Research Article

Use of metabolomics and lipidomics to evaluate the hypocholestreolemic effect of

Proanthocyanidins from grape seed in a pig model

Paola Quifer-Rada[1,2], Ying Yng Choy[3], Christopher C. Calvert[4], Andrew L. Waterhouse[3], Rosa M. Lamuela-Raventos[1,2].

[1]Department of Nutrition, Food Science and Gastronomy -XARTA-INSA-UB, School of Pharmacy and Food Science, University of Barcelona, Barcelona,Spain

[2]CIBEROBN del Instituto de Salud Carlos III, ISCIII, Spain

[3]Department of Viticulture and Enology, University of California, Davis, CA, USA

[4]Department of Animal Science, University of California, Davis, CA, USA.

Correspondence: Rosa M Lamuela Raventos (lamuela@ub.edu).

Abstract

Scope: This work aims to evaluate changes in the fecal metabolomic profile due to grape seed extract (GSE) intake by untargeted and targeted analysis using high resolution mass spectrometry in conjunction with multivariate statistics.

Methods and results: An intervention study with six crossbred female pigs was performed. The pigs followed a standard diet for 3 days, then they were fed with a supplemented diet containing 1% (w/w) of MegaNatural® Gold grape seed extract for 6 days. Fresh pig fecal samples were collected daily. A combination of untargeted high resolution mass spectrometry, multivariate analysis (PLS-DA), data-dependent MS/MS scan and accurate mass database matching was used to measure the effect of the treatment on fecal composition. The resultant PLS-DA models showed a good discrimination among classes with great robustness and predictability. A total of 14 metabolites related to the GSE

2

consumption were identified including biliary acid, dicarboxylic fatty acid, cholesterol metabolites, purine metabolites, and eicosanoid metabolites among others. Moreover, targeted metabolomics using GC-MS showed that cholesterol and its metabolites fecal excretion was increased due to the proanthocyanidins from grape seed extract.

Conclusion: The results show that oligomeric procyanidins from GSE modifies bile acid and steroid excretion, which could exert a hypocholesterolemic effect.

1 Introduction

Humans consume 10 times more polymeric polyphenols than monomeric ones. However, until recently, the research on polyphenols and health has been mainly focused on the monomeric fraction, since polymers are not bioavailable and their analysis is more complex. However, the role of polymers is gaining much more interest because these compounds may protect the intestinal cells, and also are converted, at least in part, to small phenolic acids metabolites that are readily absorbed by the host. Proanthocyanidins (PACs) are structurally diverse, including with regard to the number of monomer units involved. The PACs that consist exclusively of epicatechin and catechin units are called procyanidins and these are the most abundant type of PACs in plants. Grape seed extract (GSE) is a good source of PACs containing abundant oligomers and polymers of monomeric units of (-)-epicatechin or (+)-catechins, including the gallated forms.

The interest of studying the healthy effect of dietary PACs on human nutrition has increased in the last years, since PACs might be able to modulate the anti-inflammatory response by molecular

3

mechanisms, including the regulation of the arachidonic acid pathway by inhibiting COX (cyclooxygenase) and LOX (lipooxygenase) enzymes, inhibition of gene transcription and protein expression, regulation of the enzymatic activity of eicosanoid-generating enzymes, inhibition of the biosynthesis of inflammatory mediators (cytokines and nitric oxide), inhibition of mitogen-activated protein kinase (MAPK) pathway and modulation of the gene expression of the nuclear factor NF-κβ [1, 2]. Moreover, previously reported studies showed that the existence of galloyl moieties in the chemical structure may be crucial for the inhibitory activity of COX and LOX enzymes[3, 4].

Pigs are a good model for human digestion and metabolism and are an accepted and extensively used model for specific types of nutritional studies[5].

