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A LC–MS metabolomics approach to investigate the effect of raw apple intake in the rat plasma metabolome

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Abstract Fruit and vegetable consumption has been associated with several health benefits; however the mechanisms are largely unknown at the biochemical level. Our research aims to investigate whether plasma metabolome profiling can reflect biological effects after feeding rats with raw apple by using an untargeted UPLC–ESI–TOF–MS based metabolomics approach in both positive and negative mode. Eighty young male rats were randomised into groups receiving daily 0, 5 or 10 g fresh apple slices, respectively, for 13 weeks. During weeks 3–6 some of the animals were receiving 4 mg/ml 1,2-dimethylhydrazine dihydrochloride (DMH) once a week. Plasma samples were taken at the end of the intervention and among all groups, about half the animals were 12 h fasted. An initial ANOVA-simultaneous component analysis with a three-factor or two-factor design was employed in order to isolate potential metabolic variations related to the consumption of fresh apples. Partial least squares-discriminant analysis was then applied in order to select discriminative features between plasma metabolites in control versus apple fed rats and partial least squares modelling to reveal possible dose response. The findings indicate that in laboratory rats apple feeding may alter the microbial amino acid fermentation, lowering toxic metabolites from amino acids metabolism and increasing metabolism into more protective products. It may also delay lipid and amino acid catabolism, gluconeogenesis, affect other features of the transition from the postprandial to the fasting state and affect steroid metabolism by suppressing the plasma level of stress corticosteroids, certain mineralocorticoids and oxidised bile acid metabolites. Several new hypotheses regarding the cause of health effects from apple intake can be generated from this study for further testing in humans.

Keywords Apple · Plasma · Rats · LC/MS · ASCA · PLS-DA

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1 Introduction

Regular fruit and vegetable consumption has been associated with several positive health effects with regard to cardiovascular diseases (Bazzano et al. 2003; Liu et al. 2000; Joshipura et al. 2001) and cancers (Block et al. 1992; Steinmetz and Potter 1996; Van Duijnhoven et al. 2009). Biologically active phytochemicals and nutrients present in the plant food, indeed, interact within the metabolic pathways that potentially lead to a reduction in the risk of diseases. Particularly, apple represents the most consumed fruit throughout the European Union (Ministers 2003) and generally in the western countries. It has a high concentration of free phenolic compounds (Sun et al. 2002) thus, they might be more available for absorption into the

bloodstream, with potential positive health effects (Boyer and Liu 2004). Furthermore, it has also been shown that intake of apples may affect the composition and activity of the gut microbiota, leading to a positive impact in the immune system (Cunningham-Rundles et al. 2000), obesity (Turnbaugh et al. 2006) and cancer prevention (Rafter 2003). However the mechanisms behind is still not well known. This could partially be due to the content of pectin and other cell wall components which form important substrates for the gut microbiota.

Recently, metabolomics has arisen as a powerful approach in nutritional studies, providing a comprehensive overview of the metabolic alterations in response to specific food intake. In a previous metabolomics study, the analysis of urine samples from a rat model by LC–MS revealed exposure markers of apple and apple pectin intake (Kristensen et al. 2012). Plasma samples may provide another perspective to uncover the metabolic variations after apple consumption related to their biological effect. We employ here for the first time an LC–MS based metabolomics approach to reveal underlying patterns in the plasma metabolome when rats are fed with fresh apple at different dosages (0, 5 and 10 g/day). The findings from this rat model may ease the interpretation of potential effects of apple intake in future human studies.

2 Experimental section

2.1 Design of experiment

The study was carried out on 80 male Fisher 344 rats (4 weeks old) obtained from Charles river (Sulzfeld, Germany) during 13 weeks. The experiment was carried out according to an approval by the Danish National Agency for Protection of Experimental Animals.

After 1 week run-in period of adaptation to the purified diet, the animals were randomized by weight into five groups of 16 rats as shown in Table 1 and fed three different diets which were adjusted with regard to fresh apples, micro- and macro nutrient content, as described previously (Poulsen et al. 2011). The fresh apples from a single harvest of the minimally browning Sampion variety were stored in a modified atmosphere (2.5 % CO_2/O_2) at 0–2 °C and delivered in batches biweekly for the experiment. Apple slices with peel but without seeds and were given beside the purified diets. The rats preferred the apple for the purified diets and generally consumed all apple provided within a few hours. Rats receiving 5 and 10 g apple/day were eating less of the purified diets but as the diets for these groups were more nutrient dense the overall intake of nutrients and calories between groups was comparable. A dose of 10 g apple daily to rats may be compared

with ∼500 g/day in humans and was found to be a level acceptable to the rats. During week 3–6, some of the animals were receiving the colon carcinogen, DMH (4 mg/ml), once a week (4 doses in total) by gavage (20 mg/kg body weight), as shown in Table 1.

Before sacrifice, 56 of the animals were fasted for 12 h and 24 of the animals were given access to food up until termination.

Blood samples were collected into a heparinized funnel after sacrifice by decapitation and the heparin plasma samples were prepared for analysis by precipitation in 80 % methanol of all protein and removal using a filter (Sirocco, Waters) as described previously (Gürdeniz et al. 2012). The filtrate was dried in vacuum.

2.2 Liquid chromatography-mass spectrometry analysis

The samples were analysed by ultra performance liquid chromatography (UPLC) coupled to a premium quadrupole-time of flight mass spectrometer (QTOF MS) (Waters, Milford, MA) as described in detail previously (Gürdeniz et al. 2012). The separation was achieved on a reverse phase C_{18} BEH column (1.7 μm, 2.1 mm × 100 mm; Waters, Milford, MA) delivering a linear gradient (flow rate 0.4 ml/min) from 0.1 % aqueous formic acid to 0.1 % formic acid acetonitrile/acetone (80:20, v/v) in 5.0 min with 1 min re-equilibration time.

The LC was interfaced with an Electrospray Ionisation (ESI)-Q-TOF-Premier (Waters, Milford, MA) operated in positive and negative ion mode, in the scan range 50–1,000 m/z.

The capillary voltage was set at 2.8 or 3.2 kV for negative and positive MS mode, respectively. Ion source and desolvation gas (nitrogen) temperatures were 120 and 350 °C, the sampling cone and desolvation gas flow rates were 30 and 800 l/h. The sampling cone voltage was set at 30 kV and the Ar cell collision energy was 16 keV. Leucine enkephalin (500 ng/ml) was infused intermittently every 10 s and utilized as a lock mass for accurate on-line mass calibration.

