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A high fat, high cholesterol diet leads to changes in metabolite patterns in pigs – A metabolomic study

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ABSTRACT

Non-targeted metabolite profiling can identify biological markers of dietary exposure that lead to a better understanding of interactions between diet and health. In this study, pigs were used as an animal model to discover changes in metabolic profiles between regular basal and high fat/high cholesterol diets. Extracts of plasma, fecal and urine samples from pigs fed high fat or basal regular diets for 11 weeks were analysed using ultra-high performance liquid chromatography with high-resolution mass spectrometry (UHPLC–HRMS) and chemometric analysis. Cloud plots from XCMS online were used for class separation of the most discriminatory metabolites. The major metabolites contributing to the discrimination were identified as bile acids (BAs), lipid metabolites, fatty acids, amino acids and phosphatidic acid (PAs), phosphatidylglycerol (PGs), glycerophospholipids (PI), phosphatidylcholines (PCs) and tripeptides. These results suggest the developed approach can be used to identify biomarkers associated with specific feeding diets and possible metabolic disorders related to diet.

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1. Introduction

Nutritional metabolomics is a rapidly developing sub-branch of metabolomics, used to profile small-molecules to support integration of diet and nutrition in complex bio-systems research (Jones, Park, & Ziegler, 2012). Recently, the concept of "food metabolome" was introduced and defined as all metabolites derived from food products (Fardet et al., 2008). Chemical components in foods are absorbed either directly or after digestion, undergo extensive metabolic modification in the gastrointestinal tract and liver and then appear in the urine and feces as final metabolic products (Primrose et al., 2011). It is well known that diet has a close relationship with the long-term health and well-being of individuals. Hence, investigation of the "food metabolome" in biological samples, after feeding specific diets, has the potential to give objective information about the short- and long-term dietary intake of individuals, and to identify potential biomarkers of certain dietary patterns (Primrose et al., 2011). Previous studies have identified potential biomarkers after consumption of specific fruits (Carvalho et al., 2013; Pujos-Guillot et al., 2013), vegetables (Bernal et al., 2013; Pujos-Guillot et al., 2013), cocoa (Llorach, Urpi-Sarda, Jauregui, Monagas, & Andres-Lacueva, 2009; Moco, Martin, & Rezzi, 2012) and juices (Knab et al., 2013; van Dorsten et al., 2010). More metabolites were revealed by using metabolomic approaches compared with the detection of pre-defined chemicals found in those foods.

Eating a high-fat and high cholesterol diet is strongly associated with conditions of obesity, diabetes and metabolic syndrome, that are increasingly recognised as worldwide health concerns (Li & Chiang, 2012). For example, a high fat diet is a major risk factor for childhood obesity (Johnson, Mander, Jones, Emmett, & Jebb, 2008), cardiovascular diseases (Burgueno, Gianotti, Mansilla, Pirola, & Sookoian, 2013) and hyperlipidemia (Aubin et al., 2010; Ma et al., 2012). Little is known on the extent to which changes in nutrient content of the human diet elicit changes in metabolic profiles. There are several reports of metabolomic profiling studies on plasma, serum, urine and liver from high fat-diet induced obese mice (Cheng et al., 2010; Kim et al., 2011; Spagou et al., 2011), rats (Kim et al., 2009; Song et al., 2013) and humans (Lehtonen et al., 2013). Several potential biomarkers of obesity and related diseases, including lysophosphatidylcholines (lysoPCs), fatty acids and branched-amino acids (BCAAs) have been reported.

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Levels of endogenous and exogenous metabolites in plasma, urine and tissues reflect different dietary treatments. These differences in metabolite patterns can provide insight into underlying molecular mechanisms related to diet (Gu et al., 2007; Jones et al., 2012). Experimental platforms that are sensitive to both individual and environmental variations in diet are needed to produce reliable and robust data. Pigs have been well-recognised in biomedical research for physiological and anatomical similarities to humans (Leucht, Leuthold, & Stier, 1977; Simon & Maibach, 2000). To model the metabolite response to diet in humans, pigs were fed a high fat diet for 11 weeks and the metabolite profiles in plasma, urine and feces were analysed. Non-targeted ultra high performance liquid chromatography tandem with high resolution mass spectrometry (UHPLC–MS) was utilised for metabolomic profiling. Bile acids (BAs), lipid metabolites, fatty acids, amino acids and phosphatidic acid (PAs), phosphatidylglycerol (PGs), glycerophospholipids (PI), phosphatidylcholines (PCs), tripeptides and isoflavone conjugates were found to be the final dietary metabolites that differentiated pigs fed a high-fat and high cholesterol diet versus a basal diet. The results of this study illustrate the capacity of this metabolomic profiling approach to identify new metabolites and to recognise different metabolic patterns associated with diet.