Metabolomics is the latest "omics" approach that has provided new insights on the influence of dietary compounds in the endogenous and exogenous metabolomic profile of biological fluids, such as urine, plasma, and feces[6–9]. Liquid chromatography coupled to high resolution mass spectrometry (LC-HRMS) has been applied in metabolomics studies due to its high sensitivity, resolution and mass accuracy. LC-HRMS has then demonstrated to be a powerful tool for biomarker discovery[8] and elucidation of metabolic profiles related to diseases[10, 11].

In this work, we aim to evaluate the metabolic changes in healthy pig feces due to GSE consumption by non-targeted LC-HRMS metabolomic approach in order to understand a mechanism by which GSE could affect health. Furthermore, we also embarked a targeted metabolomics approach by GC-MS in order to confirm the results obtained from the untargeted metabolomics.

2 Materials and Methods

2.1 Chemicals and reagents

4

Kaempferol-3-O-glucoside, hesperidin, 3-hydroxybenzoic acid, p-coumaric acid, caffeic acid, gentistic acid, homovanillic, naringenin, naringenin-7-O-glucoside and hippuric acid (97-99% purity, all) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Methanol (MeOH) and acetonitrile (MeCN) of HPLC grade were obtained from Sigma-Aldrich (St. Louis, MO, USA). HPLC formic acid were purchased from Panreac Quimica S.A (Barcelona, Spain). Ultrapure water (MilliQ) was generated by the Millipore System (Bedford, MA, USA).

2.2 Animals and sample collection

The study protocol for the pigs was conducted in accordance with the ILAR Guide for the Care and Use of Laboratory Animals, and the University of California Davis Animal Welfare Assurance on file with the US Public Health Service, with approval from the University of California Davis Institutional Animal Care and Use Committee (Protocol#:17257). Six crossbred female pigs (7 months old) weighing 130-150 kg were held in standard shared pen throughout the experiment. The animals were fed a standard diet (2 kg/day) composed of corn soy, and wheat (65% corn, 20% soy, 10% wheat and balanced with vitamin and mineral supplements). The intervention diet was the standard diets supplemented with 1% w/w Grape Seed Extract (GSE). The composition of the GSE was determined previously[12] and the major PAC are were polymers (23%), trimmers (19%), dimers (14%) and monomers (12%). The pigs were fed with standard diet for 3 days, and then the animals were fed the treatment diet for a period of 6 days, followed by 3 days of post treatment control-feeding which they were back on the standard diet. Feces samples were freshly collected daily during their feeding. The whole fecal material was collected and the feces were put on ice and stored at -80°C within an hour of collection before analysis.

2.3 Liquid Chromatography-High resolution mass spectrometry

5

Fecal metabolites were extracted with methanol using an extraction method that has been shown to effectively isolate a range of metabolites, including water-soluble and lipophilic compounds in blood samples [13] . 1 mL of cold methanol was added to 100 mg of fecal sample and incubated at −20 °C overnight. Samples were centrifuged at 13,000 rpm for 5 min, the supernatant was collected and dried in a vacuum concentrator (Eppendorf, Germany) at room temperature. Samples were then reconstituted with 300 μL of water:acetonitrile (1:1) with 0.1% formic acid and filtered with 0.22 μm PTFE filters. The 1st day of control period, the 3rd and 6th day of GSE intervention and the 3rd of the post treatment control-feeding period samples were used to perform the metabolomics analysis.

Liquid chromatography analysis was performed using an Accela chromatograph (Thermo Scientific, Hemel Hempstead, UK) equipped with a quaternary pump and a thermostated autosampler. Fecal metabolites were eluted using an Atlantis T3 column 2.1x100 mm, 3μm (Waters). Gradient elution was performed using water 0.1% formic acid as aqueous (A) mobile and acetonitrile 0.1% formic acid as organic mobile phase (B): 0 min, 2% B; 2 min, 2% B; 5 min, 8% B; 14 min, 20% B; 18 min, 30% B; 22 min, 100% B; 24 min, 100% B; 25 min, 2% B; 30 min, 2% B. Flow rate was set at 350 μL/min and the injection volume was 10 μL.