The quadrupole analyser was set to cover the 50–1,000 Da range and samples were analysed in full-scan mode on the TOF with a scan time 0.08 s and interscan time 0.02 s. Positive and negative mode were analysed in separate runs and the spectra were acquired in centroid mode. Blank (aqueous 0.1 % formic acid) and a metabolomics standard containing 44 different physiological compounds (Barri et al. 2012) were analysed in triplicates throughout the batch analysis in order to check mass error and retention time shift. The 80 samples were analysed in duplicate (Gürdeniz et al. 2012).

Table 1 Group assignment

Group	Fresh slices apple per day (g)	Fasting	Initiated with DMH	N_{total}	N (ESI$^+$)	N (ESI$^-$)
Control	0	+	−	32	10	10
		−	−		−	6
		+	+		12	12
		−	+		−	4
Apple low	5	+	+	16	10	10
		−	+		−	6
Apple high	10	+	−	32	11	12
		−	−		−	4
		+	+		10	12
		−	+		−	4

N_{tot} no. of rats in each group, N (ESI$^+$) no. of rats in each sub-group for positive MS mode dataset, N (ESI$^-$) no. of rats in each sub-group for negative MS mode dataset

2.3 Raw data and data preprocessing

The raw data was converted to NetCDF format and pre-processed by MZmine. The peak detection parameters were chosen by inspecting the raw data whereas for the alignment step a minimum of ten samples were overlapped to decide the retention time shifts, as described previously (Gürdeniz et al. 2012).

2.4 Authentic standards

Indoxyl sulphate, 2-hydroxy-3-methylbutyric acid, hippuric acid, leucine enkephalin, 3-Indole propionic acid, *ortho-*, *meta-*, *para-* cresol, pyridine, chlorosulfonic acid, uridine 5′-diphosphoglucuronic acid*TRIS, adenosine 3′-phosphate 5′-phosphosulfatase, cytidine, corticosterone, L-lactic acid, L-leucine, L-tryptophan, L-phenylalanine, nicotinamide, PC[20:5(5Z,8Z,11Z,14Z,17Z)/20:5(5Z,8Z,11Z,14Z, 17Z)] were purchased from the local branch of Sigma Aldrich (Brøndby, Denmark).

LysoPC(18:1(9Z)/0:0), PC(O-18:1(1Z)/0:0) and PC(18: 2(9Z,12Z)/18:2(9Z,12Z)) were purchased from Avanti Polar Lipids, Inc. (Alabaster, Alabama, USA), and2,7,8-trimethyl-2-(β-Carboxy-Ethyl)-6-Hydroxychroman (γ-CEHC) was purchased from Cayman Chemical Company (Michigan, USA).

The sulfate conjugated *para*-cresol was synthesised by recrystallization with chlorosulphonic acid added to a solution of *p*-cresol in pyridine (Feigenbaum and Neuberg 1941). The sulfate conjugated *ortho*- and *meta*- cresol and γ-CEHC were synthesised from standards according to (Mammalian liver cytosol—guidline for use 1996) using a combined microsomal/cytosolic rat liver fraction prepared using optimised conditions (Nelson et al. 2001).

The glucuronide conjugated *meta*- and *para*-cresol were similarly synthesised using the microsomal/cytosolic rat

liver preparation (Mammalian Liver Cytosol—Guidline for Use; Z. Liu and Franklin 1984). The LysoPC(18:2/0:0) and LysoPC(20:5/0:0) were synthesised from standards, respectively PC(18:2(9Z,12Z)/18:2(9Z,12Z)) and PC(20:5/ 20:5) by treatment with Phospholipase A$_1$ enzyme from thermomyces lanuginosus (Sigma Aldrich) in TRIS (10 mM (pH 7.5) buffer (Pete and Exton 1996).

2.5 Identification

The level of chemical identification was graded into four levels according to published guidelines for metabolomics studies (Sumner et al. 2007).

Level 1 designates direct matching orthogonal data, such as retention time and mass spectrum using an authentic chemical standard.

Level 1 was also used in the case of some lysophosphatidylcholines (LysoPCs), where authentic standards were not purchased. Indeed, an in-house algorithm was developed in order to predict LysoPCs species based upon their physicochemical properties and their orthogonal data—retention time and *m/z*, including the presence of characteristic formic acid and sodium adducts. The isomeric differences for some of the compounds result in the presence of two retention time (RT) values (Creer and Gross 1985). The predicted RTs of LysoPCs using this algorithm is shown in Fig. 1 for the negative mode dataset with similar results for the positive MS mode dataset. The two datasets completely agree on every LPC identification and we also further verified the model using the five LysoPC standards purchased or synthesized, as described above.

For level 2, MS spectral data match a specific compound in electronic libraries (human metabolome database (HMDB), Scripps, Mass Bank) but no standards are available. Level 3 identification only gives chemical class
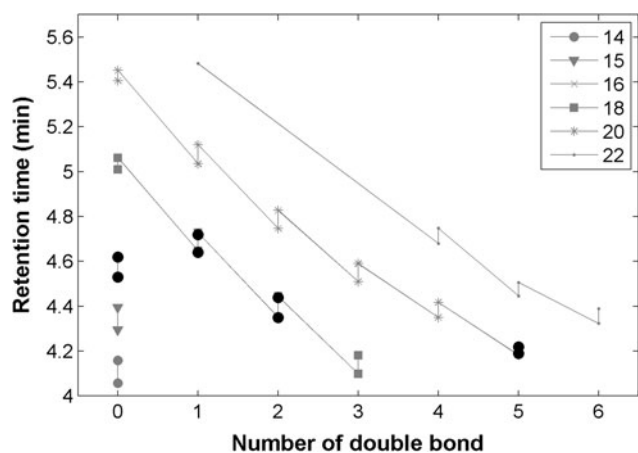
Fig. 1 LysoPC structure prediction plot. LysoPC chain length and unsaturation can be predicted by an in-house algorithm based on their retention time and mass, here shown in the negative MS mode, using the formic acid adducts as m/z values. The same results were found using the positive MS mode dataset and using pseudo-molecular ions and/or Na^+ adducts. The full black dots represent the authentic standards used to verify these relationships

assignments where the exact identity of the compound cannot be verified, e.g. compounds with typical steroid, glucuronide or sulphate patterns where the exact assignment is not possible due to ambiguity in the available compound information.

2.6 Data analysis

The feature set preprocessed by MZmine software was imported to MATLAB (Version 7, the Mathworks, Inc., MA, USA) for chemometric analysis.

To compensate for an observed drift in the analytical sensitivity during batch analysis, the two datasets resulting from positive and negative MS acquisition were preprocessed prior to the successive data analysis. In particular, each row of the data matrix, corresponding to the complete set of features measured on a sample, was normalized to unit length: operationally, this was accomplished by dividing each row by the square root of the sum of its squared elements.

