5-2016

Effects of Carboxymethyl-Lysine in Heat Processed Foods on the Plasma Metabolome in Mice

Zhou Fang
Utah State University

EFFECTS OF CARBOXYMETHYL-LYSINE IN HEAT PROCESSED FOODS ON THE PLASMA METABOLOME IN MICE

by

Zhou Fang

A thesis submitted in partial fulfillment
of the requirements for the degree

of

MASTER OF SCIENCE

in

Nutrition and Food Sciences

Approved:

Robert E. Ward Major Professor	Korry Hintze Committee Member
Jerrad Legako Committee Member	Mark R. McLellan Vice President for Research and Dean of the School of Graduate Studies

UTAH STATE UNIVERSITY
Logan, Utah

2016

ABSTRACT

Effects of Carboxymethyl-lysine in Heat Processed Foods on

the Plasma Metabolome in Mice

by

Zhou Fang, Master of Science

Utah State University, 2016

Major Professor: Dr. Robert E. Ward
Department: Nutrition Dietetics and Food Sciences

The effect of dietary carboxymethyl-lysine (CML) on the metabolite profile of plasma was investigated. Mice were fed one of five diets including: AIN93 diet (negative control), a 45% kcal from fat Diet Induced Obesity diet (DIO; positive control); CML0, Total Western Diet (TWD) with low CML; CML1, TWD with medium CML, and CML2, TWD with high CML. In addition, plasma glucose across the five diet groups was also quantitatively measured in this study.

According to the results, 93 compounds were detected in the mouse plasma samples using Gas Chromatography-Mass Spectrometry (GC-MS). Among all 93 detected compounds, 49 of them were amino acids, fatty acids, organic acids, or other organic molecules, while 44 of them could not be identified and were considered to be "unknowns". Four identified metabolites and 10 unknown metabolites were significantly

different between the five diets. In addition, only one metabolite, lactic acid, was significantly different between the three CML diets. A principal component analysis (PCA) showed a clear separation of the CML2 diet, or the diet high in CML, from the other diets along the second principal component. The DIO and AIN93 diets were mostly separated by the third principal component. In addition, both PC1 and PC 3 affected CML0 and CML2.

Overall, the metabolic profile of plasma was affected by the amount of CML in diet more than the differences between diets. However, further study is warranted to elucidate the specific mechanisms involved in the changes to the metabolome.

(65 pages)

PUBLIC ABSTRACT

Effects of Carboxymethyl-lysine in Heat Processed Foods on

the Plasma Metabolome in Mice

Zhou Fang

Carboxymethyl-lysine (CML), an advanced glycation end product (AGEs), is formed during the cooking process and may induce toxic effects on human health. The effect of dietary CML on the plasma metabolite profile was investigated. Mice were fed with one of five different diets: AIN93 diet (negative control), a 45% kcal from fat Diet Induced Obesity diet (DIO; positive control); CML0, TWD (Total Western Diet) with low CML; CML1, TWD with medium CML, and CML2, TWD with high CML for 8 weeks, and the plasma metabolome was determined by Gas Chromatography-Mass Spectrometry (GC-MS). In addition quantitative analysis of plasma glucose level across the five diet groups was also measured using internal standard.

In the mouse plasma samples 93 compounds were detected using GC-MS. Among all 93 detected compounds, 49 of them were identified, while 44 of them could not be identified and are considered as unknowns. Four identified metabolites and 10 unknown metabolites were significantly different between the five diets. Only one metabolite, lactic acid, was significantly different between three CML diets. A principal component analysis

(PCA) provided a clear separation of CML2 diet from the others. The DIO and AIN93 diets were mostly separated by PC3. Both PC1 and PC 3 affected CML0 and CML2. Glucose levels did not differ, however, CML0, CML1, and CML2 groups did showed higher glucose levels (CML0: 107.91 mg/dL, CML1: 108.46 mg/d, and CML2: 105.61 mg/dL) compared to AIN93 (104.85 mg/dL) and DIO (105.31 mg/dL).

ACKNOWLEDGMENTS

I would like to thank to my major professor, Dr. Robert E. Ward, to give me this precious opportunity to work with him, and being so helpful and patient to guide me throughout my whole project and degree program. I would also like to thank my committee members, Dr. Korry Hintze and Dr. Jerrad Legako, for their valuable time and suggestions to my research.

I could not have accomplished this without the support of my parents, Simin Fang and Linfeng Geng. I would give a big thanks to them for always being there for me and giving me confidence and encouragement. Finally, I want to thank my family and all my friends, especially to my beloved grandfather, Guoliang Geng.

Zhou Fang

CONTENTS

ABSTRACT iii

PUBLIC ABSTRACT v

ACKNOWLEDGMENTS vii

CONTENTS viii

LIST OF TABLES x

LIST OF FIGURES xi

LIST OF SYMBOLS, ABBREVIATIONS, AND DEFINITIONS xiii

Literature Review 1

- Maillard Browning 1
- Advanced Glycation Endproducts 2
- Dietary AGEs, Animal Studies 4
- Dietary AGEs, Human Studies 5
- Total Western Diet 6
- Effect of Heat Processing Foods on Protein Digestibility 6
- Fasting Glucose and Insulin Sensitivity 7
- Short Chain Fatty Acids 12
- Metabolomics Analysis 15
- Gas Chromatography and Mass Spectrometry 16
- Automated Mass Spectral Deconvolution and Identification System 16
- SpectConnect 17

Hypotheses and Objectives 19

Materials and Methods 20

- GC-MS Analysis of Mice Plasma Metabolites 20
  - Statistical Analysis 21

Results 22

Metabolomics Analysis of Mice Plasma 22
Metabolites Principal Component Analysis 37
Glucose Content in Plasma 42

Discussion 44

Conclusion 47

References 48

LIST OF TABLES

Table		Page
1	Identified metabolites significantly different in AIN93, DIO, CML0, CML1, CML2	24
2	Unknown metabolites significantly different in AIN93, DIO, CML0, CML1, CML2	25
3	Identified metabolites significantly different in CML0, CML1, CML2	26
4	Identified metabolites in AIN93, DIO, CML0, CML1, CML2	27
5	Unknown metabolites in AIN93, DIO, CML0, CML1, CML2	30
6	Identified metabolites in CML0, CML1, CML2	32
7	Unknown metabolites in CML0, CML1, CML2	35
8	Eigenvalues of the correlation matrix for the metabolites principal component analysis	38
9	Eigenvectors of principal component 1, principal component 2, and principal component 3 for the metabolites principal component analysis	40

LIST OF FIGURES

Figure Page

1 The pathways of Maillard reaction (Nursten, 2005) 1

2 CML content in the AIN, DIO, CML0 (TWD low), CML1 (TWD med), and CML2 (TWD high) diets compared to previous human and rat studies. The comparison was based on the amount of total CML in the diets divided by the calorie content of the diets 9

3 Fasting glucose (A), area under the curve (AUC) for the oral glucose tolerance test (OGTT; B) for all five diet groups. Fasting glucose (C) and incremental AUC (D) for the mice fed the TWD with increasing levels of CML 10

4 Fasting glucose (A) and area under the curve (AUC) for the insulin tolerance test (ITT; B) for all five diet groups. Fasting glucose (C) and incremental AUC (D) for mice fed the TWD diets with different CML levels 11

5 Cecal short chain fatty acid content (SCFA) from mice fed AIN, DIO and TWD diets with differing amounts of CML. SCFA are arranged in highest to lowest concentration. SCFA with different letter designation are significantly different 14

6 SpectConnect working process (Styczynski et al., 2007) 18

7 Content of lactic acid (A), phosphoric acid (B), 2-pyrrolidone-5-carboxylic acid (C), oleic acid (D). Values were normalized by the area of internal standard. Different lettered columns indicate a significant difference between diets ($p < 0.05$), which were determined by one-way ANOVA and Tukey post hoc analysis 23

8 Scree plot for the metabolites principal component analysis 37

9 Three-dimension score plot for metabolites principal component analysis (PC1, PC2, and PC3) 42

10 Mean plasma glucose content in AIN93, DIO, CML0, CML1, and CML2 ± standard error. Different lettered columns indicate a significant

difference between diets ($p < 0.05$), which were determined by one-way ANOVA and Tukey post hoc analysis. 43

11 Mean plasma glucose content in CML0, CML1, and CML2 ± standard error. Different lettered columns indicate a significant difference between diets ($p < 0.05$), which were determined by one-way ANOVA and Tukey post hoc analysis. 43

12 Mice final body weight after 8 weeks feeding for Temoin (AIN93), Hight fat (DIO), CML0, CML1, and CML2 diet groups. Different lettered columns indicate a significant difference between diets ($p < 0.05$), which were determined by one-way ANOVA and Tukey post hoc analysis. 45

LIST OF SYMBOLS, ABBREVIATIONS AND DEFINITIONS

MRP	Maillard Reaction Product
AGE	Advanced Glycation Product
GC-MS	Gas Chromatography-Mass Spectrometry
dAGE	Dietary Advanced Glycation Product
CML	Carboxymethyl Lysine
TWD	Total Western Diet
DIO	Diet Induced Obesity
SCFA	Short Chain Fatty Acid
UC	Ulcerative Colitis
BSA	Bovine Serum Albumin
NIST	National Institute of Standards and Technology
ANOVA	Analysis of Variance
RI	Retention Index
PCA	Principal Component Analysis
T2D	Type 2 Diabetes
AUC	Area Under the Curve

OGTT	Oral Glucose Tolerance Test
ITT	Insulin Tolerance Test
MSTFA	N-Methyl-N-(trimethylsiyl)trifluoroacetamide
AMDIS	Automated Mass Spectral Deconvolution and Identification System
CSF	Cerebrospinal Fluid

Literature Review

Maillard Browning

Maillard reaction, which is also known as nonenzymatic browning or glycation, is the result of the reaction between a reducing sugar and a free amino group of a protein, or nucleic acid. Figure 1 shows a general overview of the Maillard reaction.