An LTQ Orbitrap Velos mass spectrometer (Thermo Scientific, Hemel Hempstead, UK) equipped with an ESI source working in negative and positive mode was used for accurate mass measurements. Mass spectra were acquired in profile mode with a setting of 30,000 resolution at m/z 400. Operation parameters were as follows: source voltage, 4 kV; sheath gas, 20 (arbitrary units); auxiliary gas, 10 (arbitrary units); sweep gas, 2 (arbitrary units); and capillary temperature, 275 °C. Default values were used for most other acquisition parameters (FT Automatic gain control (AGC) target 5·105 for MS mode and 5·104 for MSn mode). Fecal samples were analyzed in full scan mode and data-dependent

6

MS/MS events were acquired at a resolving power of 15,000. The most intense ions detected during full scan MS triggered data-dependent scanning. Data-dependent scanning was carried out without the use of a parent ion list. Ions that were not intense enough for a data-dependent scan were analyzed in MS2 mode with the Orbitrap resolution also set at 15,000 at m/z 400. An isolation width of 100 amu was used and precursors were fragmented by collision-induced dissociation C-trap (CID) with normalized collision energy of 35 V and an activation time of 10 ms. The mass range in FTMS mode was from m/z 100 to 1000. The data analysis was achieved using XCalibur software v2.0.7 (Thermo Fisher Scientific). An external calibration for mass accuracy was carried out before the analysis.

Quality controls (QC) were prepared by pooling ten phenolic compounds in mobile phase including kaempferol-3-*O*-glucoside, hesperidin, 3-hydroxybenzoic acid, p-coumaric acid, caffeic acid, gentistic acid, homovanillic, naringenin, naringenin-3-*O*-glucoside and hippuric acid. QC were injected randomly during the sequence and once the sequence was finished.

2.4 Data management and multivariate analysis

Metabolites contributing to the discrimination between GSE treated samples and control samples were identified through a multiple-step procedure. First, feature detection, peak alignment and retention time correction were performed using XCMS-R package [14, 15] and the following parameters were set: mass tolerance 2.5 ppm, peak width range 10-60 seconds, 3 minimum scans, minimum intensity of 5000 arbitrary units, and retention time window 0.3 min. Data were filtered using a fold-change of 1.5 and a p-value of 0.01.

The preprocessed data obtained by XCMS in negative and positive ionization were separately exported to SIMCA 13.0.3 software (Umetrics) to perform multivariate analysis. Principal component analysis (PCA) was used to check the quality of the data acquisition including the three classes,

7

control, GSE and post-intervention. Then, the supervised model partial least square discriminate analysis (PLS-DA) was used to analyze fecal metabolomic differences between the GSE intervention and the control and post-intervention period. Three PLS-DA analysis were conducted, two pair-wise analysis to compare control versus GSE intervention and post-intervention versus GSE intervention, respectively. And one meta-analysis to compare the three classes together, control, GSE and post-intervention. The data sets were normalized and Pareto-scaled before PCA and PLS-DA analysis.

The quality of the model was evaluated using the goodness-of-fit parameter (R^2X), the proportion of the variance of the response variable that is explained by the model (R^2Y) and the predictive ability parameter (Q^2).

Metabolites that were discriminating between the GSE treated samples and control samples (VIP> 1.3) were identified comparing the exact mass with the METLIN Metabolite, Human Metabolome, Lipid MAPS and Mass Bank databases [16–20] with a mass accuracy of 5 ppm. Metabolites were further confirmed by MS/MS experiments and comparing the spectra with data from the databases and from the literature. Molecular formula was calculated using the exact mass and it was further confirmed by matching with the isotopic pattern.

2.5 Sterols quantification by gas-chromatography coupled to mass spectrometry

Sterols in feces were quantified using targeted metabolomics by GC-MS. The sterols that were quantified were cholesterol, coprostanol (5β-cholestan-3β-ol), and cholestanol.

