessaloniki according to the Helsinki Declaration and National standards.

The experimental design included two groups of rats, a carob-treated group and a control one ( $n = 8$  for each group). Before the beginning of the protocol all rats were acclimatized for 7 days. In the carob treated group, carob powder (10 g), purchased from a local market of Cyprus, was diluted freshly every other day into rats' water throughout the experiment (final concentration 10  $\mu$ g/mL). In addition, all rats were fed ad libitum with standard chow and allowed to have free access to water (Control group) or carob-water (Carob-treated group). Body weight and food consumption were also recorded.

Both specimens (urine, feces) were collected in 5 different time points (TP) (Day 0, Day 1, Day 5, Day 10 and Day 15), strictly at a specific time of the day. For urine collection, each rat was placed onto a glass surface and was left to urinate. The sample was then collected by a pipette into Eppendorf tube and stored at  $-80$  °C until analysis. Feces samples were collected from another glass surface to avoid contamination. Between the collection of each sample, cleaning of the glass surfaces was performed using organic solvent until evaporation to dryness. The study design is graphically described in Fig. 1.

Rat urine collection was a difficult task to perform. Thus, only 6 animals were kept in each group that gave sufficient amount of urine sample ( $> 50$   $\mu$ L) or sample in general in all 5 time point. Respectively, 7 rats were employed from each group for the fecal analysis.

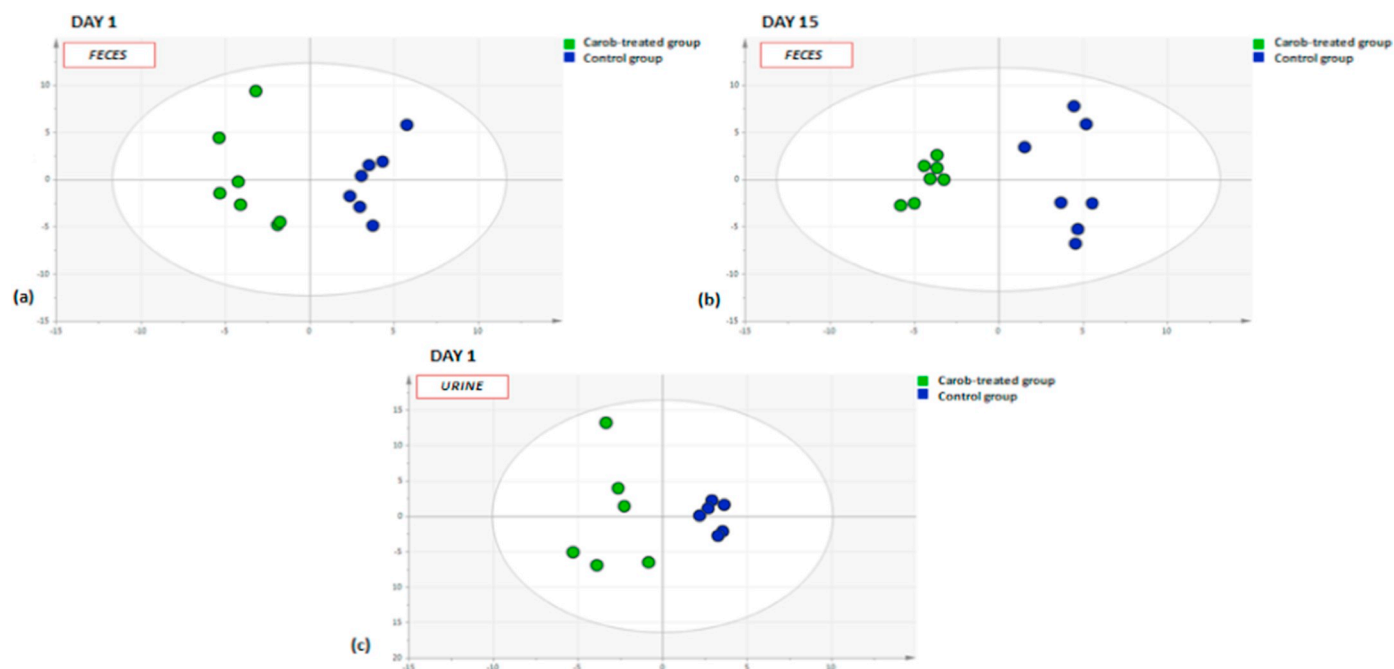
### 2.3. Urine and feces samples preparation

Urine samples left to thaw in room temperature and after vortexing, 50  $\mu$ L were diluted with 150  $\mu$ L of ice cold acetonitrile for protein precipitation. The samples were subsequently vortex mixed, centrifuged at 10,000 rpm for 15 min and 4 °C, transferred to a LC/MS vial and finally placed on the autosampler tray at 4 °C.

Fecal samples preparation was based on a previously developed protocol for efficient optimized fecal extraction for metabolomics studies by Deda et al. [31]. Briefly, extracted fecal sample was mixed with 1-propanol:H<sub>2</sub>O 50:50 v/v in a ratio of 1:4 sample weight to solvent volume, vortex-mixed and then sonicated for 10 min. Ultra-centrifugation was followed for 30 min at 20,000 rpm and 4 °C. The supernatants were filtered through 0.22  $\mu$ m syringe PTFE filters and subjected to LC-MS analysis.