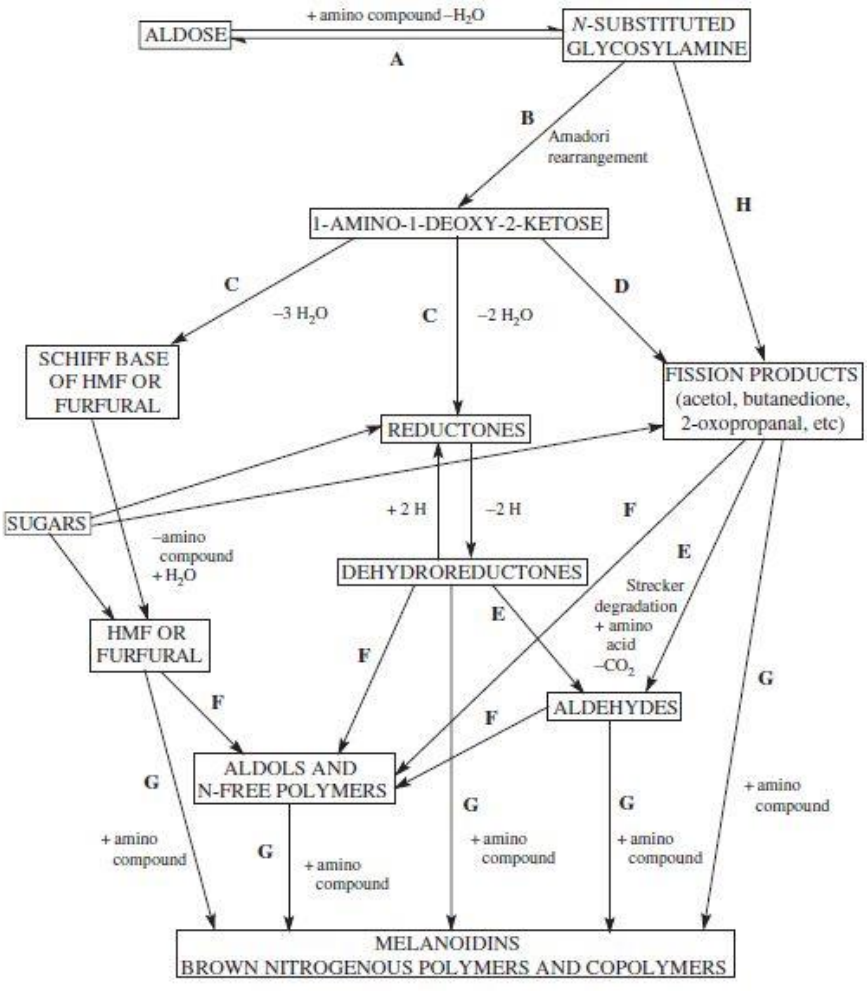


Figure 1. The pathways of Maillard reaction (Nursten, 2005).

The carbonyl group of the aldose sugar reacts with the amino group of the amino acid to form N-substituted glycosylamine and water. Then glycosylamine undergoes amadori rearrangement, forming ketosamine, which is also known as amadori rearrangement product (ARP). The ketosamine then either dehydrates into reductones and dehydro reductones, or results in short chain hydrolytic fission products such as acetol, diacetyl, or pyruvaldehyde which then undergo the strecker degradation (Nursten, 2005).

Maillard reaction has an important role in food chemistry because of its unique contribution to form specific flavors and colors to foods (Waller & Feather, 1983). However, it may also lead to some toxic byproducts and can decrease the nutritional value of foods by degrading essential amino acids, such as lysine. Maillard reaction products (MRPs) usually are classified into three groups: early MRPs, advanced MRPs (also known as AGEs) and melanoidins (Furniss, Vuichoud, Finot, & Hurrell, 1989).

Advanced Glycation Endproducts

Advanced glycation end products (AGEs) are a complex group of compounds that are produced through the Maillard reaction. AGEs are also known as glycotoxins, which occur both exogenously in food and endogenously in the body (Foerster & Henle, 2003). In addition, AGEs naturally exist in uncooked animal-derived foods, and the formation of AGEs exists in all types of cooking, such as boiling, baking, and frying (Assar, Moloney,

Lima, Magee, & Ames, 2009). Modern diets are largely heat processed. These diets are potentially leading to the formation of high levels of AGEs (Uribarri et al., 2010). Dietary advanced glycation end products (dAGEs) are known as an important source of the total AGEs in the human body, which contribute to the risk of developing the increased oxidant stress and inflammation (Uribarri et al., 2005). The formation of AGEs is a part of normal metabolism, but if the content of AGEs are excessively high in tissues or the circulation they have possibilities to become pathogenic, which may cause the development and progression of chronic diseases, such as diabetes, Alzheimer's disease, cancer and aging (Vlassara H, 1994). Due to this fact, the scientific interest in AGEs has increased in recent years. N-carboxymethyl-lysine (CML) is one of the well-characterized AGEs that typically is measured in these investigations (Bosch et al., 2007). Some AGEs such as CML can be also formed via lipid peroxidation (Kanner, 2007).

In modern Western diets, there is a wide range of food products that are exposed to cooking or other heated processes to gain some sensory properties and to enhance food safety. The formation of AGEs is related to temperature used in products processing and methods of cooking applied as well as the nutrient composition of foods (Assar et al., 2009). Fats or meat-derived foods cooked under high temperature lead to more AGEs formation than carbohydrates boiled for a longer cooking time (Yamagishi, Ueda, & Okuda, 2007). Uribarri and et al, found using high temperature for cooking, such as frying,

broiling, grilling, and roasting foods produced a higher level of dAGEs compared to low or mild temperature, such as boiling, stewing, and steaming foods (Uribarri et al., 2010). Microwaving and dry heat cooking for short times had no significant effect on the formation of dAGEs. Hull et al (Hull, Woodside, Ames, & Cuskelly, 2012) found dairy products to have an extremely high CML concentration (5143.7 mg/kg protein) compared with the other foods products, such as bread (178.4 mg/kg protein) and cereals (281.3 mg/kg protein).

Dietary AGEs, Animal Studies

Animal studies have shown that high intakes of AGEs result in increased plasma levels of CML, carboxyethyllysine, and pentosidine. Dietary consumption of AGEs also significantly increased the renal excretion of CML compared with a diet with a lower AGE load (Somoza et al., 2005). In addition, rats fed high AGEs diets gained more weight and had higher liver, heart, kidney, and lung weights. Moreover, rats on AGE-rich diets showed signs of increased lipid peroxidation. Another mouse study showed that a high-fat, high-AGE diet increased plasma AGE levels and significantly increased body weight. Mice consuming the high AGE diet also had higher fasting glucose and fasting insulin and developed diabetes (Sandu et al., 2005).

Dietary AGEs, Human Studies

Some human studies also have been conducted to study the effect of dAGEs. It has been revealed that consumption of diets rich in AGEs results in significantly higher plasma AGEs levels (increased by 64.5%, $p = 0.02$) and increased mediators (tumor necrosis factor α, IL-1β, IL-6, and vascular adhesion molecule) of vascular dysfunction (Vlassara et al., 2002). In another study, 21 healthy volunteers were assigned for one week to consume either a heated or an unheated high protein diet (3 g/kg/day) with a 1 week wash-out period in between. The heated and unheated diets were comparable in regard of protein, carbohydrate, and energy intake. Daily CML intake of the heated diet was much higher than unheated diet group which was almost negligible. The results of this study showed that the fasting serum CML level and urinary excretion significantly increased on the heated diet group compared to unheated diet control In addition, volunteers on the heated diet gained more weight than the unheated diet group (Šebeková & Somoza, 2007).

