CERES 2014 Final Report-
Ebru Selin Selen, Porter Lab

Investigation of GMO vs Organic Diet Effects on Metabolome Profiles in Dairy Cows by NMR Spectroscopy

Introduction.

There is concern that GMO crops may induce health related changes in domestic animals and humans; however, there is little direct evidence for GMO exposure consequences. Previously, we analyzed plasma and milk samples collected from two different farms about 20 miles apart, with different management approaches; organic (Miller Farms) and GMO fed cows (University Farm) to investigate the diet effect on cow metabolic biomarkers in milk and plasma. GMO group showed fundamental metabolic differences compared to organic fed diet (ORG) dairy cows. Identified biomarkers indicate increased inflammatory response and oxidative stress in GMO group. However, we could not exclude the possibility that those differences were solely due to the feeding regime or due to different farm practices. To test whether the differences we observed were due to GMO diet, we designed a second 'side by side' experiment in which all cows were kept in adjacent organic and GMO farms. We collected plasma and milk samples weekly from 13 April through 18 May, 2013. The timeline and experimental design are summarized in Figure 1. Time course we allowed sampling while all cows were off grass and enabled sampling when the organic cows transitioned to grass. Our ***objectives*** were to conduct this type of metabolome experiment with cows fed organic versus GMO diets to demonstrate: 1) that these metabolome effects are not unique to management type or location, but rather diet type.

One of the arguments that arises frequently with GMO versus organic is that the presence or absence of grass (different management practices) in the diet are really what makes the difference and it has nothing to do with GMO. We now know from our cow experiments than grass provides important diet components that provide the optimal metabolome profiles and milk content.

Organic agriculture needs to increase market share. One way to do is building public demand, that provides new market opportunities and promoting additional farmer transitions to organic agriculture to meet increased demand. Creating awareness of the facts that the GMO diet can induce detrimental changes in normal bodily function, constitute a risk factor for chronic and long-term diseases by promoting oxidative stress and low-level inflammation in the public will help to grow public demand for organic products. This will also creates a strong economic advantage to farmers, which has historically been a primary driver of farmer transitions to organic production methods. In our study we aimed to show concrete evidence of how diet influences cow' metabolism and milk product. We can now strongly reinforce the argument for organic transition, since we are able to present reasons why organic cows live at least twice as long as GMO fed cows and organic cow veterinary bills are approximately half those of GMO farm cows. Once the public recognizes that GMO produced milk has unfavorable health benefits, it should accelerate transitions to a healthier method of production.

Methodology

The IRB protocol was approved for this study. 1H 1D nuclear magnetic resonance (NMR) technology was used to profile and quantify the metabolome profile of cows under different diets. Data analysis of plasma and milk metabolome was conducted with the Metabolome Dynamics Platform, developed by Dr. Fariba Assadi-Porter, founder of MetResponse, LLC.

In the side-by-side experiments, all animals (a total of 20 Holstein cows) were kept in adjacent organic and GMO farms located near Columbus, WI. The owner of the farms cares for and feeds the animals in these side-by-side dairies. In the current design, we collected samples from the new side-by-side organic and GMO dairies that offer a unique 'bipartite' dairy operation. This provided an exceptional opportunity to demonstrate that the original biomarkers results are not location specific or due to different husbandry methods.