2.6.1 ASCA modelling

To analyse whether the consumption of fresh apples can trigger significant metabolic variations, and to investigate the contribution of the fasting or fed conditions and DMH induction, an experimental design where these three factors were controlled was used in this study, as described in the design of experiment (DoE). Therefore, to evaluate and interpret the effect of the controlled factors, ANOVA simultaneous component analysis (ASCA) (Smilde et al. 2005) which is a multivariate version of ANOVA, was

used. The core issue of ASCA is an ANOVA-like decomposition of the experimental data matrix into the individual contributions of controlled factors and their interactions, followed by simultaneous component analysis (which is analogous to PCA) of the effect matrices. In particular, when three factors are controlled, as in this study, the ANOVA-like decomposition of the total variability in the mean centred data matrix $\mathbf{X}_m$ can be expressed as:

$$\mathbf{X}_m = \mathbf{X}_a + \mathbf{X}_b + \mathbf{X}_c + \mathbf{X}_{ab} + \mathbf{X}_{ac} + \mathbf{X}_{bc} + \mathbf{X}_{abc} + \mathbf{X}_{res} \tag{1}$$

where $\mathbf{X}_a$, $\mathbf{X}_b$, and $\mathbf{X}_c$ are the matrices associated to the main effects of the three factors; $\mathbf{X}_{ab}$, $\mathbf{X}_{ac}$, and $\mathbf{X}_{bc}$ matrices corresponding to the binary interactions while $\mathbf{X}_{abc}$ is accounting for the ternary interaction and $\mathbf{X}_{res}$ includes the residuals. Accordingly, the effect of each of the terms in Eq. (1), i.e. the contribution of the factors and their interactions to the total variability, is estimated by means of the sum of squares (SS) of the corresponding matrix. For instance, in the case of the main effect of factor a, SS is defined as:

$$SS = \|X_a\|^2 = \sum_i \sum_j X_{a,ij}^2 \tag{2}$$

where $x_{a,ij}$ is the ith row jth column element of matrix $\mathbf{X}_a$.

In the present study, as the experimental design used was unbalanced, type III evaluation of the SS had to be used. This way of computing the SS allows taking the unique variation for each factor into account, but it has the drawback that the interaction terms cannot be properly estimated. Accordingly, the total variation associated with the three controlled factors was decomposed as:

$$\|\mathbf{X}_m\|^2 = \|\mathbf{X}_{apple}\|^2 + \|\mathbf{X}_{fasting}\|^2 + \|\mathbf{X}_{DMH}\|^2 + \|\mathbf{X}_{res}\|^2 \tag{3}$$

where the neglected interaction terms were included in the residual matrix. A similar approach, to the one proposed here, has been described by others (Stanimirova et al. 2011) using a two-factor ANOVA model.

Equations 2, 3 indicate a way of evaluating the effect of the controlled factors on the experimental variability; however, to check whether an observed effect is significant or not, non-parametric statistical testing based on permutation tests was used (Anderson and Ter Braak 2003). Indeed, through the use of permutation tests, it is possible to estimate in a non-parametric way (i.e. without assuming any defined density function) the probability distributions of the SS in Eq. (3), under the null hypothesis. Then, if the experimental value of the SS is higher than the 95th percentile of the distribution of the null hypothesis, the

corresponding effect is considered to be statistically significant.

In this framework, when analysing the negative MS mode dataset, all the three controlled factors (apple diet, fasting/fed condition and DMH induction) were included, while for the positive MS mode dataset, low data quality caused by instrumental errors forced us to use a two-factor ANOVA model by leaving non-fasting rats out from the dataset. Indeed, removing these data strongly improved the ASCA model revealing more distinct effects from the apple diet.

In the present study, since the extracted features varied over different ranges, the columns of the data matrices were scaled to unit variance prior to ASCA analysis.

To interpret the results, simultaneous component analysis (Ten Berge et al. 1992) was applied to the effect matrix obtained from the ANOVA decomposition to describe the unique variation associated to the apple diet factor. At this stage, for the sake of better visualization and interpretation of the results, a modified score plot was built, according to what was recently proposed by (Zwanenburg et al. 2011) to evaluate the magnitude of the differences between the factor levels compared with the natural variation. In particular, to display the variation among the replicates in the PC score plot, the residuals (i.e. the matrix X_{res}) was projected onto the principal component subspace of the effect matrix.

2.6.2 Partial least squares discriminant analysis (PLS-DA)

In order to reveal differences between plasma metabolites in control rats vs. apple rats, PLS-DA was performed in autoscaled datasets using PLS_Toolbox (Version 5.3, Eigenvector Research, Inc., MA, USA). A permutation test (with 2,000 iterations) was applied to assess the classification performance of the PLS-DA model. The validation of the PLS-DA model was done by double cross validation applying 100 iterations (Smit et al. 2007). An eightfold single cross-validation was performed for the negative and a fourfold for the positive dataset (using random subsets) for each component (inner loop). The number of latent variables (LV) was chosen as the smallest number giving a mean classification error rate below 0.02 or, alternatively, the lowest error rate (inner loop). For the test set we randomly selected 25 and 10 % of the total negative and positive datasets, respectively (outer loop).

For variable selection, the rank of each feature is recorded based on its absolute regression coefficients from each calculated sub-model (each iteration). Then, for each feature the rank product from all sub-models is calculated which is used to demonstrate the overall importance of the feature.

To be able to decide the number of significant ranked features, a cut-off was defined as follows. (1) PLS-DA models were progressively built by including ten ranked variables each time. (2) The classification error rate of cross-validation was recorded for each model and successively plotted. (3) The number of significant ranked variables was defined by the first stable minimum (at the baseline level) in the classification error rate distribution.

2.6.3 Partial least squares modelling (PLS)

Among the significant ranked variables a dose effect was also investigated by using PLS regression analysis. The three dosages of apple (0, 5 and 10 g/day) were used as the Y matrix. The analysis was applied including only the apple diet observations (N) receiving DMH induction in order to compare equally all the three groups, since all the rats receiving 5 g/day of apple were DMH initiated.

The cross-validated R^2cv and RMSECV (root mean square error of cross validation, based on PRESS statistics) were used to assess the prediction performance of the model. The R^2cv was used to evaluate the data-fitting abilities of the model and the RMSECV value to choose the optimal number of LV.

Cross-validation was performed using different random observations held-out-at-a-time. For the negative ($n = 96$) and positive ($n = 32$), respectively, eightfold and threefold were employed.

The variable selection was performed based on the variable importance for projection (VIP) score.

2.6.4 Effect sizes

The apparent effect size was calculated for the quasi-molecular (or major adduct or fragment) ion of plasma compounds from the extracted apple-effect matrix. The effect size was calculated as the difference between the means of the treatment groups (Control—Apple) divided by the pooled standard deviation.