Determining the content of AGEs and finding methods to reduce the content of AGEs during food processing is becoming important to food and nutritional scientists. However, not many studies have investigated AGEs, and there are few reports in the literature concerning the effect of food processing on AGEs formation.

Total Western Diet

Many studies using rodent models have investigated the effect of AGEs on health. The diets used in most of these studies, however, does not accurately reflect the American diet pattern which contains high calories and a suboptical micronutrient profile. Thus, Dr. Ward and colleagues developed a new diet, Total Western Diet (TWD), which matches the macronutrient and micronutrient profile of American Diets (Hintze, Benninghoff, & Ward, 2012). Compared to diets used in previous studies, the TWD has a higher content of sugar and fat, which likely favors the formation of AGEs with heat treatment. Moreover, TWD contains low levels of some micronutrients, such as thiamin, choline, vitamin D, and vitamin K, which may exacerbate the pathological effects of ingested AGEs.

Effect of Heat Processing Foods on Protein Digestibility

Although the Maillard reaction is important in food chemistry, such as giving foods color and flavor, it may also form toxic by-products and reduce the nutritive value of protein by degrading essential amino acids. And the loss of protein value includes a reduced protein digestibility and biological value (Oste, Dahlqvist, Sjoestroem, Noren, & Miller, 1986).

Seiquer (Seiquer et al., 2006) conducted a crossover trial to investigate the effect of diets rich in MRP on protein digestibility in adolescent males. They found that higher intakes of MRP negatively affects protein digestibility. Compared with control group, a

higher fecal nitrogen excretion and lower absorption of nitrogen were detected in high MRP diet group.

Fasting Glucose and Insulin Sensitivity

Type 2 diabetes (T2D) currently is the most prevalent type of diabetes and it is considered to be influenced by the intake of dietary AGE (Ames, 2007). The association between dietary AGE and diabetes has caught the attention of scientists and several studies have been conducted to investigate the role of dietary AGEs in T2DM etiology. Recent studies have demonstrated that dietary patterns have a correlationare to insulin resistance and metabolic syndrome (Kellow, Coughlan, Savige, & Reid, 2014). The Western dietary pattern which is considered to contain a significantly higher frequency of processed food is found to be associated with a higher risk of insulin resistance and metabolic syndrome (Esmaillzadeh et al., 2007). Birlouez-Aragon fed 62 human volunteers with either a high or a low CML diet for 1 month in a randomized, crossover experimental design. Diet with high-heat treatment significantly reduced insulin sensitivity, plasma concentration of vitamins C and E, and omega-3 fatty acids (Birlouez-Aragon et al., 2010).

Typically rodent studies with CML have been conducted with either a chow or a high fat diet background. However, these diets are not accurately representative of the US dietary pattern, and may miss possible interactions of the dietary pattern with CML and

T2D development. In the Ward Lab, an investigation into the metabolic effects of CML on the TWD background diet was conducted. Mice were randomly assigned to one of five experimental diets and feeding for 8 weeks (n = 10 mice/diet group). These diets included the AIN93 diet (negative control), a 45% kcal from fat Diet Induced Obesity diet (DIO; positive control); CML0, TWD with low CML; CML1, TWD with medium CML, and CML2, TWD with high CML. Figure 2 showed the content of CML in CML0, CML1 and CML2 compared to a human study (Birlouez-Aragon et al., 2010) and an animal study (Somoza et al., 2006). At week 6 and 8 of feeding process, oral glucose tolerance test (OGTT) and insulin tolerance test (ITT) were performed. After 8 weeks of feeding, mice were euthanized via carbon dioxide. Plasma, liver, gastrocnemius muscle, epididymal adipose tissue, cecal contents, colon, ileum, duodenum, and jejenum were collected for further studies.

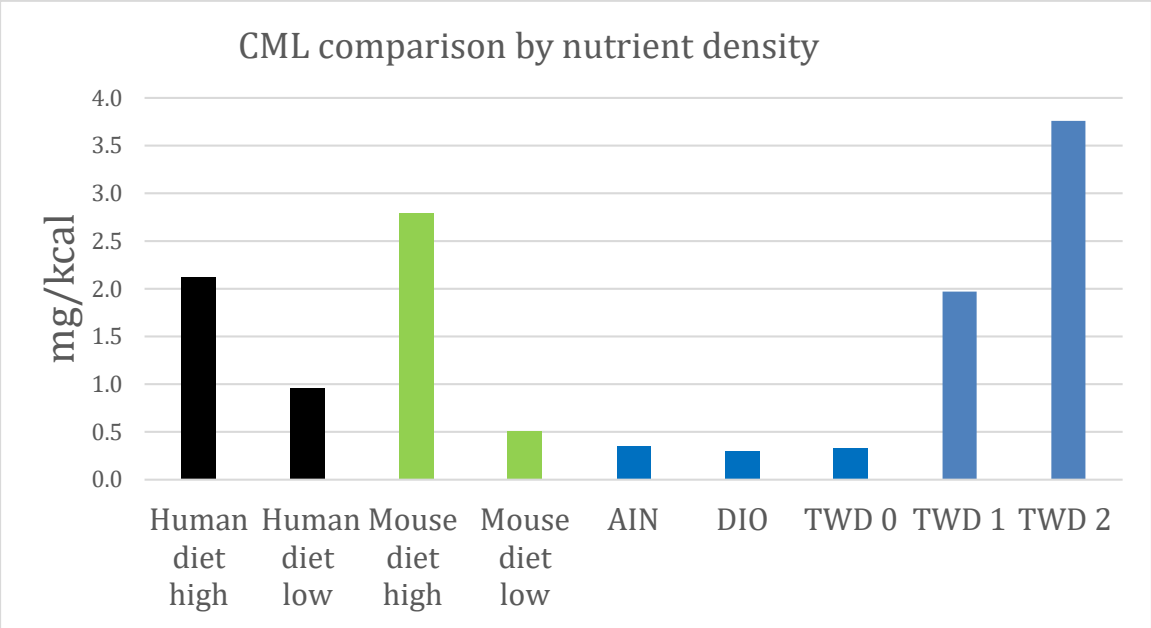


Figure 2. CML content in the AIN, DIO, CML0 (TWD low), CML1 (TWD med), and CML2 (TWD high) diets compared to previous human and rat studies. The comparison was based on the amount of total CML in the diets divided by the calorie content of the diets.

An OGTT was administered at weeks 6 of feeding. Figure 3A shows the fasting glucose levels in mice prior to the OGTT. Mice fed the CML2 diet had the highest fasting glucose compared to other four diets, and there were significant differences between the CML2 diet and CML1 and AIN93 diets ($p < 0.05$). In Figure 3B the area under the curve (AUC) for the OGTT is shown. This analysis indicates the CML2 diet led to significantly better glucose metabolism compared to the DIO diet. In a second analysis, the statistical comparisons were limited to the three CML diets as they are identical with the exception that the CML1 and CML2 diets had increased the level of CML compared to the CML0

diet. In Figure 3C it is clear that high CML diet was associated with a higher fasting glucose. In Figure 3D the incremental AUC is shown for the CML diets. Incremental AUC normalizes the OGTT to the fasting glucose level. Interestingly, this analysis indicates that dietary CML appears to simultaneously increase fasting glucose and increase glucose sensitivity.