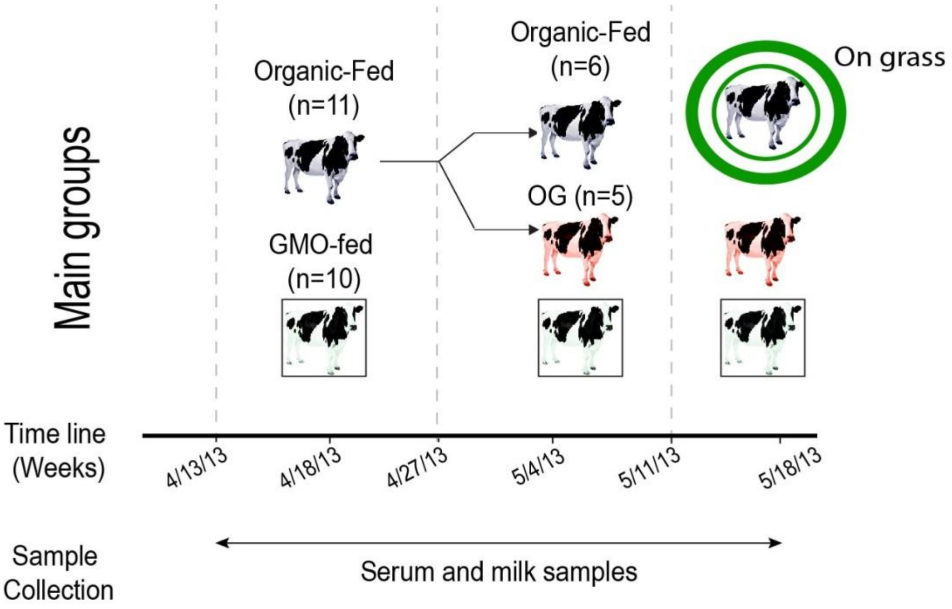

Figure 1: Experimental approach that was used in this study. ORG, GMO and COG groups are defined in the chart. The X axis shows the exact sample collection dates. The green circle around the ORG fed cow indicates that only the ORG group go on grass between the weeks 5/11/13 and 5/18/13.

Our experimental procedure had two stages. The first stage lasted one week to establish baseline sample sets for each group. The second stage lasted 5 weeks; we cross-switched diets for 5 cows from each group. Five of the organic fed cows were put on a GMO diet and five of the GMO fed cows were put on the organic diet. Accordingly, in the beginning of the second week, we had four groups of cows; five GMO fed, five Organic fed to GMO fed, five GMO fed to organic fed and five Organic fed cows. This stage allowed us: 1) to evaluate the observed diet effects on metabolism especially the oxidative stress and mild inflammation that GMO had caused on the animals,2) to test whether the observed effects on biochemical metabolism of GMO diet can be reversed by putting the animal on an organic diet, 3) to test whether the effects of GMO diet can alter the biochemical profile of organically fed cows,4) to test whether organic food is immuno-protective against GMO. ***Sample collection***. The first week, 5 ml milk and blood samples were collected approximately two hours apart. Samples from both groups were collected once per week for five weeks prior to the organic herd being moved to pasture. Blood samples were collected by using heparinized tubes, left to rest at least one hour on ice, followed by centrifugation to separate red blood cells from serum, then serum was frozen at -80°C until NMR data collection time. Milk samples were frozen until it was processed for NMR data collection. ***NMR Sample Preparation.*** We collected serum and milk samples to investigate how diet affects the cow metabolic pathways. Body fluids included metabolites or small molecules (<1000kD) such as carbohydrates, amino acids and fatty acids resulting from metabolic activities of organisms, that provide insights into the biochemical state of an organism's metabolism. Each metabolite has a unique signal that can be identified through NMR spectroscopy. Proteins and/or lipids were removed by one-step organic solvent extraction method for serum samples and processed according to our previous published procedure [13]. ***NMR Data Analysis.*** NMR data were collected at the NMR facility at Madison (NMRFAM), using 600 MHz Varian NMR equipped with auto-sampler. Collected spectra were transformed into peaks which were then be quantified by Chenomx software version 6 (http://www.chenomx.com) using an internal reference, formate. Two-way ANOVA was used to assess significance of changes between groups for each time points. Significance was determined by p-value; p values lower than 0.05 were considered significant.

Results

Lipid and antioxidant metabolism biomarkers showed different profiles between groups, during the diet effect time course. ***Temporal view of antioxidant levels.*** Hippurate, an glycine conjugate of benzoic acid, has diverse roles in metabolic reactions including; tumor growth inhibitor [1], fatty acid metabolism [2], and regulator and stimulator of ammoniagenesis [3]. Plasma and milk metabolome profiles found in organically fed cows consistently show the highest levels of the antioxidant molecule hippurate, whereas the GMO fed cow milk samples contain the least amount of hippurate. The converted organic group (COG) hippurate antioxidant level is between the concentration levels from the organic (ORG) and the GMO groups (Figure 2 A and B). ***Temporal view of lipid intermediates in milk.*** Diet regime has enormous effects on milk profiles. Milk fatty acids profile is an indicator of healthful benefits of milk to the consumer. Due to the limitations of NMR we are able to detect only short to medium chain fatty acids and ketone bodies, 3-Hydroxybutyrate (3-HB) and acetone. 3-HB is a marker of increased fatty acid oxidation. Our study identified higher levels of 3-HB in both plasma and milk samples in the ORG group then COG, with the GMO levels being the lowest among the groups. Acetate is another key intermediate in lipid metabolism which showed higher levels in the organic group than in GMO, in plasma and milk samples.