2. Materials and methods

2.1. Animals and sampling

All animal experiments and procedures were conducted in accordance with guidelines established and approved by the Beltsville Area Animal Care and Use Committee. Eleven 4-week-old female pigs were obtained from the experimental farm at the Beltsville Agricultural Research Center, Beltsville, MD. Pigs were derived from boars from a four-way crossbred composite BX line (Duroc X maternal Landrace X terminal Landrace X Yorkshire) designed by scientists at the USDA/ARS/US Meat Animal Research Center, Clay Center, NE to be genetically similar to genetics in the commercial swine industry at the time they were born; the genetics of the gilts are predominantly of the BX composite line. Pigs were from a herd screened yearly for porcine reproductive and respiratory syndrome virus (PRRSV), influenza (H1N1 and H3N2), pseudorabies and brucellosis by the Veterinary Services Group at the Beltsville Agricultural Research Center and have been negative for these infections. They were individually housed in stalls with a non-absorptive concrete floor with *ad libitum* access to water. Pigs were weighed and randomized into two groups of eleven, and seventeen pigs fed either A) a basal regular diet containing 21% of total kcal from protein, 68% from carbohydrates, and 11% from fat; or B) a high fat/high cholesterol diet containing 16% of total kcal from protein, 40.0% from carbohydrates, and 44% from fat including 2% cholesterol (Table S1). Corn starch was used as a carbohydrate source, and soy protein, L-threonine, tryptophan and methionine were used as protein sources in all diets. Soybean oil was used for diet A and soybean oil, coconut oil, lard and cholesterol were used for diet B. Pigs received a fixed amount (0.6 kg) of feed. This amount was increased gradually up to 1.2 kg to maintain linear growth. Pigs on all diets received an equivalent volume of feed for the 11 weeks of the study. Pigs consumed all feed each day with no appreciable waste.

The four-week old pigs were weighed at 0, 3, 6, 9 and 11 weeks of the study. Fecal samples were collected each morning after release by the pigs at weeks 0, 2, 4 and 6 and from the distal colon and proximal colon at necropsy on week 11. Blood samples were drawn from pigs fasted for 24 h into serum separation tubes or into EDTA containing tubes for the collection of plasma on week 11. Urine was collected aseptically at necropsy from the bladder using a 50 ml syringe and 18 gauge needle. Fecal, plasma, serum and urine samples were stored at −80 °C until analysis. Serum triglycerides (TG) and cholesterol (CHOL) levels were analysed using a commercial clinical diagnostic laboratory service under GLP standards (Antech Diagnostics GLP, Morrisville, NC).

2.2. Sample preparation

Frozen aliquots of urine were thawed, centrifuged at 7000*g* for 10 min and then filtered with a 0.20 μm PVDF filter. Two microliters were injected into UHPLC–MS for analysis.

Plasma samples were prepared for UPLC–MS analysis by methanol protein precipitation. Cold methanol (150 μl) was added to 50 μl of plasma, vortexed for 30 s, incubated at −20 °C for 20 min, centrifuged at 14,000 rpm for 10 min, and the supernatant transferred to a HPLC vial. Two microliters were injected into UHPLC–MS for analysis.

Fecal and intestinal content samples and were lyophilized and then 1 g of each sample was ground and extracted in 3-fold of methanol. The mixture was vortexed strongly and subsequently centrifuged at 14,000 rpm at 4 °C for 10 min. Two microliters were injected into UHPLC–MS and analysed.

2.3. Materials and chemicals

Cholic acid, lithocholic acid, deoxycholic acid, chenodeoxycholic acid, hyodeoxycholic acid, ursodeoxycholic acid and sodium glycochenodeoxycholate were purchased from Sigma–Aldrich (St. Louis, MO, U.S.A.). Standards were dissolved in a water/methanol solution (50/50 v/v) to obtain solutions of 50 μg ml^{-1} before LC–MS analysis. Formic acid, HPLC grade methanol and acetonitrile, were purchased from VWR International, Inc. (Clarksburg, MD). HPLC grade water was prepared from distilled water using a Milli-Q system (Millipore Lab., Bedford, MA).

2.4. UHPLC-PDA-ESI/HRMS/MS^n conditions

The UHPLC–HRMS system consisted of an LTQ Orbitrap XL mass spectrometer with an Accela 1250 binary Pump, a PAL HTC Accela TMO autosampler, a PDA detector (ThermoFisher Scientific, San Jose, CA), and a G1316A column compartment (Agilent, Palo Alto, CA). Separation was carried out on a Hypersil Gold AQ RP-C_{18} UHPLC column (200 mm × 2.1 mm i.d., 1.9 μm, ThermoFisher Scientific) with an UltraShield pre-column filter (Analytical Scientific Instruments, Richmond, CA) at a flow rate of 0.4 ml/min. The mobile phase consisted of a combination of A (0.1% formic acid in water, v/v) and B (0.1% formic acid in acetonitrile, v/v). The linear gradient was from 2% to 20% B (v/v) at 10 min, to 50% B at 25 min and to 98% B at 30 min, and held at 98% B to 32 min. Both positive and negative ionisation modes were used and the conditions were set as follows: sheath gas at 80 (arbitrary units), aux and sweep gas at 15 (arbitrary units), spray voltage at 4.5 kV for positive mode and 4.0 kV for negative mode, capillary temp at 250 °C, capillary voltage at 15 V and tube lens at 70 V. The mass range was from 100 to 2000 *m*/*z* with a resolution of 15,000, FTMS AGC target at 2e5, FT-MS/MS AGC target at 1e5, isolation width of 1.5 amu, and max ion injection time of 500 ms. The most intense ion was selected for the data-dependent scan to offer their MS^2 to MS^4 product ions, respectively, with the a normalisation collision energy at 35%.

2.5. Data analysis

2.5.1. LC–MS data pre-treatment and handling

Acquired UHPLC–HRMS raw files were converted into mzXML format using Proteowizard 3.0. 3569 (http://proteowizard.