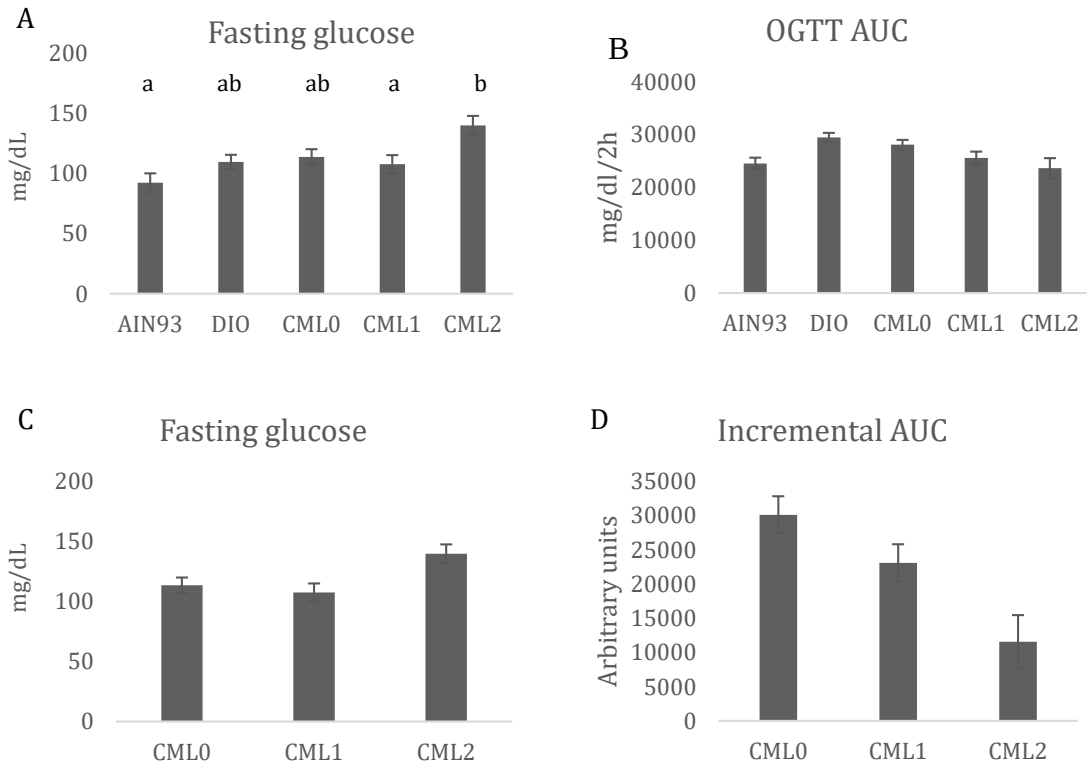


Figure 3. Fasting glucose (A), area under the curve (AUC) for the oral glucose tolerance test (OGTT; B) for all five diet groups. Fasting glucose (C) and incremental AUC (D) for the mice fed the TWD with increasing levels of CML.

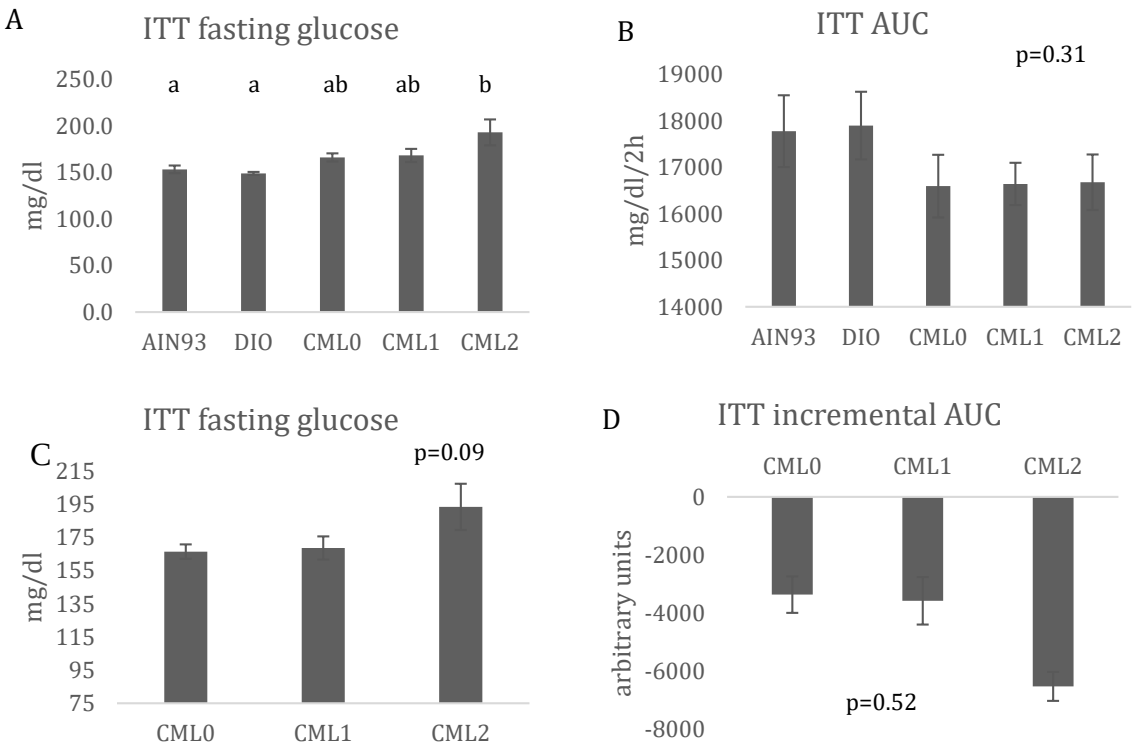


Figure 4. Fasting glucose (A) and area under the curve (AUC) for the insulin tolerance test (ITT; B) for all five diet groups. Fasting glucose (C) and incremental AUC (D) for mice fed the TWD diets with different CML levels.

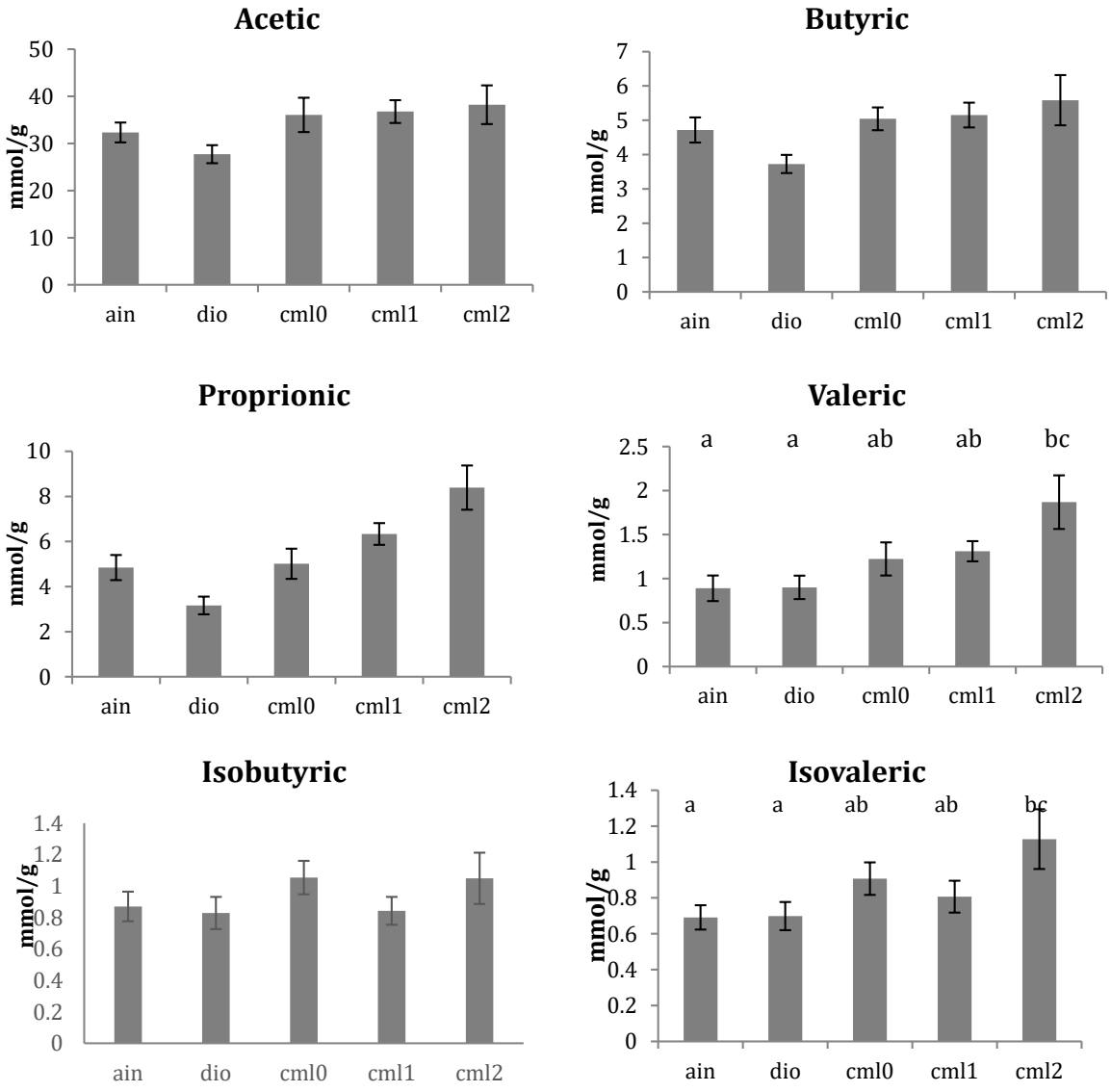
An ITT was performed in week 7 and the results are shown in Figure 4. In the ITT, mice are fed injected with insulin and plasma glucose is monitored for 120 min. The test is designed to induce hypoglycemia, and the goal of homeostasis is the return the glucose to pretest levels. As can be seen in Figure 4A, mice fed the CML2 diet had higher fasting glucose than mice fed the AIN93 and DIO diets ($p < 0.05$). The AUC for the ITT was not different (4B), but there did appear to be a trend with mice in the CML0, CML1, and

CML2 diets compared to the AIN93 and DIO diets. When restricted to the TWD diets (CML0, CML1, and CML2; Figure 4C), there was a trend for mice fed the CML2 diet to have a higher fasting glucose ($p = 0.09$). In addition, mice fed the differences in incremental AUC for the ITT test approached statistical significance for mice fed the TWD diets (Figure 4D). This data indicates that mice fed high levels of CML were less able to restore glucose to pretest levels after insulin injection.