3 Results

The pre-processing of the raw data detected 4,015 features in the negative MS mode dataset in 180 samples (including replicates) whereas 3,427 features were detected in the positive MS mode dataset considering 53 samples (excluding one replicate and any sample obtained from the postprandial subset). As in previous studies with the current method we cover a polarity range from highly water soluble compounds such as arginine to polar lipids such as LysoPC (17:0) as shown by our external metabolome standard (Barri et al. 2012).

3.1 ASCA modelling

As previously mentioned, the total variation in the samples was split into three main effects: fasting/fed condition (only for the negative MS mode dataset), apple diet, and DMH treatment.

In order to assess the significance of each factor a permutation test was applied. The type III SS distribution of the permutated data was compared with the non-permutated data. As expected, the effect of the fasting versus fed condition was highly significant for the negative MS mode dataset ($p = 6.0$ e-5) being the strongest effect present in the dataset, however the effect of diet was also significant ($p = 0.02$), as shown in Fig. 2.

The DMH treatment in this model was not significant ($p = 0.3$).

In order to exclude a possible effect due to the order of calculation, the type III ANOVA was also calculated by inverting the order of calculation of the main effects and no change was revealed.

Simultaneous component analysis (SCA) was later applied on the apple diet matrix and the residual matrix $\mathbf{X}_{res}$ was projected onto the principal component subspace of the effect matrix, Fig. 3 for the negative MS mode dataset (the SCA plot for the positive MS mode dataset is shown in Fig. SF1, supplementary material). The scores for the two principal components extracted explain the total variation of the data and the three diet groups are separated. However, the loadings from the SCA model were not meaningful since the unbalanced design made the interpretation difficult. The treatment effect (control vs. diet) was investigated by PLS-DA analysis.
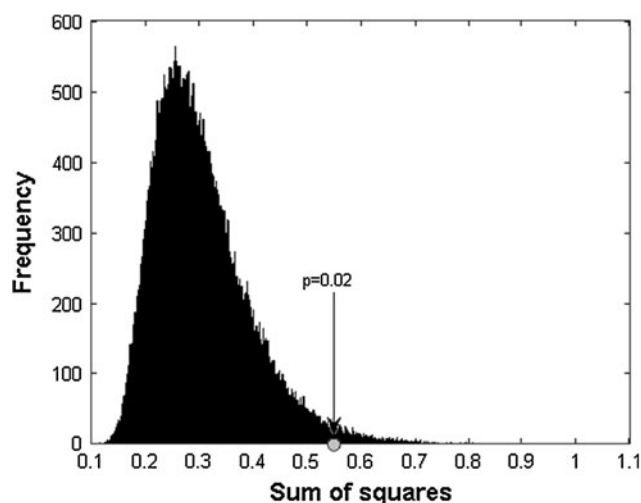
Fig. 2 Permutation test. The type III of the SSQ distribution of the permutated negative MS mode dataset, 100,000 permutations, (histogram) is compared with the SSQ distribution of the experimental data (*full point*) for the diet effect ($p = 0.02$)
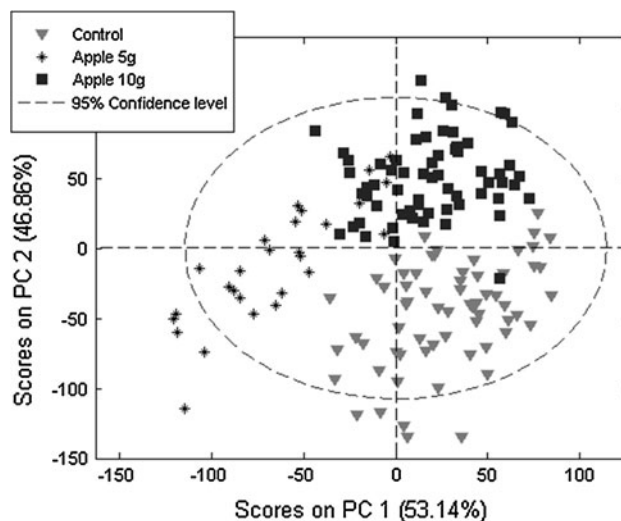
Fig. 3 SCA score plot of the apple diet matrix for the negative dataset. SCA model was applied to the effect matrix obtained from the ANOVA decomposition. To display the variation among the replicates in the PC score plot, the residuals matrix was projected onto the principal component subspace of the apple-effect matrix

## 3.2 Partial least squares discriminant analysis (PLS-DA)

The validation results showed that the classification accuracy for the original datasets was very good and, in particular, that the classification error obtained was significantly lower ($p \ll 0.0005$) than the one which could be obtained on the randomly permuted dataset (Fig. 4 for the MS negative and Fig. SF3 in supplementary materials for the MS positive datasets).

All the variables were ranked according to their rank product based on the 100-times iterated PLSDA model.

In order to decide a threshold for the ranked variables, the PLSDA models were progressively built, as described in the method section. A total of 230 features resulted for the positive MS mode dataset and 300 for the negative MS mode dataset (Figs. SF4, SF5, respectively in supplementary material).

Among these selected features, the ones we could identify or putatively identify are reported in Table 2 and in Table ST1 (supplementary material). Although these are compounds as part of a multivariate pattern, we have calculated the apparent univariate effect sizes for the quasi-molecular (or major adduct or fragment) ions as shown in Table 2 and in Table ST1 (supplementary material).

Aromatic, branched-chain and derivative amino acids, such as, tryptophan and leucine (or isoleucine, no discrimination by RT or mass) were higher in the apple fed group, except for phenylalanine, whereas several carboxylic acids, including hydroxyphenyllactic acid, 2-hydroxy-3-methylbutyric acid and lactic acid as well as some amides, e.g. 2-phenylacetamide were found to be lower.
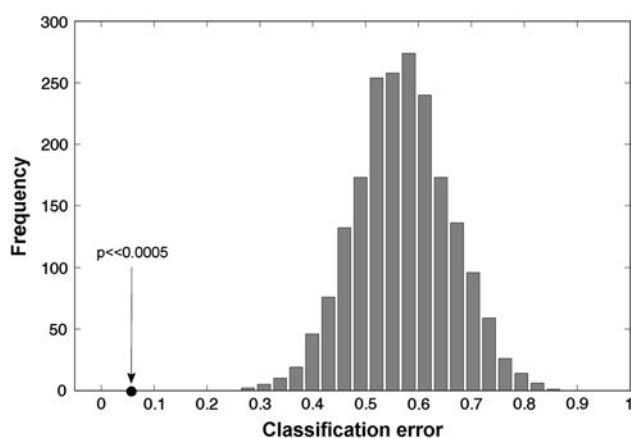
Fig. 4 Permutation test for a PLS-DA model of control versus apple diet. The histograms show the number of double cross-validation errors in 2,000 permutations for the negative dataset. The *arrow* and the *black full point* indicate the classification error in the original dataset

Phenolic and indolic products from protein fermentation, like indoxyl sulphate, *p*-cresol sulfate, *p*-cresol glucuronide and indoleacetic acid were observed increased in the control group, except for 3-indole propionic acid which was more prevalent in the apple fed group.