### 2.4. LC-MS/MS analysis

All samples were analyzed followed a previously developed and validated Hydrophilic Interaction Liquid Chromatography tandem Mass Spectrometry (HILIC-MS/MS) method, performed in an ACQUITY UPLC H-Class chromatography system combined with a Xevo TQD mass spectrometer (Waters Corporation, Millford, USA), operating in both positive and negative mode, where 101 hydrophilic metabolites are identified [32]. The column was an Acquity BEH Amide Column



**Fig. 2.** (a) OPLS-DA scores plot of rat fecal samples of carob-treated group (green) and control group (blue) at the second time point (TP 2). (b) OPLS-DA score plots of rat fecal samples of carob-treated group (green) and control group (blue) at the fifth time point (TP 5). (c) OPLS-DA scores plot of rat urine samples of carob-treated group (green) and control group (blue) at the second time point (TP 2). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(2.1 mm × 150 mm, 1.7 m), with an Acquity UPLC Van-Guard pre-column (Waters Ltd., Elstree, UK), and the mobile phase was consisted of A) Acetonitrile:H<sub>2</sub>O 95:5 v/v and B) Acetonitrile:H<sub>2</sub>O 30:70 v/v, both containing 10 mM ammonium formate. Regarding MS conditions, capillary voltage was ± 3500 V, desolvation temperature was 350 °C, while desolvation and cone gas flow was set at 650 L/h and 50 L/h, respectively. Cone voltage and collision energy for the multiple reaction monitoring (MRM) mode were optimized for each compound separately by direct infusion. System's stability and analytical performance were

evaluated with the use of pooled urine or fecal samples, as Quality Control (QC) samples, every seven samples [33]. QC samples were prepared by mixing equal aliquots of all samples and were further treated in the same ways as studied samples.

## 2.5. Data analysis

LC-MS/MS data were obtained using MassLynx® (Waters, Milford, MA, USA) and peak integration was performed by TargetLynx® (v4.1).

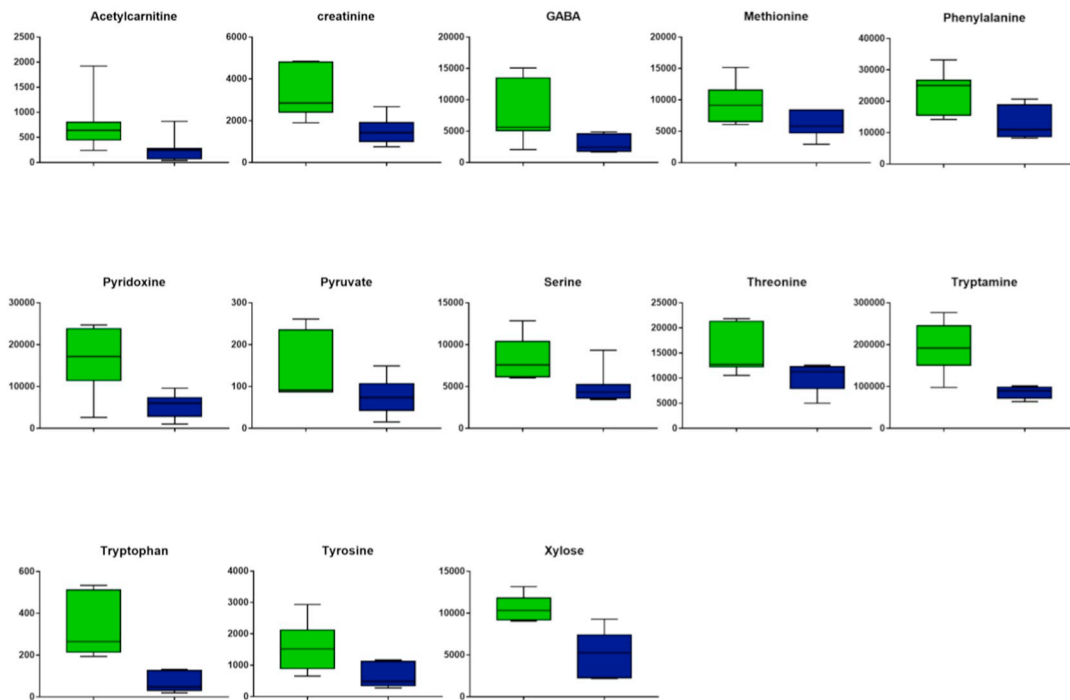
**Table 1**

Significant fecal metabolites contributing to the differentiation between the two studied groups on days 1 and 15: *p*-value, the area under the curve (AUC) and the variable importance for the projection (VIP) of each metabolite are also demonstrated.