100 mg of fecal samples were weighted and 50 µL of 1000 mg/L 5α-cholestane was added to each sample as internal standard. Samples were extracted with 2 mL of chloroform/methanol 2:1 (v/v) (3 times) and sonicated for 10 min. Then 2 mL of NaCl 0.9% was added to clean the extract and

8

sonicated again for additional10 min. The organic phase was then transferred to a clean tube through a Filter-Lab 1300/80 Qualitative filter paper (100 mm) containing anhydrous sodium sulfate. The extract was evaporated to dryness under a nitrogen stream and suspended with 2 mL of KOH/MeOH 2M for saponification (overnight). The reaction was stopped by adding 2 mL of MilliQ water and sterols were extracted with 5 mL of n-hexane (3 times). The organic phase was transferred to small glass vials, dried completely under a stream of nitrogen and derivatized with 150 µL of BSTFA. Samples were then incubated at 150 °C for 60 min, evaporated and suspended with 1 mL of n-hexane.

Samples were analyzed on a Shimadzu QP2010 gas chromatograph coupled to mass detector (Kyoto, Japan). The injector temperature was held at 280 °C throughout the analysis while transfer line temperature was 260 °C. Separation was performed on a HP-5MS 5% phenyl methyl silicone (Agilent, USA) 30 m capillary column (0.25 mm ID, 0.25 µm film thickness), using helium as carrier gas at 1 mL/min. The initial column temperature of 50 °C was held for 1 min, then programmed at 6 °C/min to 320 °C and held for 10 min giving a total run time of 56 min. The splitless mode injector was maintained at 280 ℃. The mass spectrometer operated in the electron impact mode at 70 eV, with an ion source temperature of 200 °C. Mass spectra in the full scan mode were recorded in the mass range of 50–550 amu. Peak identification was based on comparison with standards of retention times and mass spectra fragmentation.

Non-parametric tests were used in the statistical analysis of the data. A Wilcoxon test for related samples was performed to compare sterol changes in response to the GSE intervention.

3 Results

3.1 Quality control

9

Quality controls were performed during the mass spectrometry analysis to evaluate the quality of the acquisition. Intra- and interbatch retention time shifts were lower than 0.1 min, peak areas variation was lower than 18% and mean mass accuracy was 2 ppm. Principal component analysis of the quality control injections was also performed to dismiss clustering among batches.

3.2 Multivariate analysis

The resultant PLS-DA models showed a good discrimination among the three classes (control, GSE intervention, and post-feeding) with great robustness and predictability to explain the differences between GSE intervention versus control and versus the post-intervention diet ($R^2X> 0.65$, $R^2Y>0.95$, $Q^2>0.90$) (Table 1). The PLS-DA model including the three classes was used to evaluate which ions were differentiating between GSE intervention, control and post-intervention. PLS-DA including GSE intervention and control classes was able to show which fecal metabolites were affected by the GSE consumption and provided the most useful information about how GSE can modulate changes in the fecal metabolome (Figure 1). Moreover, the analysis performed between GSE and post-intervention classes was set to evaluate carry-over effect on the fecal metabolome after three days of not consuming GSE.

3.3 Metabolite identification

A total of 14 metabolites related to the GSE consumption were identified (Table 2), only one metabolite was exogenous, 4-hydroxyphenylvaleric acid which is a microbial metabolite of PACs. The rest of the metabolites were endogenous: one metabolite was related to the biliary acid metabolism, three metabolites were associated with dicarboxylic fatty acids, two metabolites were linked to cholesterol metabolism, two metabolites were related to purine metabolism, two metabolites belonged to vitamin D metabolism, and one to vitamin A metabolism, and finally, two metabolites

10

were related to docosahexanoic acid metabolism. Moreover, 12 metabolites which were up- or down-regulated during the GSE intervention (VIP≥1.6) were not identified (Table 2) due to lack of a match in the metabolomics databases.

The multivariate analysis performed between the GSE and post-intervention periods revealed that the concentrations of sebacic acid, 7-hydroxy-3-oxo-5β-cholanoic acid, hypoxanthine and 4-hydroxyphenylvaleric acid were reestablished to baseline levels, suggesting that the metabolic routes were restore after 3 days no consuming GSE.