Acylcarnitines, including some long-chain (oleoyl- and linoeyl- carnitine) and medium-chain (2-octenoyl-, valeryl- and isovaleryl- carnitine) species were predominant in the control group as well as corticosterone and some of its metabolites (allo-tetrahydrocorticosterone and 18-hydroxycorticosterone) and cortisol metabolites.

A primary bile acid, nutriacholic acid, and a secondary bile acid, 7-ketodeoxycholic acid, were also affected showing a lower level after apple intake.

Another main contribution to the PLS-DA model separation between control and apple fed rats was related to the group of LysoPCs.

Overall, the long chain polyunsaturated fatty acids containing phosphatidylcholine were found at a higher level in plasma in the controls as opposed to some short-chain unsaturated and longer-chain mono/di-unsaturated ones, LysoPC(15:0), LysoPC (18:1), LysoPC (20:1), LysoPC(20:2).

Furthermore, the level of γ-CEHC sulfate was lower after apple intake while the levels of nicotinamide, cytidine and deoxycytidine were higher.

3.3 Partial least squares modelling (PLS)

A possible dose response was further investigated by employing PLS analysis on the selected features (*n*) from the PLS-DA analysis within the negative MS mode (*n* = 300) and positive MS mode (*n* = 230) datasets. The statistics for these models are reported in supplementary material (Table ST2).

For both the models the dosage effect was explained by the first latent variable, LV1, with the most discriminating features, selected on the basis of highest VIP values (see example in SF6, supplementary material), showing response in either direction.

Leucine/isoleucine, 3-indolepropionic acid and acetyl-leucine seemed to increase with the amount of apples consumed; in contrast, the observed plasma levels of 2-phenylacetamide, cortisone and other unknown compounds (see supplementary material) and the polyunsaturated long chain LysoPC(22:4), LysoPC(22:5) and LysoPC (P-18:0) decreased with increasing apple dose.

4 Discussion

We have previously published data related to neoplasia and to the metabolomics of fasting from this rat study (Poulsen et al. 2011; Gürdeniz et al. 2012). The present metabolic profiling study was directed to explore the change in the blood plasma after apple feeding through 13 weeks. We have also previously shown that apple feeding in rats through 4 weeks strongly affects the composition of the caecal microbiota (Licht et al. 2010) as well as the composition of urine indicating that apple feeding had a major effect on gut microbial metabolism (Kristensen et al. 2012).

The presence of two factors in addition to apple feeding in the present study design, namely animals in the fasting versus fed condition and treatment with a colon carcinogen, required a more elaborate statistical approach in order to minimise their influence. The multivariate ASCA model (Smilde et al. 2005) forms a good approach for this kind of data structure, even though the unbalanced number of samples in each group required elimination of part of the data from the analysis.

The initial ANOVA decomposition reduced the complexity, demonstrated by a clear separation between the three factors in the SCA model thereby simplifying further data analysis.

The focus of the data analysis here was investigation of the metabolites influenced by or directly originating from whole fresh apple feeding. PLS-DA analysis of the isolated data matrix related to apple feeding revealed a couple of 100 features (including quasi-molecular ions, fragments and adducts) in each ionisation mode, spanning from the polar (e.g. including cytidine) to relatively unpolar (including many phospholipids) range, as the most influential for the discrimination between the control and apple fed groups. We discuss each of the different classes of identified discriminating metabolites below and especially discuss the previously observed effects related to altered gut functionality and to changes in plasma cholesterol.

Table 2 Identified plasma metabolites discriminating control and apple diet groups and dose response

Measured m/z	Suggested ion	t_R (min)	Monoisotopic mass	Annotation	High response group and effect size
266.0770	$[M + Na]^+$	0.78	243.0855	[a]Cytidine	Apple: 0.67
112.0495[d]	$[M–C_5H_8O_4 + H]^+$				
228.0996	$[M + H]^+$	0.87	227.0906	[a]Deoxycytidine	Apple: 0.43
455.1908	$[2 M + H]^+$				
112.0518	$[M–C_5H_8O_3 + H]^+$				
89.02284	$[M–H]^-$	0.90	90.0317	[a]Lactic acid or lactate	Control: 0.68
123.0564	$[M + H]^+$	1.0	122.0480	[a]Nicotinamide	Apple: 0.75
130.0863	$[M–H]^-$	1.37	131.0946	[a]Isoleucine or Leucine**	Apple: 0.41
132.1028	$[M + H]^+$				
120.0822	$[M–CHO_2 + H]^+$	1.47	165.0790	[a]Phenylalanine	Control: 0.67
205.0992	$[M + H]^+$	1.57	204.0899	[a]Tryptophan	Apple: 0.28
146.0616	$[M–C_2H_2O–NH_3 + H]^+$				
159.0934	$[M–CH_2O_2 + H]^+$				
188.0723	$[M–NH_3 + H]^+$				
132.0823	$[M–C_2O_2–NH_3 + H]^+$				
181.0496	$[M–H]^-$	1.61	182.0579	[a]Hydroxyphenyllactic acid	Control: 1.15
117.0546	$[M–H]^-$	1.70	118.0630	[a]2-Hydroxy-3-methylbutyric acid	Control: 1.22
71.04976	$[M–CO_2H_2]^-$				
246.1719	$[M + H]^+$	1.74	245.1627	[b]Isovalerylcarnitine	Control: 1.08
85.0200[d]	$[C_4H_5O_2]^+$				
212.0005	$[M–H]^-$	1.76	213.0096	[a]Indoxyl sulfate	Control: 1.55
79.95683	$[SO_3]^-$				
132.0449	$[M–SO_3]^-$				
246.1720	$[M + H]^+$	1.78	245.1627	[b]Valerylcarnitine	Control: 1.37
85.0200[d]	$[C_4H_5O_2]^+$				
134.0601	$[M–H]^-$	1.80	135.0684	[c]2-phenylacetamide**	Control: 1.08
178.0504	$[M + CH_2O_2–H]^-$				
187.0056	$[M–H]^-$	2.05	188.0143	[a]p-Cresol sulphate	Control: 1.32
107.0494	$[M–SO_3–H]^-$				
79.95693	$[SO_3]^-$				
283.0815	$[M–H]^-$	2.09	284.0896	[a]p-Cresol glucoronide	Control: 1.15
113.0237	$[M–C_7H_8O]^-$				
175.0236	$[C_7H_8O]^-$				
130.0658	$[M–C_2H_4O_2 + H]^+$	2.46	175.0633	[a]Indoleacetic acid	Control: 1.38
176.0721[d]	$[M + H]^+$				
286.2033	$[M + H]^+$	2.46	285.1940	[c]2-Octenoylcarnitine	Control: 1.37
343.0843	$[M–H]^-$	2.47	344.0930	[a]γ-CEHC sulfate**	Control: 1.63
365.0673	$[M + Na–H]^-$				
188.0706	$[M–H]^-$	2.73	189.0790	[a]3-Indolepropionic acid**	Apple: 1.01
190.0883	$[M + H]^+$				
172.0773	$[M–H_2O + H]^+$				
130.0664	$[M–C_2H_4O_2 + H]^+$				
347.2236	$[M + H]^+$	2.93	346.2144	[b]Cortexolone	Control: 1.16
381.1828	$[M + Cl–H]^-$	3.11	346.2144	[a]Corticosterone**	Control: 1.48
391.2108	$[M + CO_2H_2–H]^-$				
347.223	$[M + H]^+$				
329.2134	$[M–H_2O + H]^+$				