Compound	Day 1				Day 15			
	AUC	<i>t</i> -Test	Log <sub>2</sub> FC <sup>a</sup>	VIP	AUC	<i>t</i> -Test	Log <sub>2</sub> FC <sup>a</sup>	VIP
Acetylcarnitine	0.82	0.050	1.11	1.11	0.96	0.014	-0.74	1.22
Alanine	–	–	–	–	0.90	0.012	-0.57	1.22
Creatine	–	–	–	–	0.86	0.046	-1.10	1.02
Creatinine	0.92	0.006	1.16	1.49	–	–	–	–
γ-Aminobutyric acid	0.92	0.044	1.25	1.21	–	–	–	–
Glucose	–	–	–	–	0.90	0.014	-1.10	1.17
Guanine	–	–	–	–	0.98	0.008	-1.19	1.29
Leucine	–	–	–	–	1.0	0.0005	-1.11	1.46
Lysine	–	–	–	–	0.94	0.007	-1.11	1.32
Methionine	0.82	0.040	0.65	1.17	1.0	0.0008	-1.08	1.47
Pantothenate	–	–	–	–	0.88	0.029	-1.64	1.10
Phenylalanine	0.88	0.009	0.83	1.40	1.0	0.002	-1.24	1.49
Pyridoxine	0.88	0.008	1.59	1.50	–	–	–	–
Pyruvate	0.88	0.050	1.11	1.12	–	–	–	–
Riboflavin	–	–	–	–	0.92	0.007	-1.37	1.31
Serine	0.90	0.020	0.77	1.31	0.92	0.004	-0.64	1.31
Threonine	0.86	0.034	0.59	1.21	0.96	0.006	-0.98	1.32
Tryptamine	0.96	0.003	1.10	1.66	0.92	0.003	-1.18	1.42
Tryptophan	1.0	0.003	2.42	1.63	0.98	0.003	-1.67	1.41
Tyrosine	0.90	0.014	1.38	1.39	1.0	0.002	-1.48	1.45
Xylose	0.96	0.001	1.28	1.66	–	–	–	–

<sup>a</sup> Fold change was calculated by using the logarithm of the carob treated group-control group ratio (mean of each group) for all significant metabolites on Day 1 and Day 15.

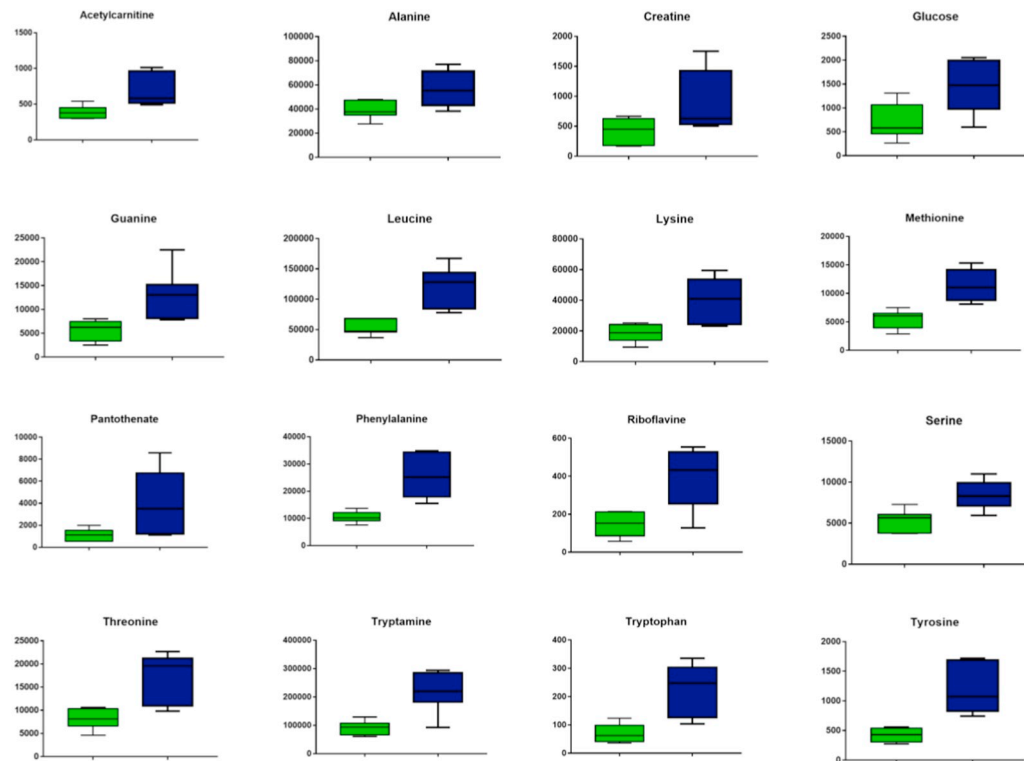
**Day 1\_Feces**



**Carob-Treated group**

**Control group**

**Day 15\_Feces**



**Carob-Treated group**

**Control group**

Fig. 3. Box plots of the most significant compounds found in feces and urine on Day 1 and 15.

The metabolites selected for further statistical evaluation were either present in the 80% of the samples analyzed or they had a relative standard deviation (RSD) < 30% in the QC samples. Analytes with higher value of RSD, were excluded based on the analytical criteria for the system stability. Multivariate statistical analysis, as well as biomarker evaluation by VIP plots (Variable Importance for the Projection), loading plots, S-plots, p(corr) and hotelling's lines was performed using SIMCA 13.0 (Umetrics, Umea, Sweden) in UV scaling. Also, PCA scores plot of QC samples using SIMCA 13.0 was constructed, in order to evaluate systems' suitability. Models validation was evaluated using permutation plots and CV-ANOVA value. Two-tailed *t*-test, with unequal variance and a threshold of  $p < 0.05$ , and ANOVA were performed in Microsoft Excel. GraphPad Prism 7.0 for Windows (GraphPad Software, La Jolla California USA, [www.graphpad.com](http://www.graphpad.com)) was used to evaluate the accuracy of potential biomarkers by Area Under the Curve-Receiver Operating Characteristic (AUC-ROC) curve analysis and also for the illustration of ROC-curve and box-plots graphs. MetaboAnalyst 4.0 was used for the biochemical pathway annotation [34].