3.4 Sterols excretion

In order to verify that GSE could reduce cholesterol absorption we performed a targeted metabolomics using GC-MS to study the sterol excretion after a mid-term GSE consumption. As it is shown in Table 4, the fecal excretion of cholesterol and its metabolites, coprostanol and cholestanol, significantly increase during the GSE intervention (p-value<0.05) suggesting that GSE may indeed reduce the absorbance of sterols. A mean increase in feces of 436%, 322%, and 305% of cholesterol, coprostanol, cholestanol, respectively, was observed after the GSE intervention.

4 Discussion

The results show that oligomeric procyanidins from grape seed extract modifies bile acid and steroid excretion. This observation may provide a mechanistic explanation for the cholesterol lowering activity of polyphenols that has been reported previously [21, 22] ; Yasuda et al. 2008 [23] also showed that cacao procyanidins reduced plasma cholesterol and increased fecal biliar acids excretion in rats. The results of this study also suggest that oligomeric procyanidins, such as dimers and trimers, are the principal components responsible for the hypocholesterolemic effect, the principal

11

procyanidins of GSE [12]. A microbiota metabolite from secondary biliary acids, 7-hydroxy-3-oxo-5β-cholanoic acid, increased during the GSE intervention period. Moreover, the fecal excretion of deoxycholic acid and lithocholic acid also increased 34% and 52%, respectively. These two results suggest that primary bile acids excretion increased and that they were further metabolized to secondary bile acids and derivatives by gut bacteria. Furthermore, in another study, Ngamukote et al. 2011 [24] showed that gallic acid, catechin and epicatechin had a cholesterol-lowering activity by inhibiting pancreatic cholesterol esterase, by binding bile acids and reducing the solubility of cholesterol in micelles. Moreover, intermediate metabolites in the cholesterol biosynthesis, like zymosterol, were also increased during GSE intervention suggesting that endogenous cholesterol biosynthesis was stimulated due to lower absorption of cholesterol [21].

Targeted metabolomics approach by GC-MS confirmed the increased excretion of major levels of sterols in pig feces after a mid-term intervention with the standard diet supplemented with GSE. In this analysis, we have identified and quantified cholesterol, coprostanol, and cholestanol. Coprostanol and cholestanol are cholesterol metabolites form in the gut by intestinal microbiota [25, 26]. It has been reported that unmetabolized cholesterol is liable to enterohepatic circulation, whereas coprostanol is poorly absorbable and excreted[27], thus the metabolization of cholesterol into the gut may facilitate the elimination of cholesterol from the body. In this work, we demonstrate that GSE increases the excretion of cholesterol probably by increasing biliary excretion and reducing micellar solubility and these results agrees with previously published works as mentioned above. Dietary fiber has also similar cholesterol-lowering activity than GSE, , since water-soluble fiber increases bile acids and cholesterol fecal excretion[28, 29].

12

Sebacic, suberic and undecanedoic acids are 8- to 11-carbon medium chain dicarboxylic acids. Dicarboxylic acids are naturally formed by cytochrome P-450 ω-oxidation which is a minor metabolic pathway in the liver of mammals and it can be an alternative to β-oxidation [30]. However, it is well known that dicarboxylic acids can also be produced by microbial transformation of fatty acids through ω-oxidation [31, 32]. In a previous report, we showed that in this same experiment, the GSE diet caused an ecological shift in the colon microbiome of pigs, increasing Lachnospiraceae, Clostridales, Lactobacillus and Ruminococcacceae[33], and therefore the decrease in concentration of dicarboxylic acids may be associated with the microbiome shift due to lower microbial metabolic activity of fatty acids of certain bacterial species, inhibition of ω-oxidation, or impaired regulation of β- and ω-oxidation.