Table 2 continued

Measured m/z	Suggested ion	t_R (min)	Monoisotopic mass	Annotation	High response group and effect size
405.2636	$[M-H]^-$	3.47	406.2719	[b]7-Ketodeoxycholic acid	Control: 1.67
451.2686	$[M + CO_2H_2-H]^-$				
473.2511	$[M + CO_2H_2 + Na-H]^-$				
389.2686	$[M-H]^-$	3.66	390.277008	[c]Nutriacholic acid	Control: 0.6
435.2686	$[M + CO_2H_2-H]^-$				
383.1845	$[M + Na-2H]^-$	3.75	362.2093	[c]18-Hydroxycorticosterone	Control: 1.17
315.1955	$[M-CH_2O_2-H]^-$				
317.2117	$[M-CH_2O_2-H]^-$	3.98	364.2250	[c]Dihydrocortisol	Control: 0.98
385.1987	$[M + Na-2H]^-$				
424.3438	$[M + H]^+$	4.10	423.3349	[c]Linoleyl carnitine	Control: 1.56
518.3271	$[M + H]^+$	4.18	517.3168	[a]LysoPC (0:0/18:3)	Control: 0.57
474.2612	$[M-C_3H_6-H]^-$				
562.3143	$[M + CO_2H_2-H]^-$	4.23	517.3168	[a]LysoPC (18:3/0:0)	Control: 1.01
502.2935	$[M-CH_3-H]^-$				
518.3271	$[M + H]^+$				
426.3598	$[M + H]^+$	4.28	425.3505	[a]Oleoyl L-carnitine	Control: 1.48
526.3133	$[M + CO_2H_2-H]^-$	4.29	481.3168	[a]LysoPC (0:0/15:0)	Apple: 0.23
612.3292	$[M + CO_2H_2-H]^-$	4.32	567.3325	[c]LysoPC (0:0/22:6)	Control: 0.51
520.3422	$[M + H]^+$	4.35	519.3325	[a]LysoPC (0:0/18:2)	Control: 0.88
502.3252	$[M-H_2O + H]^+$				
564.3296	$[M + CO_2H_2-H]^-$				
526.3137	$[M + CO_2H_2-H]^-$	4.40	481.3168	[a]LysoPC (15:0/0:0)	Apple: 1.03
482.3256	$[M + H]^+$				
504.3210	$[M + Na]^+$				
612.3298	$[M + CO_2H_2-H]^-$	4.39	567.3325	[a]LysoPC (0:0/22:6)	Control: 0.64
568.3425	$[M + H]^+$				
590.3231	$[M + Na]^+$				
520.3412	$[M + H]^+$	4.44	519.3325	[a]LysoPC (18:2/0:0)	Control: 0.77
476.2776	$[M-C_3H_7-H]^-$				
614.3449	$[M + CO_2H_2-H]^-$	4.51	569.3481	[a]LysoPC (0:0/22:5)**	Control: 0.60
570.3588	$[M + H]^+$				
614.3458	$[M + CO_2H_2-H]^-$	4.56	569.3481	[a]LysoPC (22:5/0:0)**	Control: 1.96
570.3588	$[M + H]^+$				
592.3419	$[M + Na]^+$				
566.3468	$[M + CO_2H_2-H]^-$	4.65	521.3481	[a]LysoPC (0:0/18:1)	Apple: 0.34
616.3606	$[M + CO_2H_2-H]^-$	4.68	571.3638	[a]LysoPC (0:0/22:4)**	Control: 1.06
572.3739	$[M + H]^+$				
530.3288	$[M-COCH_2 + H]^+$				
566.3459	$[M + CO_2H_2-H]^-$	4.73	521.3481	[a]LysoPC (18:1/0:0)	Apple: 0.60
522.3574	$[M + H]^+$				
504.3468	$[M-H_2O + H]^+$				
592.3628	$[M + CO_2H_2-H]^-$	4.75	547.3638	[a]LysoPC (0:0/20:2)	Apple: 0.12
616.3616	$[M + CO_2H_2-H]^-$	4.75	571.3638	[a]LysoPC (22:4/0:0)**	Control: 2.89
572.3734	$[M + H]^+$				
594.3581	$[M + Na]^+$				
592.3611	$[M + CO_2H_2-H]^-$	4.83	547.3638	[a]LysoPC (20:2/0:0)	Apple: 0.41

Table 2 continued

Measured m/z	Suggested ion	t_R (min)	Monoisotopic mass	Annotation	High response group and effect size
552.3667	$[M + CO_2H_2–H]^-$	4.86	507.3689	[a]LysoPC (P-18:0)	Apple: 0.45
508.3766	$[M + H]^+$				
530.3645	$[M + Na]^+$				
594.3775	$[M + CO_2H_2–H]^-$	5.04	549.3795	[a]LysoPC (0:0/20:1)	Apple: 0.81
594.3766	$[M + CO_2H_2–H]^-$	5.12	549.3795	[a]LysoPC (20:1/0:0)	Apple: 0.80
572.3735	$[M + Na]^+$				
550.3897	$[M + H]^+$				
576.3998	$[M + H]^+$	5.23	575.3950	[a]LysoPC (22:2)	Control: 0.09

[a, b, c] Identified at level 1, 2 or 3, respectively

[d] Only present in the raw data and not detected by MZMine

Dose response: a significant linear increase or decrease by the dose of apple was observed by the PLS model

4.1 Amino acids and their metabolites

One of the metabolite patterns observed here after feeding the rats with apples at a moderate dose was an increase of the hydrophobic side chain amino-acids, including tryptophan and leucine/isoleucine. This is most likely caused by a delay in amino acid catabolism along with an influence on the colonic bacteria responsible for the degradation of proteins and/or amino acids.