### 3. Results

#### 3.1. LC-MS/MS data

Seventy urine and 80 fecal samples were analyzed, in 5 different time points in order to investigate the nutritious effects of carob in everyday diet. In total, 67 and 63 hydrophilic metabolites were detected in the urine and fecal samples respectively, that also matched the %RSD (< 30%) criteria from the respective pooled QC samples. The *in-house* method is able to determine endogenous compounds including amino acids, organic acids, carbohydrates, vitamins and other derivatives of energy metabolism.

Fecal samples' data statistical analysis of Day 0 (TP1) by unsupervised Principal Component Analysis (PCA) showed no discrimination between the two examined groups. Looking into the obtained data of Day 1 (TP2), Orthogonal Partial Least Squares-Discriminant Analysis (OPLS-DA) models provided strong differentiation in the respective fecal samples with R2Y 0.911 and Q2Y 0.759, and CV-ANOVA value < 0.05 (Fig. 2a). In order to find the metabolites affected by the carob consumption, different visualization scheme from multivariate analysis and evaluation tools from univariate analysis were performed. More specific, s-plots and loading plots, constructed from the respective OPLS-DA models, showed a plethora of variables contributing to the separation of the two groups, mostly in feces. These variables were cross-checked with raw data, VIP plots and *p*-values from Student's *t*-Test and ANOVA. In Table 1, all the altered fecal metabolites are given along with their AUC-ROC and log2 fold change values. As it can be noticed, all affected metabolites, are found to be in higher concentration level in the carob-treated group compared to the control group. Respective box plots and ROC-AUC curves of the significant metabolites are illustrated in Figs. 3 and 4. However, that was not the case when datasets from Day 5 and Day 10 (TPs 3 & 4) were examined, as no statistically valid models could be built. PCA or OPLS-DA score plots were not able to separate the carob treated group from the control group. Group separation was also found statistically significant on Day 15 (TP5). OPLS-DA scores plot in t1 vs. t2 space (R2Y 0.939, Q2Y 0.745), permutation plots and CV-ANOVA ( $p = 0.02$ ) revealed a statistically valid model (Fig. 2b). The constructed multivariate model combined with univariate statistical analysis enabled highly significant metabolites to be revealed. Overall, 16 metabolites were noted to alter significantly during the first 15 days of carob treatment (Table 1). Unlike Day 1, all important metabolites were found to be significantly lower in the carob treated group, as it can also be seen in

their respective box plots in Fig. 3. Also, the ROC-AUC curves of the significant metabolites found in Day 15 are presented in Fig. 4.

Regarding the urine samples the same statistical approach was performed. Again, PCA score plot of TP1 showed no differences between the two groups. Moving to Day 1 (TP2) a mild differentiation between the two groups (CV-ANOVA = 0.045) was observed (Fig. 2c). In Table 2, all the affected metabolites and their respective *p*-values, AUC values and log2foldchange are presented. It is clear that also in this specimen, all the significant metabolites appear to be in higher concentration on the carob-treated group. Respective box plots and ROC-AUC curves of the significant metabolites are given in Figs. 3 and 4. However, no other discrimination between the two groups was observed in the other 3 time points tested.

### 4. Discussion

The last step in every nutritional study is the interpretation of the findings, in order to evaluate the biological effect of the specific dietary intervention. The exact mechanism through which dietary components affect the levels of measured endogenous metabolites is difficult to be revealed. Nutrient or nonnutrient signals involved together in the biochemical profile configuration. In addition, gut microbiota may have a great metabolic impact [35].

Since the study of bioavailability and absorption demands balance in the cycle of dose and excretion, the evaluation of bioavailability and absorption of specific compounds relies on their presence in the studied biospecimens [17].

Urine samples are preferably used to monitor the metabolism of nutrients and to reveal biomarkers of specific food intake [36], however some concerns remain with regard to the utility of the obtained results [37].

Fecal samples' metabolic profiling is also a particularly valuable tool, able to reveal the metabolic impact of nutritional intervention [38]. Metabolites detected in fecal samples reflect host and gut microbiome metabolism, as well as the symbiosis. The symbiotic relation between the host and gut microbe is essential not only for the intestinal metabolism, but also for the health state of the organism [39].

Very low concentrations even below of limit of quantification, intestinal and liver metabolism, as well as renal clearance, could complicate the interpretation of a nutritional intervention [17].

Although there is a plethora of studies in which raw carobs or carob products were analyzed by applying either liquid chromatography or gas chromatography with mass spectrometry or tandem MS [1,4,6,10,40–44] and QTOF [45], only one published study is dealing with metabolomics-based analysis of biospecimens [17].