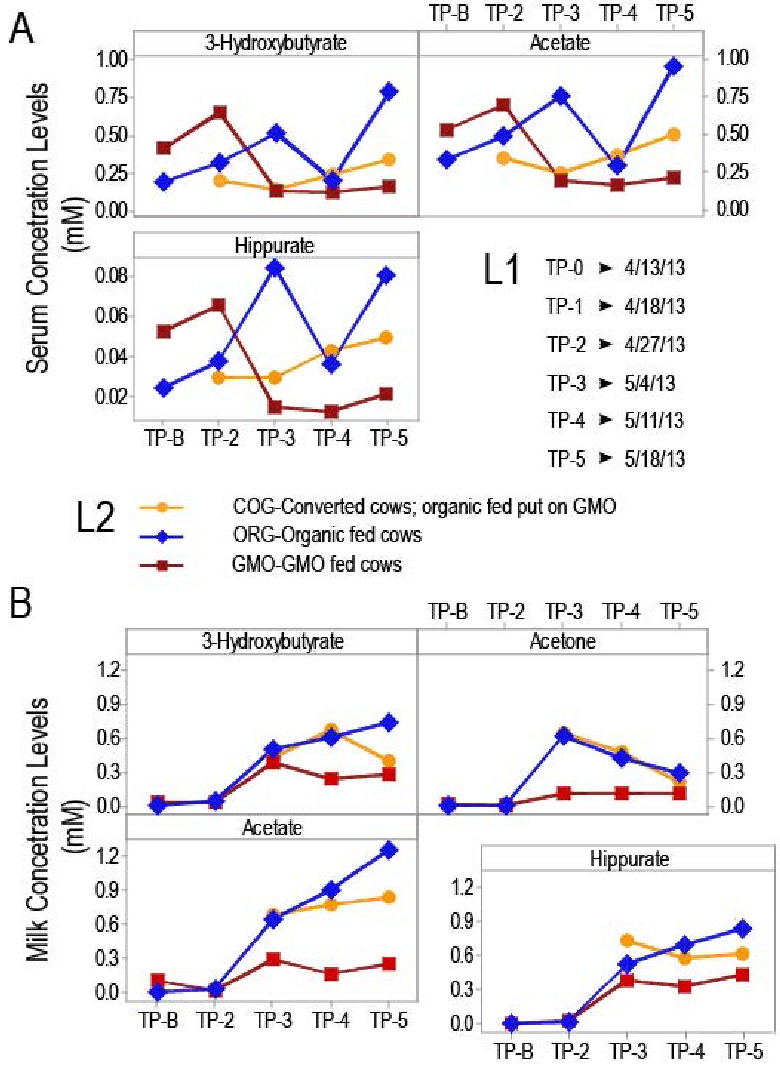

Figure 2: Temporal pattern of selected metabolites. The Y axis shows the concentration levels in mM units. The X axis shows the time points which are indicated in L1 (TP-1 through TP-5) as well as the corresponding date of sample collection. TP-B in each metabolite graph represents the arithmetic mean of samples collected for baseline (TP0 and TP1). Red, orange and blue lines stand for GMO, COG and ORG groups respectively and are summarized in L2. Panel A shows the metabolite concentrations in plasma samples collected from cows, Panel B shows concentration levels in milk samples.

Lipid profiling by gas chromatography coupled with mass spectroscopy (GS-MS)

Milk samples of organic (ORG), GMO (GMO) and organic to GMO (COG), converted- fed, cows were profiled with gas chromatography (GC) since NMR will not easily detect them. We profiled 21 fatty acid species including short chain, medium chain, long chain poly-unsaturated fatty acids (PUFA) and mono-unsaturated fatty acids (MUFA). Long chain fatty acids, chains longer than 13C, are the most abundant class in milk fat with a 76.2% share, followed by medium chain, 19% and short chain 4.8% (Figure 3A). Among the long chain fatty acids, PUFA has the highest share with 72.7%, followed by MUFA (27.3%) (Figure 3A).To investigate the differential fatty acid content in the three feeding regimes at three time points, we used a multivariate approach, PLSDA. Our results showed clear effects of diet regimes on the milk fatty acid composition (Figure 3B). Palmitic acid, lineloic acid, capyrilic acid, capric acid, alpha linoleic acid and butyric acid are revealed as important species, due to the higher variances of those species between diet regimes. Important metabolites and the direction of changes of fatty acid contents in ORG and COG group as compared to GMO group are provided in Table 1. Palmitic and oleic acid are two highly abundant fat species in milk fat. The grass fed organic group showed the lowest ratio (healthiest) among all studied groups and decreased palmitic acid in the grass-fed organic group compared to other groups which supports the benefits of grass feeding in the ORG fed group.