Furthermore, the results suggest that other metabolic routes were also altered: purine metabolism was down-regulated, agreeing with a previous study in which the fecal metabolome was affected by moderate red wine intake in humans [9]. In that study, results showed a lower concentration of xanthine in feces after 4 weeks of daily wine intake. Hypoxathine and xanthine are purine bases generated by ATP degradation under low oxygen concentration conditions. Hypoxanthine is converted to xanthine by xanthine oxidase and subsequently catabolized to uric acid. During this conversion, oxygen reactive species are released, like hydrogen peroxide, superoxide radicals and hydroxyl radicals, which increase microvascular and mucosal permeability [34]. Purine bases have been reported to be a biomarker of hypoxia stress and tissue ischemia [35, 36], and so the decrease of purine bases in the gastrointestinal tract may represent an improvement of the oxygen balance between oxygen delivery and consumption in the gut. Moreover, it has been reported that flavonoids have an inhibitory effect on the activity of xanthine oxidase [37].

13

The gastrointestinal tract is believed to be a potential source of inflammation associated with diet and obesity. The results show that some eicosanoids and prostaglandins were up-regulated during the GSE intervention period, like 19,20-DiHDPA and 4,5-dehydro docosahexaenoic acid, which suggest that oligomeric procyanidins from grape seed extract may affect inflammatory signaling at the mucosal gastrointestinal barrier.

Formation of vitamin D3 (cholecalciferol) di- and trihydroxy metabolites in intestine have been reported previously [38, 39]. The higher concentration of vitamin A and D3 in pig feces during the GSE intervention period could be due to oligomeric PACs decreasing micelle solubility [23, 40], slowing fat-soluble vitamin absorption. This fact should be considered when recommendation of GSE or procyanidin supplements are given since fat-soluble vitamins absorption may be affected. This will be especially important for vitamin D since a great percentage of the population do not meet the dairy recommendation of vitamin D, particularly in winter when the outdoor activity is reduced and there is major risk of vitamin D deficiency.

4-Hydroxyphenylvaleric acid was also significantly up-regulated in feces during the GSE intervention; 4-hydroxyphenylvaleric acid is a microbial metabolite of PACs. Dietary polyphenols are exposed to microbial degradation processes such as hydrolysis, microbial deconjugation and microbial conversion when they reach the colon. It has been demonstrated that PACs are metabolized by gut bacteria into low molecular weight phenolic acids, including phenylpropionic, phenylacetic, benzoic acids and derivatives [41]. In our previous study, 4-hydroxyphenylvaleric acid was found to be the main proanthocyanidins microbial metabolite in pig feces [33].

Although 14 metabolites were successfully identified, 10 compounds that were also significantly up- or downregulated during GSE intervention period (VIP>1.6) (Table 3) could not be identified due to

14

lack of information in the metabolite databases since most of these databases are mainly focused in plasma and urine. Therefore, a fecal metabolite database is still needed to study the interaction between dietary polyphenols, host microbiota metabolism, and related health effects.

In conclusion grape seed extract modifies the fecal endogenous metabolic profile in pigs, a good model for the human system. This work provides new insights into a mechanism to explain the hypocholesteromic effect of grape seed proanthocyanidins by increasing biliary excretion and reducing micellar solubility. Moreover, the results also show that eicosanoids and prostaglandins were up-regulated during the GSE intervention suggesting a mechanism by which the proanthocyanidins could alter anti-inflammatory signaling at the mucosal gastrointestinal barrier. However, more studies including the recollection of different biological fluids such as urine and plasma are needed in order to study the effect of the GSE consumption in the whole metabolome.

15

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Conflict of interest:

Dr. Lamuela-Raventos reports serving on the board of and receiving lecture fees from FIVIN;

receiving lecture fees from Cerveceros de España; and receiving lecture fees and travel support from

PepsiCo. Dr. Waterhouse received travel support from Polyphenolics Inc.

Nevertheless, these foundations were involved in the study design, the collection, analysis and

interpretation of data, the writing of the manuscript or the decision to submit the manuscript for

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The other authors declare no conflict of interest.