Plasma, urine and cecal content untargeted metabolic profiles of seven mice treated with carobs for 3 weeks were compared with those from controls [17]. In the present study, targeted metabolomics of 15 days carob treated rats were performed. A two weeks treatment was selected in order to investigate if that time period was adequate for the metabolic alterations to be observed, in regard to the three weeks treatment followed by Jové et al. [17].

While from 63 metabolites detected in fecal samples 21 were found to differentiate, in urine samples only 7 from a total of 67 detected metabolites were altered due to the treatment.

The majority of metabolites found to be differentiated in rat fecal samples, constitutes carob' ingredients according to FooDB [46]. Particularly, amino acids L-Alanine, L-Leucine, L-Lysine, L-Methionine, L-Phenylalanine, L-Serine, L-Threonine, L-Tryptophan and L-Tyrosine, vitamins, Riboflavin, Pantothenic acid and Pyridoxine have been found in carobs.

The alteration of the specific compounds in fecal samples could be attributed to carob intake, to altered metabolism due to the

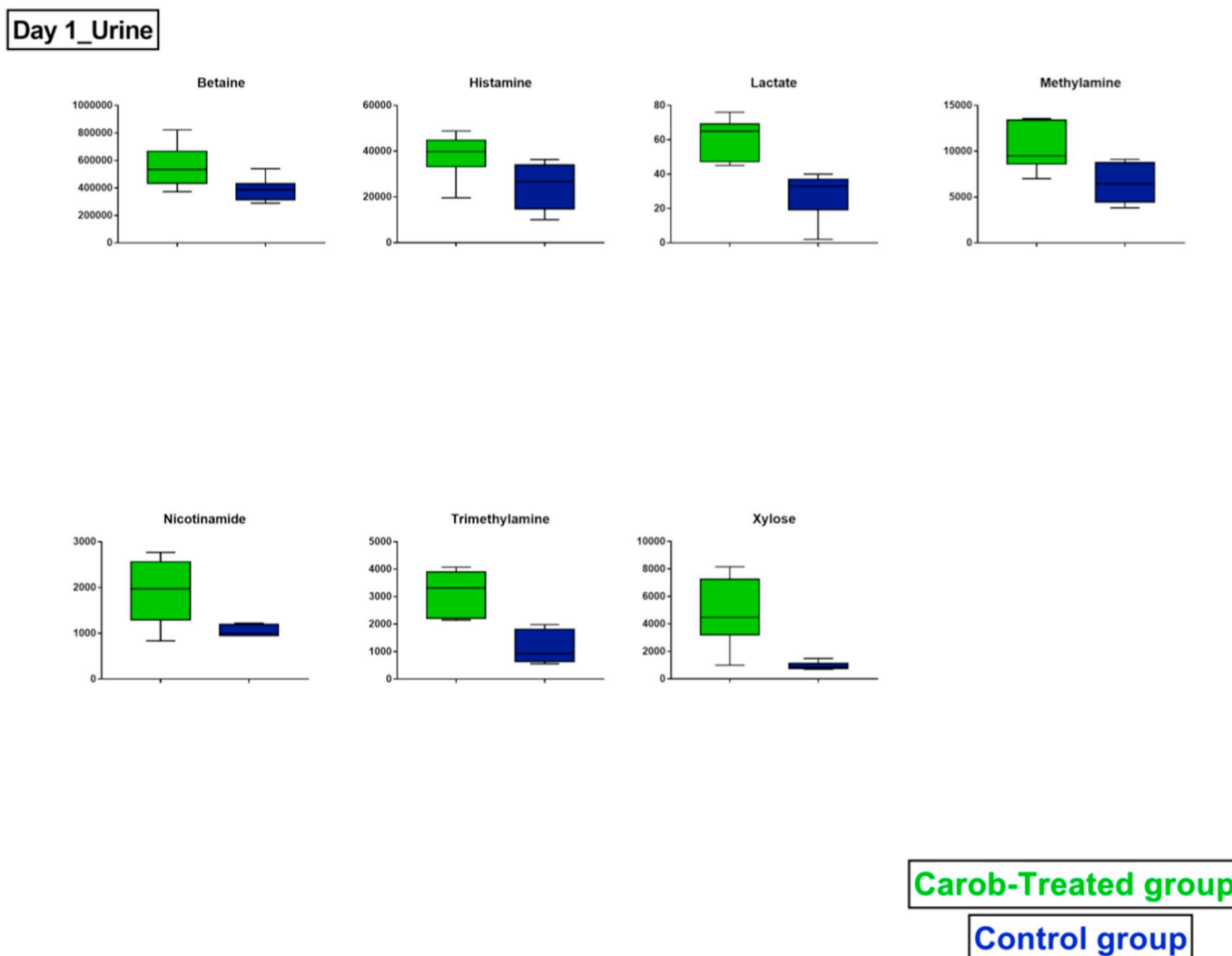


Fig. 3. (continued)

intervention, to the physiological adaptations derived from the intervention, to differentiation of gut microbiota or probably to the combination of all the above.

Diet is a strongly influential factor, capable of inducing changes in gut microbe population [47,48]. Our results, demonstrate that carob treatment likely affected fecal metabolome through gut microbiome alterations and might have favored the development of positively influential strains [18]. This also is pointed out by metabolites found to be differentiated, since they are related to intestinal symbiotic metabolism.