Table 1: Fatty acid species selected by multivariate analysis.

Number of C	Direction of change from GMO diet	Common Name
4:0	↓	Butyric acid
8:0	↓	Caprylic acid
10:0	↓	Capric acid
16:0	↓	Palmitic acid
18:2 n-6	↓	Linoleic acid
18:3 n-3	↑	alpha-linoleic acid

Diet regime showed evidence for reduced rumen metabolism relative to GMO diet and rumen short and medium chain fatty acid species, butyrate (4:0), capric (8:0) and caprylic (10:0), suggesting that ORG and COG diets are lipid metabolizing and more healthful compared to a GMO diet. Alpha-linoleic acid (LNA) is one of the unsaturated fatty acid species that is shown to have beneficial impacts on consumer health [4, 5]. Our study showed LNA levels are comparable between GMO and grass fed-organic groups while the organic to GMO converted group showed decreased LNA content in milk.

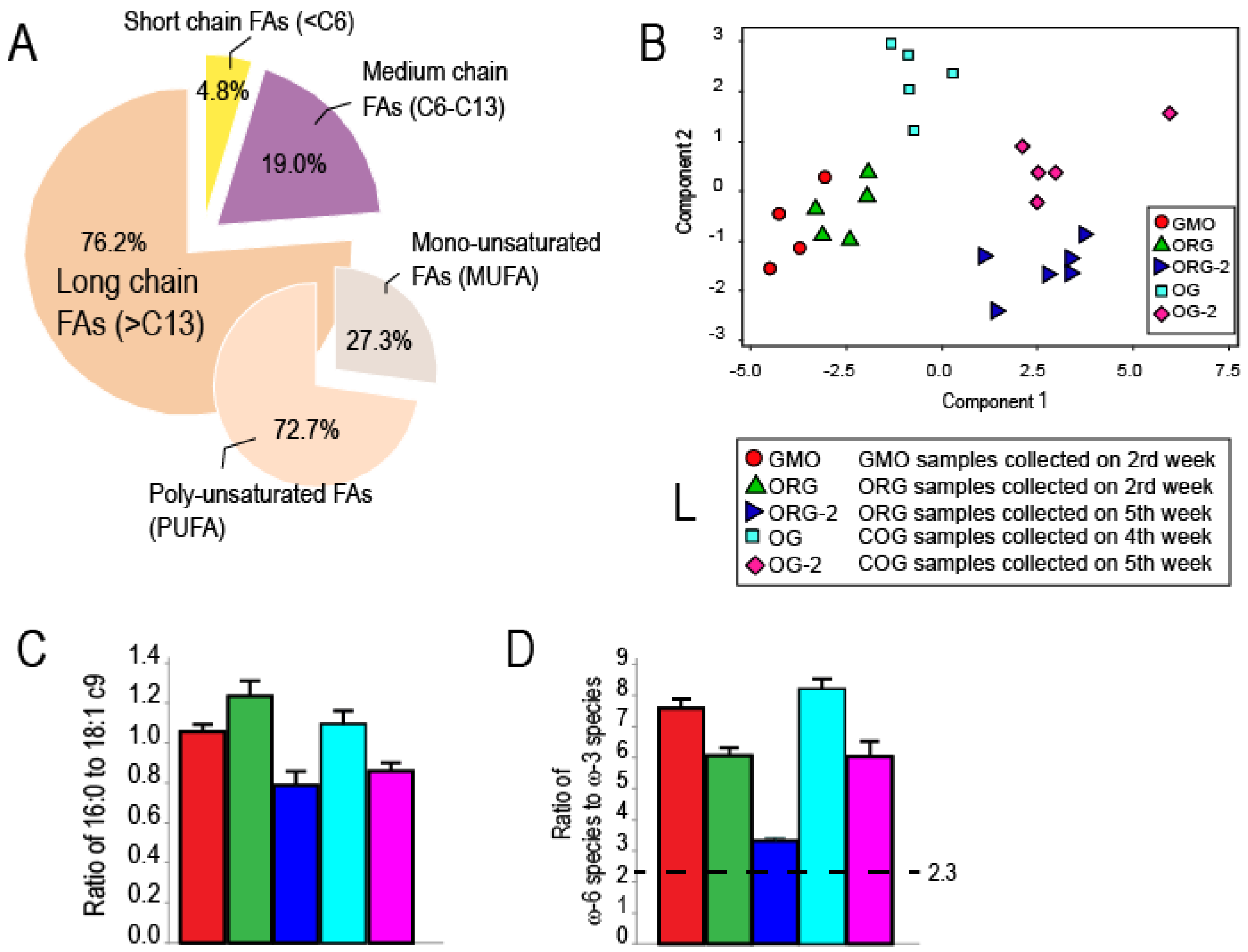

Figure 3: Milk fatty acid profiling through GC-MS. Panel A: Percentage contents of profiled fatty acid species. **Panel B** 2D-PLSDA scores plot showed clear separation between experimental groups. The X axis shows component 1 scores and the Y axis shows component 2 scores. **Panel C**. Ratio of saturated to unsaturated fatty acid; indicator of milk fat content. **Panel D.** Ratios between omega 6 and omega 3 fatty acid species, one of the indicators of the inflammatory properties of milk. Dashed line represents the optimal ratio between omega-6 and omega-3 fatty acids [6]. Ratios in Panels C and D are the ratios of peak intensities that are profiled through GC-MS. **Panel L** summarizes the color coded groups in Panels B, C and D. Red, green, blue, turquoise and purple bars stand for GMO, ORG, ORG-2, i.e. organic cows on grass (TP-5) ,COG, and COG-2 (TP-5) groups respectively.

Discussion

Higher levels of the lipid oxidation marker 3-HB in plasma and milk samples of ORG- fed cows indicate ORG cows' lipid metabolism is shifted towards catabolism of short chain fatty acids and results in less lipidic plasma and milk profiles. In the last week of the experimental study (TP-5), the anti-lipidic profile becomes more prominent when the ORG group fed on grass. The organic feeding regime is able to shift metabolism to a higher antioxidant state, hippurate level, associated with the less lipidic plasma and milk profiles (3-HB and acetone) as compared to the GMO group. When cows are fed with grass, the levels of 3-HB, acetone and hippurate are significantly increased as compared to the GMO fed group indicating beneficial effects of grass feeding. Under our experimental design, we found that the ORG diet results in more healthy metabolic and lipidic profiles. Grass feeding promotes even healthier metabolic profiles in cows.