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20

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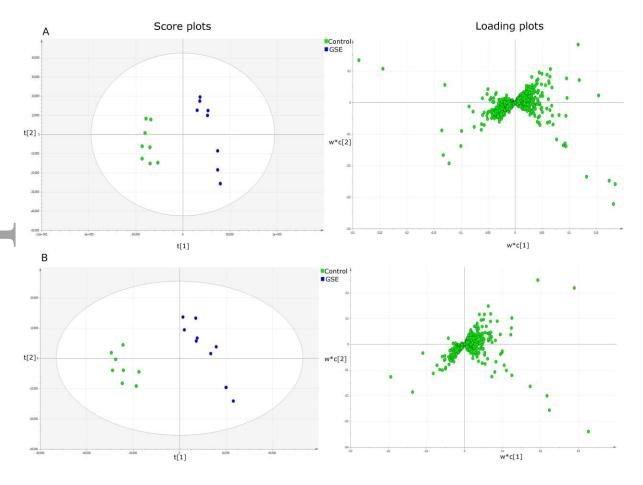
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21

Figure legends

Figure 1. PLS-DA score and loadings plots from pig fecal samples collected during the control period and after the 6-days of GSE supplemented diet.

A: Negative ionization analysis; B: Positive ionization analysis.

23

Table 1. Summary statistics of PLS-DA modeling quality

PLS-DA model	Polarity	R^2X (cum)[a]	R^2Y (cum)[a]	Q^2 (cum)[b]
Three classes	Negative	0.874	0.985	0.96
	Positive	0.74	0.957	0.916
GSE *vs* Control	Negative	0.713	0.982	0.965
	Positive	0.654	0.977	0.948
GSE *vs* PF	Negative	0.767	0.997	0.991
	Positive	0.663	0.975	0.904

[a]R^2Xcum and R^2Ycum are the cumulative explained variation in X and Y, respectively
[b]Q^2cum is the predicted variation.

24

Table 2. List of the most prominent fecal metabolites identified for discriminating between GSE treated pigs and control according to VIP value

Metabolite	Metabolic Pathway	Molecular formula	m/z detected	Exact mass	Error (ppm)	Effect	VIP	MS^2 Fragments
7-Hydroxy-3-oxo-5β-cholanoic acid	Bile acids	$C_{24}H_{38}O_4$	[M-H]$^+$ 391.2845	390.1770	0.7	Upregulated	12.1	373.2744, 355.2638, 337.2529, 319.2422, 309.2584, 159.1173
Sebacic acid	Dicarboxilic acid	$C_{10}H_{18}O_4$	[M-H]$^-$201.1127	202.1204	2.7	Downregulated	5.4	139.1125, 183.1022, 201.1129
4-hydroxyphenylvaleric acid	PAC microbial metabolism	$C_{11}H_{13}O_3$	[M-H]$^-$ 193.0863	194.0943	1.9	Upregulated	5.2	175.0759, 193.0864, 149.0967
Suberic acid (1,8-octanedioic acid)	Dicarboxilic acid	$C_8H_{14}O_4$	[M-H]$^-$ 173.0813	174.0892	3.3	Downregulated	4.4	111.0810, 173.0812
24-vinyloxy-cholest-5,23-dien-3β-ol	Chloresteol metabolism	$C_{29}H_{46}O_2$	[M-H]$^+$ 427.3569	426.3498	0.8	Upregulated	4.1	409.3467, 165.0914 427.3570
Zymosterol intermediate 1	Chloresteol metabolism	$C_{29}H_{46}O_2$	[M-H]$^+$ 427.3568	426.3498	0.8	Upregulated	4.0	409.3467, 165.0914 427.3570
Hypoxanthine	Purine metabolism	$C_5H_4N_4O$	[M-H]$^+$ 137.0462	136.0385	3.5	Downregulated	3.7	120.0525, 110.0347 94.0406
1,22,25-trihydroxy-23,24-tetradehydro-24-homo-20-epivitamin D3	vitamin D	$C_{28}H_{42}O_4$	[M-H]$^-$ 441.3002	442.3083	0.9	Upregulated	3.5	423.2899, 291.2324, 149.0605, 397.3107
Xanthine	Purine metabolism	$C_5H_4N_4O_2$	[M-H]$^+$153.0409	152.0334	0.44	Downregulated	3	137.0457
1-hydroxy-18-(4-hydroxy-4-methyl-2-pentynyloxy)-23,24,25,26,27-pentanorvitamin D3	vitamin D	$C_{28}H_{42}O_4$	[M-H]$^-$ 441.3001	442.3083	0.01	Upregulated	2.7	441.2992, 291.2315
19,20-DiHDPA	PUFA[a]	$C_{22}H_{34}O_4$	[M-H]$^+$ 363.2531	362.2457	0.5	Upregulated	2.7	345.2430, 327.2325, 363.1779, 309.2209, 299.2368
Undecanedioic acid	Dicarboxilic acid	$C_{11}H_{20}O_4$	[M-H]$^-$ 215.1281	216.1362	1.5	Downregulated	2.2	197.1177, 153.1280, 215.1281
Retinol Acetate	vitamin A	$C_{22}H_{31}O_2$	[M-H]$^+$ 329.2475	328,2402	0.02	Upregulated	2.1	255.2114, 311.2378, 329.2484, 247.1700, 161.1332
4,5-dehydro docosahexaenoic acid	PUFA	$C_{22}H_{30}O_2$	[M-H]$^+$ 327.2318	326.2246	0.3	Upregulated	1.3	253.1949, 309.2208, 281.2262