At day 1, the levels of the altered amino acids were increased. Interestingly, at the last time point, they were decreased. On the first day of carob treatment, rats probably did not adapt to the nutritional changes, in order to fully exploit received dietary components, resulting in increased excretion of nutrients in fecal samples. The capacity of digestive enzymes and amino acids transporters probably did not respond to the excess of their consumed substrates [49].

At first sight, the fact that at the last sampling time point (on 15th day) relative levels (peak areas) of the differentiated metabolites are decreased compared to controls seems to be enigmatic. Diet is such a powerful factor that could even lead to physiological changes in intestinal track [50,51]. There are studies that relate the dietary components to the regulation of epithelial permeability [52]. Moreover, Rtibi et al., discussed that immature carob pods treatment resulted in reduction of jejunal secretion and increase of absorption while with mature carobs treatment, the opposite effect was observed, due to

different phytochemical composition [53].

Additionally, reduction in amino acid levels on the last time point probably indicates reduced catabolism and efficient absorption of nutrient in small intestine derived from the necessary physiological adjustments, such as increase of intestinal villi height, or alterations of the enzymes involved in digestion. In an early study, authors suggested that the increase in length of cecum could be attributed to fiber intake during the development of the digestive tract [25].

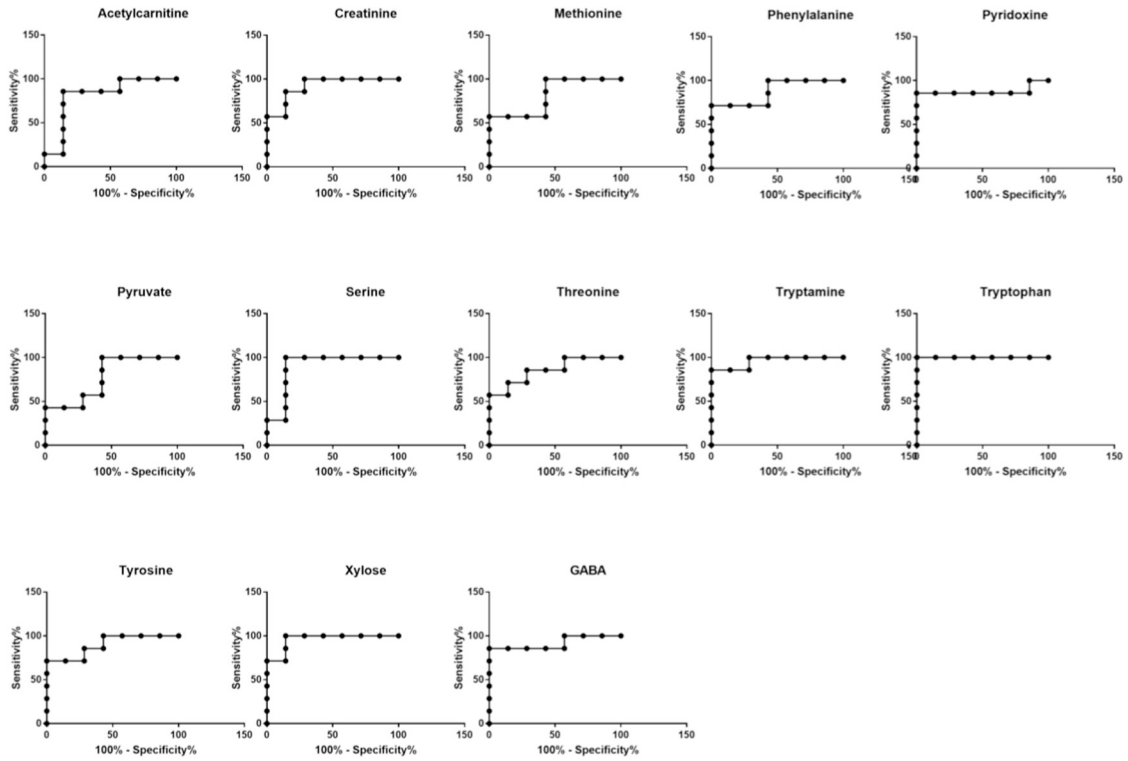
Biochemical pathways related to carob intake included (1) Glycine, serine and threonine, (2) Phenylalanine, tyrosine and tryptophan (3) Valine, leucine and isoleucine metabolism based on Pathway Analysis performed on the online platform MetaboAnalyst [34,54].

Thus, carob intake mainly affected amino acid metabolism. Jové et al. also found lipid, bile acid and amino acid altered metabolism in their carob treated mice [17].

Apart from the overall estimation of differentiated biochemical pathways, there is also a need for deeper examination of tissue-specific biochemistry. Intestinal amino acids metabolism presents high compartmentalization [55] and species specificity due to differentiations of digestive enzymes [49]. For example, tyrosine is synthesized from phenylalanine and is not degraded while, alanine, lysine, methionine, phenylalanine and threonine are degraded in rodents small intestine [56].