Short Chain Fatty Acids

Short chain fatty acids (SCFAs) are organic fatty acids with less than six carbon atoms, such as acetic acid, propionic acid, butyric acid, and valeric acid (Wong, de Souza, Kendall, Emam, & Jenkins, 2006). The production of SCFAs is typically associated with many factors. The composition of the microflora in colon, source of substrate, and gut transit time are three major factors. SCFAs are produced via bacterial fermentation of carbohydrates in colon, especially dietary fiber, to decrease intestinal pH and inhibit the growth of pathogenic microorganisms to protect the colon (Kellow et al., 2014). Different SCFA have different functions in the colon. Acetate is considered to be the primary substrate for cholesterol synthesis and can increase the colonic blood flow. Butyrate one of the most important SCFAs, provides energy for colonic epithelial cells, reduces inflammation and maintains colon health (Scheppach, 1994). One study investigated the

effect of dietary glycated protein the colonic microbiota and production of SCFA. The result showed that the SCFA levels were significantly decreased by heated and glycated bovine serum albumin (BSA) compared to the control (Mills et al., 2008).



.

Figure 5. Cecal short chain fatty acid content (SCFA) from mice fed AIN, DIO and TWD diets with differing amounts of CML. SCFA are arranged in highest to lowest concentration. SCFA with different letter designation are significantly different.

SCFA were measured in the cecal contents of the mice fed the different diets described below and the results are shown in Figure 5. There were no differences in acetic, butyric or isobutyric acids among the mice, but there were differences in proprionic, valeric and isovaleric acids. More specifically, there was more proprionic acid in the cecal contents of mice fed the CML2 diet than the AIN, DIO and CML0 diets (Figure 5). In addition, there was also more valeric acid and isovaleric acid in the ceceal contents of mice fed the CML2 diet compared to the AIN and DIO diets (Figure 5).

Metabolomics Analysis

Metabolomics is the study of the small molecules in biological samples and has become an important tool to identify and quantify all metabolites in many disciplines (Lawton et al., 2008). Recently, it has been performed in order to identify biomarkers for dietary assessment and diseases (Deng, Jones, & Swanson, 2014). For instance, the glucose level is usually used to diagnose diabetes and the cholesterol content is associated with cardiovascular disease. To investigate whether the metabolites profiles could help the prediction of diabetes, Wang and colleges (Wang et al., 2011) conducted a study based on 2422 normoglycemic individuals followed for twelve years. The result showed 201 people who developed diabetes in this study had a significantly greater plasma concentration of five branched-chain amino acids which were tryrosine, isoleucine, leucine, valine and

phenylalanine. The effect of diets can also be revealed when studying the metabolites (Bruce et al., 2009). To perform metabolomics studies analytical techniques are often utilized, such as gas chromatography and mass spectrometry (GC-MS), or liquid chromatography (LC) or nuclear magnetic resonance (NMR).

Gas Chromatography and Mass spectrometry

Gas Chromatography and Mass Spectrometry is a commonly used method for studies of metabolic profiling. Chemicals are separated in GC based on their gas volatility and affinity for the stationary phase of the column utilized in the GC system, followed by identifying them in MS depending on breaking molecules into ionized fragments and detecting the fragments with mass to charge ratio.

Automated Mass Spectral Deconvolution and Identification System

Automated Mass Spectral Deconvolution and Identification System (AMDIS) is a free computer program that was designed for extracting spectra of individual component that is detected in a GC-MS data file. It deconvolutes the GC-MS data file for detecting all separate components followed by comparing them in a target library such as National Institute of Standards and Technology (NIST) Mass Spectral Database, or the library that the user built from GC-MS data files.

SpectConnect

SpectConnet is a freely available analytical service at http://spectconnect.mit.edu. In general, analysis of profiling metabolites from GC-MS data files depends on reference libraries, however, SpectConnect can systematically find components that are conserved across samples without using manual curation or reference library. SpectConnet compares each spectrum in each sample to each spectra in every other sample to ensure which components are conserved across the replicate samples. Components are conserved and identified by comparing the retention time and spectra. If the chromatogramic peak is a real peak and not noise, the mass spectrum in difference samples will be pairwise similar to each other (Styczynski et al., 2007). The process of how SpectConnect works is showed in Figure 6.

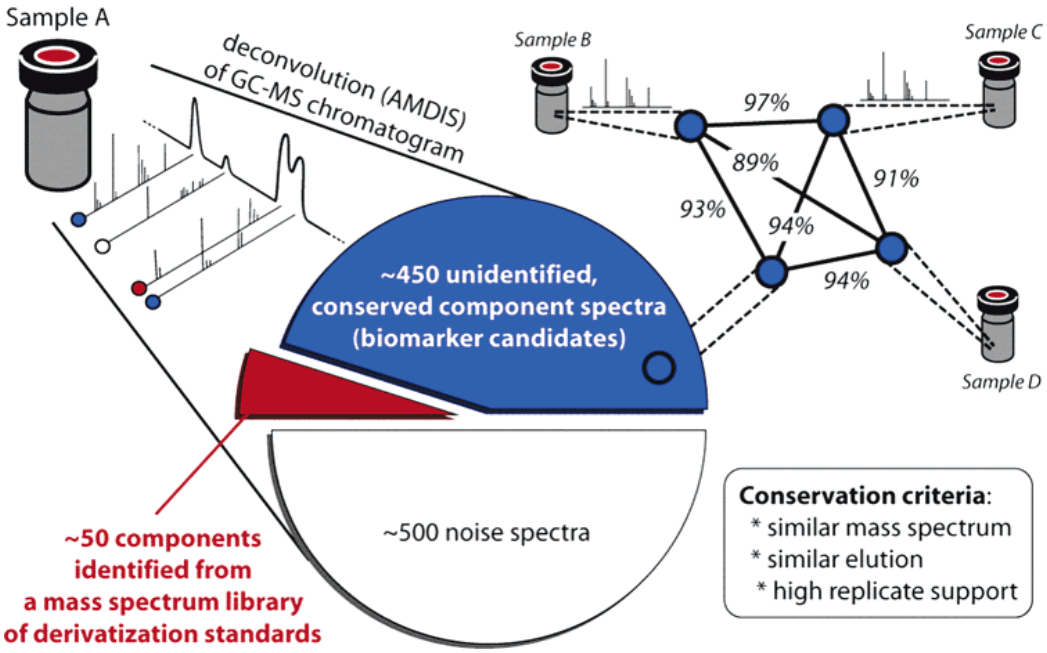


Figure 6. SpectConnect working process (Styczynski et al., 2007).

Hypotheses and Objectives

The Hypotheses of This Study:

1. Increased dietary CML will affect the plasma metabolite profile.
2. Increased dietary CML will result in an increase level of short chain fatty acids in plasma.
3. Increased dietary CML will result in an increase glucose level in plasma.
4. Increased dietary CML will result in an increase in the branched chain fatty acids in plasma.

The Objectives of This Study:

1. Determine the effect of dietary CML on the plasma metabolites profile.
2. Determine and compare the glucose levels in different experimental diet groups.

Materials and Methods

GC-MS Analysis of Mouse Plasma Metabolites

Plasma samples were collected from the mice experiment in the Ward Lab. Metabolites extraction and derivatization protocols were carried out as mentioned in Dunn et al (Dunn et al., 2011). Succinic acid (0.5 mg/mL, Sigma-Aldrich, Co) and sorbitol (0.5 mg/mL) were used as internal standards. Frozen plasma samples were thawed on ice for 30 to 60 min. Fifty micro liters of plasma were taken into 2 mL centrifuge tube followed by the addition of 10 µL of each internal standard. Then 300 µL of methanol was added and vortexed for 15 seconds to extract the metabolites. The mixture was centrifuged at room temperature and at 13,000 g for 15 min. Three hundreds microliters of the extraction mixture was transfer to a new 2 mL centrifuge tube. Then the mixture was completely dried in a centrifugal vacuum evaporator (SVC100H, Hicksville, NY) for 2 h. Twenty five micro liters of 20 mg/mL methoxyamine hydrochloride was added to the 2 mL centrifuge tube with dried sample and heated in the block heater (Fisher Scientific, Waltham, WA) at 80 °C for 15 min. The sample was removed from the block heater and 25 µL of N-Methyl-N-(trimethylsiyl) trifluoroacetamide (MSTFA) was added to each sample and vortexed for 15 s, then mixture was heated in the block heater for 15 min. Samples were cooled down after incubation and centrifuged at room temperature (13,000 g for 15 min).