An effect of apple feeding on the composition as well as the functionality of the rat caecal microbiota was previously observed (Licht et al. 2010; Kristensen et al. 2012). Furthermore, an effect of the gut microbiota on the rodent blood metabolites of amino acids has also been reported (Wikoff et al. 2009). In the colon a considerable fraction of the body pool of free amino acids are degraded by the gut microbiota into products that span from uremic toxins (e.g. indoxyl sulphate) to compounds with potentially beneficial effects, and this fermentation becomes even more efficient in the more distal part of the colon (Windey et al. 2012). Specifically, a fraction of the free aromatic amino acids from the circulation are lost and metabolised by the microbiota.

In this study, lower levels of indoxyl sulfate, *p*-cresol sulfate and *p*-cresol glucuronide were found in the plasma from the apple group. Indoxyl sulfate is a microbial metabolite of tryptophan. The other aromatic amino acids, phenylalanine and tyrosine, are degraded into *p*-cresol, which is subsequently conjugated into sulphate and glucuronide esters. The unconjugated forms of these degradation products belong to the group of protein-bound uremic toxins (Vanholder et al. 2003). Many uremic toxins are also cytotoxic, and *p*-cresol is known to be toxic to hepatocytes and to affect drug-protein binding and permeability of the cell membrane (Goodhart et al. 1987; Heipieper et al. 1991; Lascelles and Taylor 1966;

McNamara et al. 1981; Thompson et al. 1994; Yokoyama et al. 1982).

Lower levels of indoleacetic acid and hydroxyphenyl-lactic acid were also observed in the apple fed group. These are endogenous breakdown products of tryptophan and tyrosine, respectively. Both compounds are neurotoxic and high concentrations are observed in phenylketonuria, a disease characterised by a non-functional phenylalanine hydroxylase (Michals and Matalon 1985).

Another deamination product of tryptophan, 3-indole-propionic acid, produced in the gastrointestinal tract, showed a positive dose response after feeding with apples. This compound has been demonstrated to have a strong neuroprotective action and to decrease formation of β-amyloid; moreover, its radical-scavenging efficiency is stronger than that of melatonin, without generating pro-oxidant intermediates (Chyan et al. 1999).

A raised level of nicotinamide was also found after apple feeding. This compound belongs to the vitamin B group and it is the amide of nicotinic acid. The higher level might be due to the natural content in apples (Souci et al. 1991) or to an increased amidation of nicotinic acid synthesised in vivo from tryptophan, which is also found at a higher level in the apple fed group.

So overall apple feeding led to a beneficial change in amino acid turnover, favouring protective degradation products over the toxic ones and potentially decreasing loss of unpolar amino acids from circulation. These potential effects of apple intake need validation and further study in humans.

4.2 Acylcarnitines

A group of medium- and long-chain acylcarnitines was revealed as compounds for the control group. The acyl-carnitines are compounds generated by the freely reversible

reaction of acyl-CoA esters with carnitine, mediated by carnitine acyl-transferase. This mechanism occurs in order to transport the fatty acid units across the mitochondrial membrane for their subsequent β-oxidation (Bremer 1983) and increases in the fasting state (Pearson and Tubbs 1967). Generally, the acyl-carnitine esters rise in plasma during the fasting condition because of the elevated concentration of acetyl-CoA leading to formation of acetyl carnitine. In this study, a decrease of carnitine was not observed. However, the increase in long-chain acyl carnitines, such as oleoyl- and linoleyl carnitine which were recognised in this study as plasma compounds decreased by apple intake, is generally associated with an increased rate of fatty acid oxidation in the mitochondria (Bremer 1983).

We speculate that the decrease of these long-chain acylcarnitine units in the apple group might be due to a longer duration of the postprandial state compared to the rats in the control group. Both groups were exposed to the same level of sugars but a slower release of sugars from the apple matrix in the gut may have caused a slower degradation of the apple-bound sugars allowing a retarded onset of lipid degradation.

4.3 LysoPCs

A general decrease in unsaturated LysoPCs of varying chain lengths was also observed in the apple group. LPCs are produced either by phospholipases A1 and A2, endothelial lipase or lecithin-cholesterol acyltransferase (LCA) (Subbaiah and Liu 1996). The LCA catalyses the transfer of fatty acid (FA) from phosphatidylcholine to free cholesterol in plasma for the formation of cholesteryl esters. LPCs may therefore be seen as compounds of transfer of lipid chains from phospholipids into the transport pool of cholesteryl esters.

In general the LPCs with more saturated acids are formed in the plasma whereas unsaturated LPCs observed here are mainly formed from PCs in the liver. As previously reported from this study both groups of LPCs were markers of the postprandial state in rats (Gürdeniz et al. 2012).

As opposed to the more unsaturated LPCs we observe in the current study a higher plasma level of LysoPC(15:0), LysoPC(P-18:0), LysoPC(18:1), LysoPC(20:1) and LysoPC(20:2) in the apple fed groups indicating that apple affects plasma and hepatic formation of LysoPCs in different ways. Hepatic production is lower after apple feeding again supporting a longer duration of the postprandial state whereas plasma transfer (by LCAT) of lipids from PCs to form LPCs and presumably fatty acid cholesterol esters is increased, indicating an increased transfer of cholesterol esters to HDL. A lower level of HDL cholesterol was observed in a previous study on dried apple powder fed to rats (Kristensen et al. 2010). HDL cholesterol is the major lipoprotein fraction in the rat and is involved in outbound as well as reverse cholesterol transport because the rat lacks cholesterol ester transfer protein. It is possible that a lower HDL level after apple feeding increases the need for LCAT to form cholesterol esters for outbound transport.

4.4 Cytidine and deoxycytidine

Higher level of the nucleoside cytidine was found in the apple fed group. This compound is a precursor of cytidine triphosphate which is used in the biosynthesis of phosphatidylcholine; therefore, this change may be related to the observation that apple consumption affects phosphatidylcholine-related pathways.

The level of the deoxyribonucleotide form, deoxycytidine was also found higher after apple intake, possibly related to a partial reduction of excess cytidine into deoxycytidine as shown previously by others (Thomson et al. 1962).

4.5 Corticosteroids

A pattern of compounds from the corticosteroids group seemed to be less strongly expressed after apple feeding, with corticosterone showing a dose–response effect. Corticosterone is the most dominant glucocorticoid in rodents, but less abundant in humans. It is the precursor molecule for aldosterone modulated by 11β-hydroxylase, a mitochondrial enzyme. Most of the corticosterone present in the blood is excreted in urine and faeces after phase II metabolism in liver and gut (Touma et al. 2003). It is the major stress hormone with a relevant impact on the function of several key limbic regions, like hippocampus, prefrontal cortex (PFC) and amygdala and operates through mineralocorticoid and glucocorticoid receptors (nuclear receptors) (McEwen 1999; Funder 1997).