Gut microbiome is also responsible for the obtained biochemical profile. As not all the amino acids are absorbed, in order to enter blood

**Day 1\_Feces**



**Day 15\_Feces**

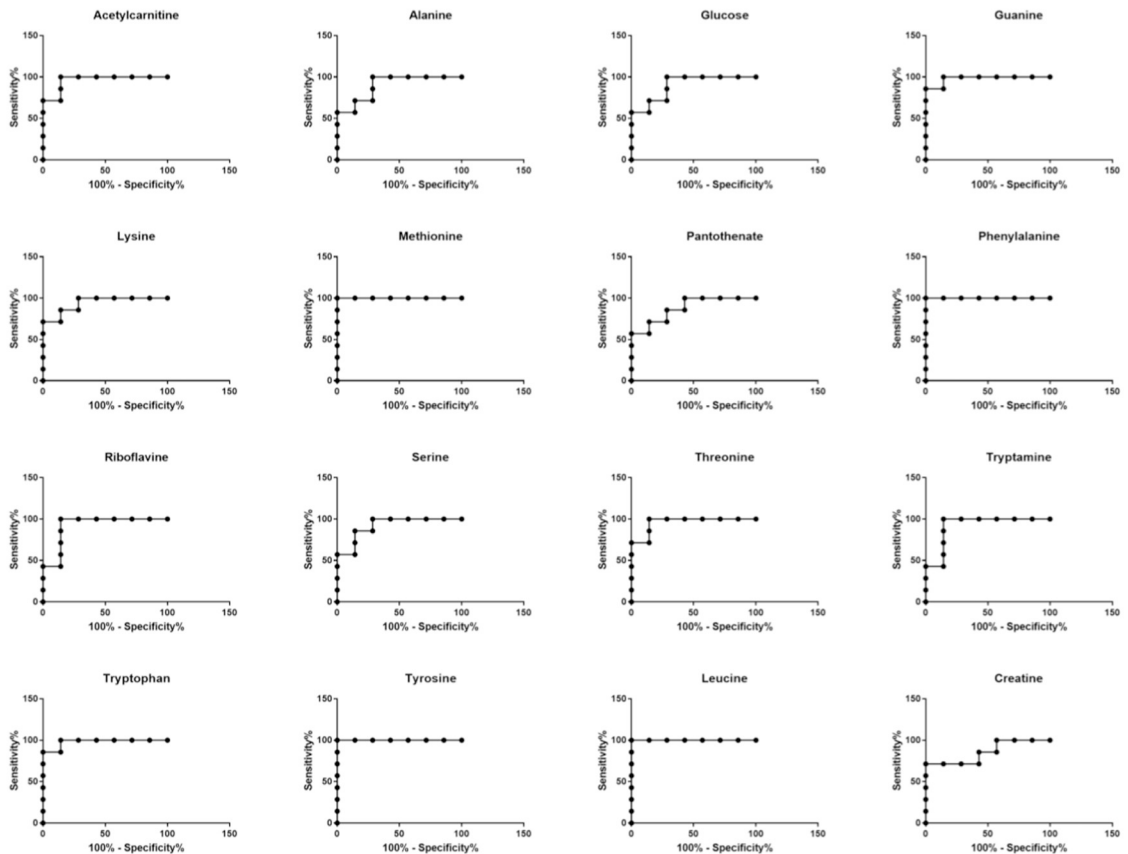


Fig. 4. ROC curves of the most significant compounds found in feces and urine on Day 1 and 15.

stream, some of them are catabolized. Some others pass in the large intestine to meet an extensive catabolism by gut microbiota, through deamination and decarboxylation [49]. Several biochemical processes are performed such as bacterial breakdown of polysaccharides, amino acids de novo biosynthesis, bacterial conversion of amino acids into polypeptides to ensure the compounds availability to the host and to maintain their microbial community.

Biosynthetic routes of several vitamins of B complex are also carried out from gut microbiome [57].

Synthesis of purine and pyrimidine nucleotides is important routes for intestinal utilization of several amino acids, mainly glutamine, glutamate and aspartate [55].

Differentiations in the levels of sugars and vitamins also occurred in tested samples. Carob is well known for its richness in sugars [53]. The levels of vitamins exhibit a similar behavior to amino acids. The elevation of vitamins on day 1 shows an excess of nutrients that were not adsorbed, while in contrast the decrease on the last day indicates effective absorption.

Based on the hypothesis that carob consumption leads to beneficial increase of gut microbiome, the excess of produced vitamins is used for the development of microbial community. Vitamins support energy production and associate to fats, carbohydrates and proteins metabolism [58].

On the 5th and 10th day, statistically significantly results were not observed. Based on these evidences, it was hypothesized that only after 10 days of treatment the rats were able to adopt the subjected physiological changes to fully exploit the abundance of nutrients.

By comparing fecal and urine rat metabolic profiles, the first was proved to be more efficient tool to capture the metabolic changes. Carob, as it is rich in fibers, amino acids, vitamins and antioxidants affects directly gastrointestinal tract. Fecal metabolome highly

correlates with intestinal mucosa. Carob's beneficial and therapeutic actions on gastrointestinal tract have recently been discussed [53,59]. For example, commercially available carob seed-based flour was effectively used in the therapy of gastro-esophageal reflux (GER), a long term and common condition in infants [59,60].

Only mild differentiations of the first sampling time point were observed based on the obtained urine profiles. Seven were found to be differentiated. Xylose, essential for human nutrition, is a urine biomarker of fruit consumption [61]. Betaine that was found altered in urine samples, derived either from endogenous metabolism of choline or from diet [62].