One micro liter sample was injected into the GC-MS. Analysis was performed with a Shimadzu GC-2010 coupled with a Shimadzu GC-MS (QP2010S, Kyoto, Japan). A ZB-5 MSI column (35 m length, 0.25 mm diameter and 0.25 µm film thickness) was used. Helium was used as the carrier gas. Injection was carried out with a split ratio of 5:1. The injector and detector temperature were 250 °C. The oven temperature ramped from 70 °C (1 min) to 110 °C at 40 °C/min, followed by a 5 °C/min-ramp to 350 °C. Metabolites were identified using AMDIS software and SpectConnect, and NIST library. Peak area was normalized by internal standard and expressed with the normalized value.

Statistical Analysis

IBM SPSS (version 22.0, Armonk, NT) was used to conduct all statistical analysis. One-way analysis of variance (ANOVA) was used to evaluate the statistical differences of metabolites between all treatments. Tukey was used as post hoc to determine which of these treatments differ from each other based on the result of one-way ANOVA. P value < .05 was applied to determine the statistical difference. Principal component analysis (PCA) was performed using SPSS to provide a visual way to analyze the correlation between all experimental treatments and metabolites in plasma.

Results

Metabolomics Analysis of Mouse Plasma

GC-MS analysis of mouse plasma samples for AIN93, DIO, CML0, CML1, and CML2 diet groups detected 93 compounds, of which 49 were identified based on the retention index (RI) and NIST library (Table 4 & 5). Most of the identified metabolites fall under the categories of amino acids, fatty acids, organic acids, and organic molecules. Table 1 and Table 2 showed in 93 detected compounds, 44 of them could not be identified and were considered as unknown compounds since those compounds could not positively match the RI to the AMDIS library, NIST database (Table 4 & 5). The result of one-way ANOVA showed in Figure 6 that these five diets had a significant different on lactic acid, phosphoric acid, 2-Pyrrolidome-5-carboxylic acid, oleic acid and 10 unknown metabolites ($p < .05$).

In addition, one-way ANOVA was also performed for comparing three CML diets since they had identical formula except the difference of CML levels. The result revealed there was only one metabolite, lactic acid, had a significant difference ($p = .048$) and none of unknown metabolites showed significant difference (Table 3 and Table 6 & 7).

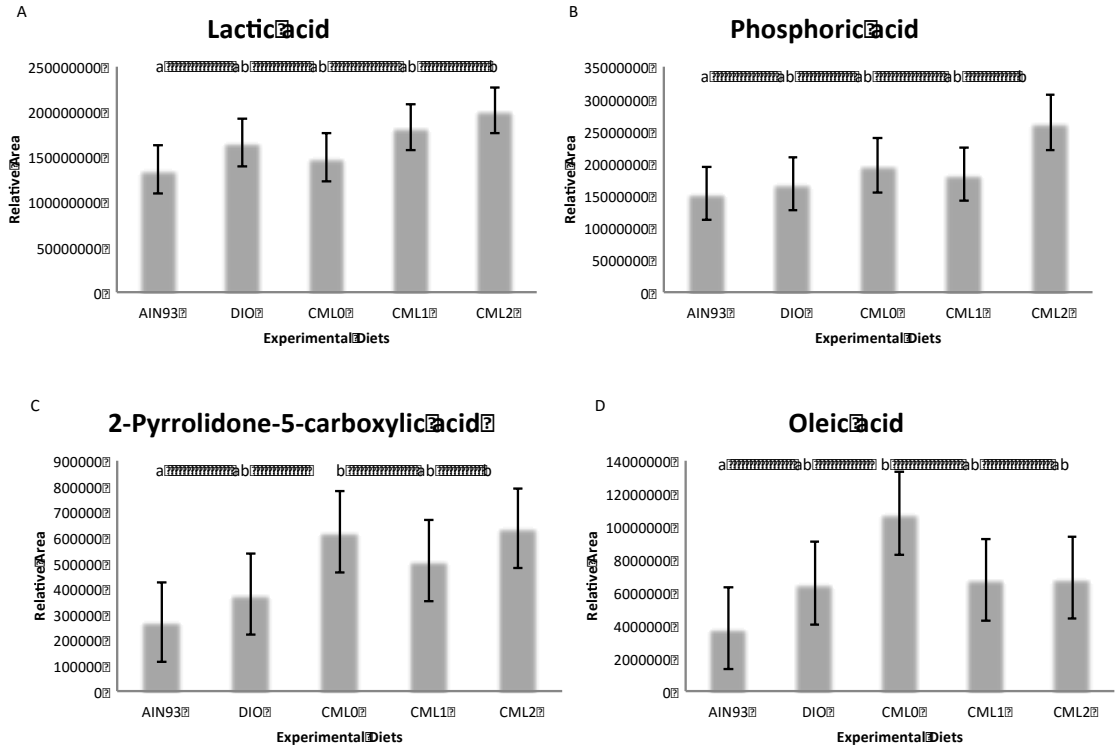


Figure 7. Content of lactic acid (A), phosphoric acid (B), 2-pyrrolidone-5-carboxylic acid (C), oleic acid (D). Values were normalized by the area of internal standard. Different lettered columns indicate a significant difference between diets ($p < .05$), which were determined by one-way ANOVA and Tukey post hoc analysis.

Table 1

Identified metabolites significantly different in AIN93, DIO, CML0, CML1, CML2

Compound	RI[1]	AIN93[2]	DIO[2]	CML0[2]	CML1[2]	CML2[2]	SEM	*p* value
Lactic acid	1078	135628037[a]	165776799[ab]	149168626[ab]	182880378[ab]	201989684[b]	26363228	0.045
Phosphoric acid	1263	15345957[a]	16791580[ab]	19690299[ab]	18321233[ab]	26281871[b]	4236228	0.027
2-Pyrrolidone-5-carboxylic acid	1478	270874[a]	377831[ab]	620736[b]	508328[ab]	636120[b]	157413	0.005
Oleic acid	2245	3777390[a]	6531323[ab]	10774657[b]	6777144[ab]	6840615[ab]	2495501	0.049

[a,b]Different lettered superscripts within a row indicate significant difference between mean values ($p < .05$) determined by one-way ANOVA and Tukey HSD post hoc analysis

[1]Rentention index

[2]Values are expressed after normalized by internal standard

Table 2

Unknown metabolites significantly different in AIN93, DIO, CML0, CML1, CML2

Compound	RI[1]	AIN93[2]	DIO[2]	CML0[2]	CML1[2]	CML2[2]	SEM	*p* value
unknown 1	1056	568720[a]	839625[a]	1504718[b]	971021[ab]	1339147[b]	378271	0.004
unknown 3	1310	780858[a]	947557[ab]	1677585[b]	1135334[ab]	1399501[ab]	357343	0.022
unknown 11	2281	1798909[a]	2697041[ab]	3901549[b]	2799166[ab]	3033786[ab]	753363	0.041
unknown 22	3419	17022486[a]	20792622[ab]	25782969[ab]	21199359[b]	25869116[b]	3743877	0.028
unknown 24	3478	646177[a]	1115902[ab]	1270658[b]	1010912[ab]	1045417[ab]	230475	0.032
unknown 33	1063	103188[ab]	106484[a]	152891[ab]	128040[ab]	212932[b]	45011	0.01
unknown 34	1171	156406[a]	337288[ab]	409558[b]	301084[ab]	336571[ab]	93563	0.032
unknown 35	1678	38930[a]	74121[ab]	119274[ab]	77441[b]	104856[ab]	31005	0.024
unknown 37	1807	277381[a]	468470[ab]	479697[ab]	408516[ab]	651873[b]	135310	0.007
unknown 43	2090	5501303[a]	10673806[b]	14450702[b]	14061218[b]	14136041[b]	3825312	0.004

[a,b]Different lettered superscripts within a row indicate significant difference between mean values ($p < .05$) determined by one-way ANOVA and Tukey HSD post hoc analysis

[1]Rentention index

[2]Values are expressed after normalized by internal standard

Table 3

Identified metabolites significantly different in CML0, CML1, CML2

Compound	RI[1]	CML0[2]	CML1[2]	CML2[2]	SEM[2]	*p* value
Lactic acid	1078	149168626[a]	182880378[ab]	201989684[b]	26744818	0.048

[a,b]Different lettered superscripts within a row indicate significant difference between mean values ($p < .05$) determined by one-way ANOVA and Tukey HSD post hoc analysis