To support our metabolomics findings that we obtained through CERES grant, we were able, with Center for Integrated Agricultural Studies (CIAS) funding, to conduct the lipid analyses described above using complementary studies by GC-MS. This collaboration with the Mark Cook Lab allowed us to profile the fat composition in the milk from all three groups in our study (CIAS, 2014). GS-MS findings on fatty acid profiles supports increased lipid oxidation we have shown through biomarkers 3-HB and acetone through 1D 1H NMR analysis from the CERES support. Decreased butyrate levels and increased lipid catabolism marker 3-HB in ORG and converted groups, compared to GMO, indicates butyrate is being used as an energy source.

Several fatty acids are preferentially utilized as energy sources in the mammary gland (e.g., 16:0 and 18:0) and the rumen of cows. Among them palmitic acid, which is utilized as an energy source during milk production, is the final product of *de novo* lipid synthesis. It has been shown that, higher palmitic acid content is proportional to a negative energy balance [7, 8], and is an indicator of metabolic disturbance in cows. While individual contributions of fatty acid species are important, ratios between several fatty acid species are also important factors that affect milk quality. The ratio between 16:0 to 18:0 (and 18:0 derivatives) is indicative of milk lipid content. Previous studies indicated a milk fat profile with a lower ratio between long chain fatty acid, 16:0 and 18:0 species in fat composition is a healthful indicator to humans [9]. Our results indicate that grass fed ORG cows have the favorable milk lipid profile with lesser saturated fatty acid content among all other groups (Figure 3C). Another ratio we used to assess the milk quality, in terms of inflammatory aspects, is the ratio between omega-6 species to omega-3 fatty acid species. The organic diet resulted in a lower ratio than the GMO diet. The results become more prominent when the ORG group was put on grass feed in the last week of study. A significant reduction in the ratio suggests that grass feed promotes anti-inflammatory omega species over pro-inflammatory species (Figure 3D).

NMR and GC-MS analysis both showed that the combination of grass feeding and organic diet in cows have the maximum beneficial and healthful effects in milk for human consumption.

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