In the present study, no statistically significant differentiations were observed between groups or days based on rats' body weight, while statistically significant differentiations were observed between weeks for both treated and control groups according to food consumption.

## 5. Conclusion

An MS-based metabolomics method was utilized to investigate the content variation of specific targeted metabolites in urine and feces from the rats after carob powder treated. In general, it is rather difficult to extract clear conclusions from nutrition studies due to the multifactorial physiological biochemistry. However, nutritional studies in combination with detailed microbiome investigations in a longitudinal manner can provide useful knowledge of the interaction between host and nutrition. Nutritional studies are dealing with some difficulties in the interpretation of the findings. Fecal metabolome was affected more in rat treated with carobs in comparison to urine metabolome. Metabolic impact on tested samples was observed on the first and last time points. In the only relevant published study, authors similarly observed that cecal content metabolome was affected more in

### Day 1\_Urine

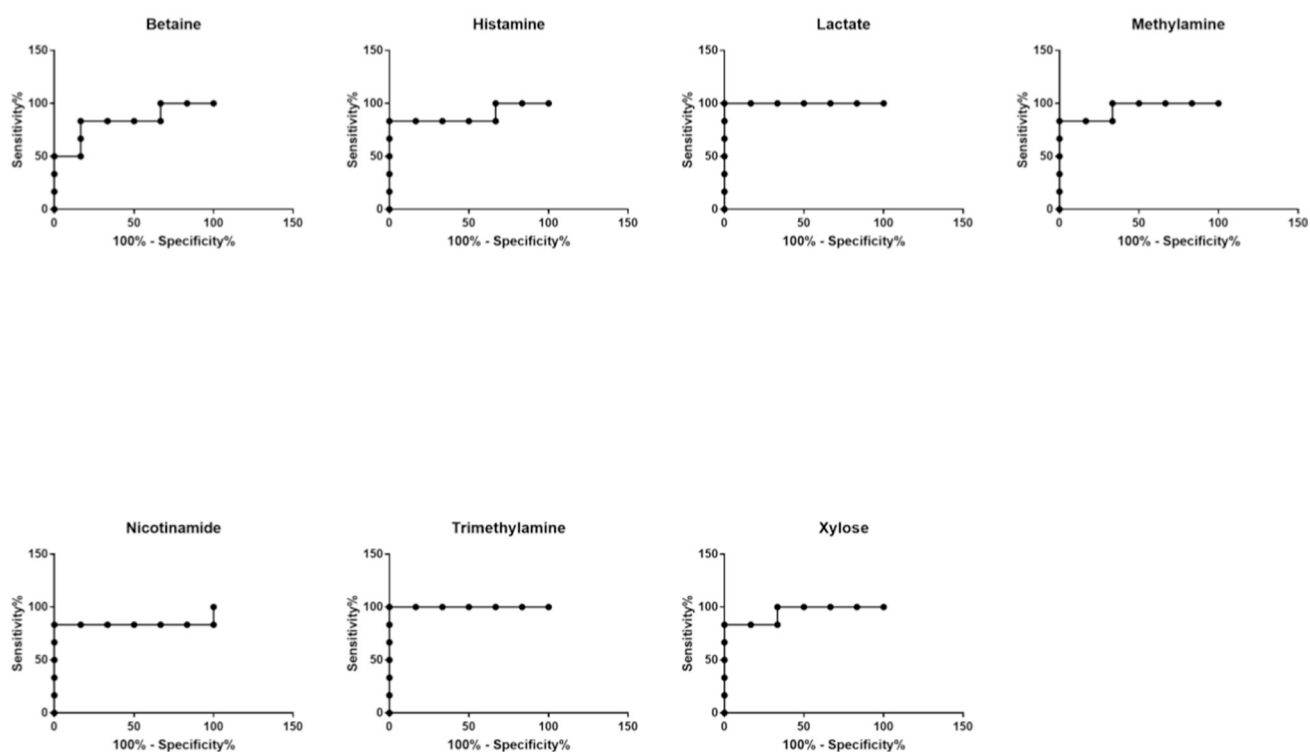


Fig. 4. (continued)



**Table 2**

Significant urine contributing to the differentiation between the two studied groups on day 1: p-value, the area under the curve (AUC) and the variable importance for the projection (VIP) of each metabolite are also demonstrated.

Compound	Day 1			
	AUC	t-Test	Log2FC <sup>a</sup>	VIP
Betaine	0.83	0.049	0.51	1.53
Histamine	0.89	0.043	0.70	1.52
Lactate	1.0	0.001	1.53	2.09
Methylamine	0.94	0.020	0.70	1.71
Nicotinamide	0.83	0.033	0.77	1.74
Trimethylamine	1.0	0.0008	1.60	2.17
Xylose	0.94	0.012	1.53	1.98

<sup>a</sup> Fold change was calculated by using the logarithm of the carob treated group-control group ratio (mean of each group) for all significant metabolites on Day 1 and Day 15.

comparison to urine and plasma metabolome in carob treated mice [17]. By performing HILIC/MS-MS metabolomics, samples were successfully differentiated according to the carob treatment.

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