[1]Rentention index

[2]Values are expressed after normalized by internal standard

Table 4

Identified metabolites in AIN93, DIO, CML0, CML1, CML2

	Compound	RI[1]	AIN93[2]	DIO[2]	CML0[2]	CML1[2]	CML2[2]	SEM	*p* value
1	Pyridine	1061	75081	108511	82728	97857	134500	23378	0.147
2	1,3-propanediol	1072	60886	75319	64531	61821	89699	12198	0.454
3	Lactic acid	1078	135628037	165776799	149168626	182880378	201989684	26363228	0.045
4	Acetic acid	1086	1079543	1425555	1560742	1619485	1605412	225081	0.065
5	L-Valine	1095	998615	1254435	923434	766624	704363	216571	0.381
6	L-Alanine	1036, 1107	28846218	33922416	26787580	15441358	19429919	7414955	0.271
7	Hydroxylamine	1119	2101774	2268580	1945883	1951787	1977862	137971	0.537
8	2-Hydroxybutyric acid	1137	517087	597635	546716	482016	585416	47827	0.972
9	Benzyl alcohol	1149	3169523	2759408	2127358	1938404	1604938	634194	0.151
10	Butanoic acid (Butyric acid)	1159	333172	411699	514854	430643	469293	67890	0.323
11	L-Norvaline	1207	860803	1322729	881182	687120	826139	239868	0.362
12	Urea	1233	29529788	44702773	42639104	50706492	40771367	7745762	0.229
13	Benzene	1239	1345285	1565211	1671042	1629903	1785178	163033	0.343
14	Glycerol	1264	11196246	14033708	12842176	11628196	15527002	1775122	0.76
15	Phosphoric acid	1263	15345957	16791580	19690299	18321233	26281871	4236228	0.027
16	L-Threonine	1280	1338219	1723209	1517584	1293857	1356148	176639	0.886
17	Ethanedioic acid	1139, 1292	26944376	35873286	38202991	35659090	33973099	4290227	0.391
18	Glycine	1293	1980721	2338118	1817069	2206776	2054044	201468	0.632
19	Butanedioic acid	1295	6067297	6788462	6534110	7101359	7295008	483320	0.907
20	2-Butenedioic acid	1327	148920	202188	221365	217834	258110	39674	0.395
21	2-Pyrrolidone-5-carboxylic acid	1478	270874	377831	620736	508328	636120	157413	0.005

	Compound	RI[1]	AIN93[2]	DIO[2]	CML0[2]	CML1[2]	CML2[2]	SEM	*p* value
22	L-Proline	1512	10755449	13572171	14886154	11880213	13709603	1633455	0.558
23	Dodecanoic acid	1644	589003	762124	980585	934678	810588	154621	0.144
24	d-Ribose	1703	551658	663851	650662	644006	739423	66887	0.826
25	1,2,3-Propanetricarboxylic acid	1852	3782003	5873103	5438073	4885899	5418213	805308	0.344
26	Tetradecanoic acid	1859	117264853	124768161	152580406	157477323	142300988	17404189	0.156
27	d-Glucose	1955	118412673	134393569	139710092	138183699	151012870	11781632	0.581
28	Hexadecanoic acid	2066	27717141	32744784	39436601	37407778	34652368	4524316	0.222
29	Inositol	2153	3204026	4454098	5288380	3812881	3900700	783810	0.111
30	Heptadecanoic acid	2168	171528	233457	243831	301327	243651	46131	0.086
31	Linoleic acid	2240	8106505	8025929	11057862	10513564	10571087	1465953	0.13
32	Oleic acid	2245	3777390	6531323	10774657	6777144	6840615	2495501	0.049
33	L-Tryptophan	2267	1834525	1927037	1794223	1435904	1481962	221172	0.504
34	Arachidonic acid	2400	3448381	3855805	4054859	3422118	3869055	280533	0.784
35	Eicosanoic acid	2469	1175130	1470884	1531402	1629787	1433910	169692	0.729
36	Bis(2-ethylhexyl) phthalate	2571	154450	182284	281104	259248	240092	53240	0.296
37	cis-4,7,10,13,16,19-Docosahexaenoic acid	2584	1434789	1937650	1929310	1706258	1754981	205321	0.674
38	Octadecanoic acid	2273, 2793	43155612	51708816	57269124	57580958	53398684	5857403	0.226
39	Oleamide	2818	57252300	70648192	64212484	66661342	72482922	5989003	0.525
40	Cholesterol	3098	22483708	33509101	40503600	38309431	33126970	6957756	0.069
41	Nonanoic acid	1338	1091032	1102458	1326360	1520003	1476630	201823	0.443
42	d-Mannose	1925	1727871	1643664	1468884	1291507	1331105	190561	0.478
43	Dibutyl phthalate	1976	327902	285070	401857	567359	419108	108148	0.011
44	1-Octanol	1301	240384	238277	281102	266212	246431	18522	0.569

	Compound	RI[1]	AIN93[2]	DIO[2]	CML0[2]	CML1[2]	CML2[2]	SEM	*p* value
45	L-Ornithine	1843	632776	799231	837654	733984	850066	89396	0.8
46	cis-13-Docosenoic acid	2634	2840640	2805401	3357336	3275883	2782418	280020	0.325
47	Aspartic acid	1467	51079	209589	303830	365967	318055	124679	0.101
48	13-Docosenamide	2778	6896654	9864222	16542256	13182188	10268165.	3658094	0.273
49	1,2-Propanediol	1039	177365	208022	14232042	277915	206967	6267572	0.323

[1]Rentention index;

[2]Values are expressed after normalized by internal standard

Table 5

Unknown metabolites in AIN93, DIO, CML0, CML1, CML2

Compound	RI[1]	AIN93[2]	DIO[2]	CML0[2]	CML1[2]	CML2[2]	SEM	*p* value
unknown 1	1056	568720	839625	1504718	971021	1339147	378271	0.004
unknown 2	1200	296514	330466	291512	306164	324221	17023	0.937
unknown 3	1310	780858	947557	1677585	1135334	1399501	357343	0.022
unknown 4	1722	684912	693543	845740	741398	939654	109330	0.384
unknown 5	1773	195512	262155	279936	245867	266917	32848	0.12
unknown 6	1878	340367	358255	437566	378729	506299	67771	0.151
unknown 7	1964	12339216	15554563	16112480	14668753	14225315	1453707	0.674
unknown 8	1981	1605002	1709160	1532908	1615690	1195051	198275	0.365
unknown 9	2022	432310	457285	522668	470754	507665	36892	0.445
unknown 10	2252	567498	359367	659276	509321	470088	111607	0.07
unknown 11	2281	1798909	2697041	3901549	2799166	3033786	753363	0.041
unknown 12	2312	550449	759368	914044	741287	671807	132715	0.233
unknown 13	2412	3005753	3188604	3694588	3296315	4023337	411551	0.092
unknown 14	2438	845239	1288599	1723518	1285785	1695472	360545	0.326
unknown 15	2579	5774296	5988665	6368482	6043814	7226318	570031	0.844
unknown 16	2658	501779	596058	581493	559777	500952	44497	0.846
unknown 17	2687	143985	140912	149399	144765	132999	6076	0.544
unknown 18	2771	1467504	1874529	2467295	1936443	1756425	364508	0.586
unknown 19	2827	7888531	8838030	9928913	8885158	9929959	859970	0.384
unknown 20	2834	4354446	5038854	5672474	5021924	4960909	466906	0.549
unknown 21	3115	250423	223921	297505	257283	217876	31702	0.369

Compound	RI[1]	AIN93[2]	DIO[2]	CML0[2]	CML1[2]	CML2[2]	SEM	*p* value
unknown 22	3419	17022486	20792622	25782969	21199359	25869116	3743877	0.028
unknown 23	3448	396854	445561	516298	452904	671427	106555	0.086
unknown 24	3478	646177	1115902	1270658	1010912	1045417	230475	0.032
unknown 25	1280	470689	764763	537770	591074	708801	121026	0.579
unknown 26	1534	1875509	2140969	2507987	2174822	2433088	252534	0.355
unknown 27	2944	111545	144841	152493	136293	121499	16756	0.222
unknown 28	2997	606512	707469	712958	675646	641503	45047	0.635
unknown 29	1790	1297802	1342936	1892709	1511149	1869294	283731	0.152
unknown 30	3479	44672	61990	90084	65582	59815	16409	0.332
unknown 31	1210	722724	798856	944879	822153	978569	106140	0.61
unknown 32	1063	103188	106484	152891	128040	212932	45011	0.01
unknown 33	1171	156406	337288	409558	301084	336571	93563	0.032
unknown 34	1678	38930	74121	119274	77441	104856	31005	0.024
unknown 35	1745	53695	55637	65429	58254	63831	5099	0.68
unknown 36	1807	277381	468470	479697	408516	651873	135310	0.007
unknown 37	2143	110695	144937	109087	127816	52149	34929	0.097
unknown 38	2422	989903	963955	1480532	1144797	1537151	270447	0.091
unknown 39	2591	593224	891187	528102	670838	457296	166906	0.055
unknown 40	2749	14974155	13610026	10476547	13020242	16198189	2163069	0.604
unknown 41	2931	430800	359007	345464	378424	400301	33873	0.754
unknown 42	2005	4266439	3370354	2566451	3401081	3861321	635580	0.056
unknown 43	2090	5501303	10673806	14450702	14061218	14136041	3825312	0.004
unknown 44	1293	19033150	23141440	26870112	24269326	25355793	2966526	0.403