[a]PUFA Polyunsaturated fatty acid.

Table 3. List of the fecal metabolites not identified that greatly discriminate between GSE treated pigs and control according to VIP value

Predicted Molecular formula	m/z detected	Exact mass	Error (ppm)	Effect	VIP	MS2 Fragments
C18H36O3	[M-H]⁻ 299.2586	300.2664	1.6	Downregulated	12.9	281.2476, 253.2529, 183.1749
C25H40O6	[M-H]⁻ 435.2745	436.2825	0.4	Upregulated	7.7	389.2660, 435.2724
C8H4O3	[M-H]⁺ 148.9954	148.0160	2.3	Upregulated	7.1	121.0283
C28H57O9P	[M-H]⁻ 567.3683	568.374	4.7	Upregulated	6.9	567.3688, 549.3586, 391.2850
C18H16O6	[M-H]⁻ 327.0864	328.0946	0.8	Downregulated	6.0	283.096, 239.1070, 309.0759, 327.0846
C25H22O4	[M-H]⁻ 385.1429	386.1518	1.2	Downregulated	5.9	259.1805, 385.1427
C25H24N5	[M-H]⁻ 393.1945	394.2031	0.9	Upregulated	5.3	311.2217, 393.1941
C30H56O14N5	[M-H]⁺ 711.3925	710.3823	4.1	Upregulated	5.1	693.3821, 623.3398
C23H34O14N9	[M-H]⁻ 659.2126	660.2224	3.1	Downregulated	4.5	329.1028, 285.1129, 241.1231, 659.2134
C18H34O3	[M-H]⁻ 297.2427	298.2507	0.2	Downregulated	3.7	

Table 4. Cholesterol and its metabolites fecal excretion in feces before and after the intervention with grape seed extract.

Metabolite	Standard diet[a] (mg/kg)	GSE supplemented diet[a] (mg/kg)	p-value
Cholesterol	170.8 (88)	605.4 (222)	<0.05
Coprostanol	218.7 (97)	540.6 (190)	<0.05
Cholestanol	174.3 (81)	480.1(200)	<0.05

[a]Concentrations expressed as mean (SD) in mg/kg

Grape seed extract (GSE) is a good source of PACs containing abundant oligomers and polymers of monomeric units of (-)-epicatechin or (+)-catechins, including the gallated forms. This work aims to evaluate changes in the fecal metabolomic profile due to grape seed extract intake by untargeted and targeted analysis using high resolution mass spectrometry in conjunction with multivariate statistics. The results show that oligomeric procyanidins from grape seed extract modifies bile acid and steroid excretion, which could exert a hypocholesterolemic effect.

27