An increase of serum corticosterone was observed in cholestatic animals and humans caused by activation of the hypothalamic–pituitary–adrenal axis by endotoxin and cytokines (Swain and Maric 1996). The intermediate, 18-hydroxycorticosterone, in aldosterone synthesis is the direct derivative of corticosterone formed by aldosterone synthase. The change in this metabolite therefore seems to follow the corticosterone levels and might indicate a lower aldosterone synthesis. Cortexolone is a precursor of cortisol with mineralocorticoid activity and its lower presence after apple feeding indicates a decreased formation of cortisol after apple feeding as a potential major mechanism. The metabolism of glucocorticoids is particularly important for the renal function. Aldosterone, indeed, is a homeostatic modulator of sodium and potassium levels in

the blood and a regulator of electrolyte transport in the distal nephron.

The 11β-HSD2 in the distal nephron inactivates corticosteroids in order to make the aldosterone functionally significant (Stewart et al. 1987; Funder et al. 1988). Therefore, it seems that the apple feeding might up-regulate this enzyme thereby lowering the mineralcorticoids responsible of sodium retention and potassium loss (Frey 2006). The net effect of apple feeding would therefore be the reverse, a better retention of K and a more favourable K/Na ratio. This would be expected to lower blood pressure, an effect not measured in rats but well known in humans who are supplied with a diet high in fruit and vegetables, such as the DASH diet (Rosamond et al. 1998).

Dihydrocortisol is a 5-β-reduced metabolite produced by the enzyme, steroid 5-β-reductase, which catalyzes the reduction of many steroids, including cortisol. It is also involved in bile acid biosynthesis, C_{21}-steroid hormone metabolism, androgen and estrogen metabolism. It has been hypothesized that it may have a potential mineralocorticoid activity (Marver and Edelman 1978). We speculate that its presence here reflects increased degradation of cortisol due to a higher plasma level of this precursor.

4.6 γ-CEHC

γ-CEHC sulfate is a side chain degradation product of γ-tocopherol in both rats and humans and was lower in plasma from rats fed with apples, showing dose–response. It has an intact chroman structure with potential antioxidant activity. Zhao et al. proposed a metabolic degradation pathway for γ-tocopherol starting by a ω-hydroxylation, catalysed by cytochrome P450 4F (CYP4F) and CYP3A, followed by oxidation to a carboxylic acid and finally β-oxidation of the main side chain to form carboxyethyl hydroxychromans (CEHCs). These are subjected to glucuronidation and/or sulfation prior to urinary excretion (Li et al. 2008; Zhao et al. 2010). We speculate, therefore, that apples might decrease the degradation of tocopherols in analogy to effects observed for sesame (Ikeda et al. 2002) leading to lower levels of degradation products although we cannot observe higher levels of γ-tocopherol in our system to confirm it.

4.7 Lactate/lactic acid

After apple feeding, lower levels of lactate/lactic acid and higher levels of acetylleucine and pyridinoline were observed. Lactate is produced from pyruvate via the enzyme lactate dehydrogenase (LDH) during normal metabolism and its blood concentration normally rises during exercise and decreases during gluconeogenesis. We speculate that liver gluconeogenesis based on lactate as a substrate in the control rats is higher than in the apple fed rats in accordance with the other observations related to fed/fasting balance offsets in the two treatment groups. We did not measure the physical activity of the rats in this study so an alternative explanation might be that a higher level of stress hormones in the control rats might have resulted in a more restless behaviour.

4.8 Bile acids

Two bile acids, 7-ketodeoxycholic acid and one more ambiguous oxo-bile acid, possibly nutriacholic acid, are found to be at a lower level after apple consumption in our study. Oxo-bile acids are generally regarded as toxic bacterial metabolites of bile acids and are normally reduced quickly by endogenous enzymes. The observation of lower levels of these toxic metabolites from the microbiota points again to beneficial changes in the microbiota as a major effect of longer-term apple feeding. However, the effect might also be secondary to a changed excretion of bile acids as a consequence of the changes in lipid- and steroid metabolism. In support of the latter explanation we have previously observed reduced cholesterol levels in a rat study with air-dried apples (Kristensen et al. 2010). A cholesterol lowering effect of apples in humans has recently been verified in a human cross-over trial with apples (Ravn-Haren et al. 2012).

Overall, apple intake changed the level of several amino acid metabolites from the gut microbiota, leading to a decrease in the plasma level of the toxic metabolites, indoxyl sulfate, p-cresol sulfate, and p-cresol glucuronide, and an increase in the more protective product, 3-indole propionic acid. Apple feeding might also delay the transition from the postprandial to the fasting state as revealed by a slower catabolism of aromatic and branched-chain amino acids, a lower level of long-chain unsaturated lysophosphatidylcholines and a slower rate of formation of acylcarnitine and/or of its excretion into the bloodstream. In this sense apples may constitute a 'slow-realease carbohydrate formulation' that delays the onset of branched-chain amino acid and lipid catabolism. Moreover, apple feeding suppressed the level of some corticosteroids, including corticosterone, and of toxic bile acids and of the vitamin E-degradation product, γ-CEHC sulfate. We hypothesize that apple feeding caused a lower metabolic, oxidative and steroid-related stress, which includes lower synthesis of gluco- and mineralocorticoid stress hormones and altered formation of bile acids. None of the compounds identified here were metabolites of apple-specific compounds and no apple-specific features were observed that were not present also in controls. We speculate therefore that our findings might be extrapolated to any other apple variety. In our

previous study on urine metabolomics after apple intake most markers were directly or indirectly derived from the apples and definite exposure markers solely present after apple feeding were observed. This difference may be explained by the difference between urine, which is a waste disposal fluid and plasma, which contains a homeostatically controlled mixture of endogenous metabolites. However, the difference may also reflect the fact that plasma was collected at single time points, mainly after fasting, whereas urine represents a full 24-h cycle of excretion.

In conclusion, our untargeted LC–MS-based metabolomics study indicates that the intake of fresh apple in rats has a considerable and specific impact on the plasma metabolite profile, reflecting altered gut microbial metabolism, retarded lipid- and protein catabolism, and lowered metabolic, oxidative and steroid-related stress. The current exploratory study of the effect of apple feeding on the rat plasma metabolome is the first of its kind and opens for a number of new hypotheses regarding the potential mechanisms behind the health-related effects of apples and possibly other fruits. These findings should be substantiated by formal testing of the new hypotheses in studies with humans.

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