[1]Rentention index; [2]Values are expressed after normalized by internal standard

Table 6

Identified metabolites in CML0, CML1, CML2

	Compound	RI[1]	CML0[2]	CML1[2]	CML2[2]	SEM	*p* value
1	Pyridine	1061	82728	97857	134500	26620	0.065
2	1,3-propanediol	1072	64531	61821	89699	15372	0.153
3	Lactic acid	1078	149168626	182880378	201989684	26744818	0.048
4	Acetic acid	1086	1560742	1619485	1605412	30670	0.956
5	L-Valine	1095	923434	766624	704363	112885	0.62
6	L-Alanine	1036, 1107	26787580	15441358	19429919	5755874	0.656
7	Hydroxylamine	1119	1945883	1951787	1977862	17016	0.987
8	2-Hydroxybutyric acid	1137	546716	482016	585416	52242	0.739
9	Benzyl alcohol	1149	2127358	1938404	1604938	264520	0.676
10	Butanoic acid	1159	514854	430643	469293	42152	0.677
11	L-Norvaline	1207	881182	687120	826139	100013	0.579
12	Urea	1233	42639104	50706492	40771367	5280118	0.615
13	Benzene	1239	1671042	1629903	1785178	80446	0.808
14	Glycerol	1264	12842176	11628196	15527002	1995108	0.636
15	Phosphoric acid	1263	19690299	18321233	26281871	4256268	0.04
16	L-Threonine	1280	1517584	1293857	1356148	115467	0.844
17	Ethanedioic acid	1139, 1292	38202991	35659090	33973099	2129396	0.796
18	Glycine	1293	1817069	2206776	2054044	196365	0.346
19	Butanedioic acid	1295	6534110	7101359	7295008	395439	0.847
20	2-Butenedioic acid	1327.	221365	217834	258110	22304	0.728
21	2-Pyrrolidone-5-carboxylic acid	1478	620736	508328	636120	69765	0.478
22	L-Proline	1512	14886154	11880213	13709603	1514739	0.462

	Compound	RI[1]	CML0[2]	CML1[2]	CML2[2]	SEM	p-value
23	Dodecanoic acid	1644	980585	934678	810588	87944	0.689
24	d-Ribose	1703	650662	644006	739423	53271	0.756
25	1,2,3-Propanetricarboxylic acid	1852	5438073	4885899	5418213	313221	0.846
26	Tetradecanoic acid	1859	152580406	157477323	142300988	7745615	0.779
27	d-Glucose	1955	139710092	138183699	151012870	7007974	0.809
28	Hexadecanoic acid	2066	39436601	37407778	34652368	2401294	0.762
29	Inositol	2153	5288380	3812881	3900700	827694	0.182
30	Heptadecanoic acid	2168	243831	301327	243651	33247	0.336
31	Linoleic acid	2240	11057862	10513564	10571087	299031	0.94
32	Oleic acid	2245	10774657	6777144	6840615	2289862	0.245
33	L-Tryptophan	2267	1794223	1435904	1481962	194945	0.429
34	Arachidonic acid	2400	4054859	3422118	3869055	325227	0.509
35	Eicosanoic acid	2469	1531402	1629787	1433910	97938	0.871
36	Bis(2-ethylhexyl) phthalate	2571	281104	259248	240092	20520	0.873
37	cis-4,7,10,13,16,19-Docosahexaenoic acid	2584	1929310	1706258	1754981	117272	0.794
38	Octadecanoic acid	2273, 2793	57269124	57580958	53398684	2329841	0.847
39	Oleamide	2818	64212484	66661342	72482922	4248290	0.712
40	Cholesterol	3098	40503600	38309431	33126970	3787852	0.605
41	Nonanoic acid	1338	1326360	1520003	1476630	101620	0.812
42	d-Mannose	1925	1468884	1291507	1331105	93106	0.8
43	Dibutyl phthalate	1976	401857	567359	419108	90982	0.08
44	1-Octanol	1301	281102	266212	246431	17393	0.682
45	L-Ornithine	1843	837654	733984	850066	63740	0.737
46	cis-13-Docosenoic acid	2634	3357336	3275883	2782418	311093	0.503

	Compound	RI[1]	CML0[2]	CML1[2]	CML2[2]	SEM	p-value
47	Aspartic acid	1467	303830	365967	318055	32554.	0.673
48	13-Docosenamide	2778	16542256	13182188	10268165	3139686	0.432
49	1,2-Propanediol	1039	14232042	277915	206967	8076977	0.308

[1]Rentention index

[2]Values are expressed after normalized by internal standard

Table 7

Unknown metabolites in CML0, CML1, CML2

Compound	RI[1]	CML0[2]	CML1[2]	CML2[2]	SEM	p-value
unknown 1	1056	1504718	971021	1339147	378271	0.367
unknown 2	1200	291512	306164	324221	17023	0.821
unknown 3	1310	1677585	1135334	1399501	357343	0.539
unknown 4	1722	845740	741398	939654	109330	0.904
unknown 5	1772	279936	245867	266917	32848	0.346
unknown 6	1878	437566	378729	506299	67771	0.695
unknown 7	1964	16112480	14668753	14225315	1453707	0.744
unknown 8	1981	1532908	1615690	1195051	198275	0.451
unknown 9	2022	522668	470754	507665	36892	0.54
unknown 10	2252	659276	509321	470088	111607	0.096
unknown 11	2281	3901549	2799166	3033786	753363	0.601
unknown 12	2312	914044	741287	671807	132715	0.374
unknown 13	2412	3694588	3296315	4023337	411551	0.34
unknown 14	2438	1723518	1285785	1695472	360545	0.993
unknown 15	2579	6368482	6043814	7226318	570031	0.778
unknown 16	2658	581493	559777	500952	44497	0.764
unknown 17	2687	149399	144765	132999	6076	0.382
unknown 18	2771	2467295	1936443	1756425	364508	0.492
unknown 19	2827	9928913	8885158	9929959	859970	0.77
unknown 20	2834	5672474	5021924	4960909	466906	0.685
unknown 21	3115	297505	257283	217876	31702	0.11

Compound	RI[1]	CML0[2]	CML1[2]	CML2[2]	SEM	p-value
unknown 22	3419	25782969	21199359	25869116	3743877	0.995
unknown 23	3448	516298	452904	671427	106555	0.424
unknown 24	3478	1270658	1010912	1045417	230475	0.157
unknown 25	1280	537770	591074	708801	121026	0.566
unknown 26	1534	2507987	2174822	2433088	252534	0.925
unknown 27	2944	152493	136293	121499	16756	0.272
unknown 28	2997	712958	675646	641503	45047	0.55
unknown 29	1790	1892709	1511149	1869294	283731	0.976
unknown 30	3479	90084	65582	59815	16409	0.498
unknown 31	1210	944879	822153	978569	106140	0.813
unknown 32	1063	152891	128040	212932	45011	0.474
unknown 33	1171	409558	301084	336571	93563	0.595
unknown 34	1678	119274	77441	104856	31005	0.653
unknown 35	1745	65429	58254	63831	5099	0.816
unknown 36	1807	479697	408516	651873	135310	0.163
unknown 37	2143	109087	127816	52149	34929	0.2
unknown 38	2422	1480532	1144797	1537151	270447	0.978
unknown 39	2591	528102	670838	457296	166906	0.771
unknown 40	2749	10476547	13020242	16198189	2163069	0.281
unknown 41	2931	345464	378424	400301	33873	0.547
unknown 42	2005	2566451	3401081	3861321	635580	0.145
unknown 43	2090	14450702	14061218	14136041	3825312	0.974
unknown 44	1293	26870112	24269326	25355793	2966526	0.842

[1]Rentention index; [2]Values are expressed after normalized by internal standard

Metabolites Principal Component Analysis

The principal component analysis (PCA) was performed using all positively identified metabolites. The eigenvalues of the correlation matrix (Table 8) and the scree plot (Figure 8) were used to determine that the first three principal components were able to sufficiently explain 76.18% of the variation in the data.

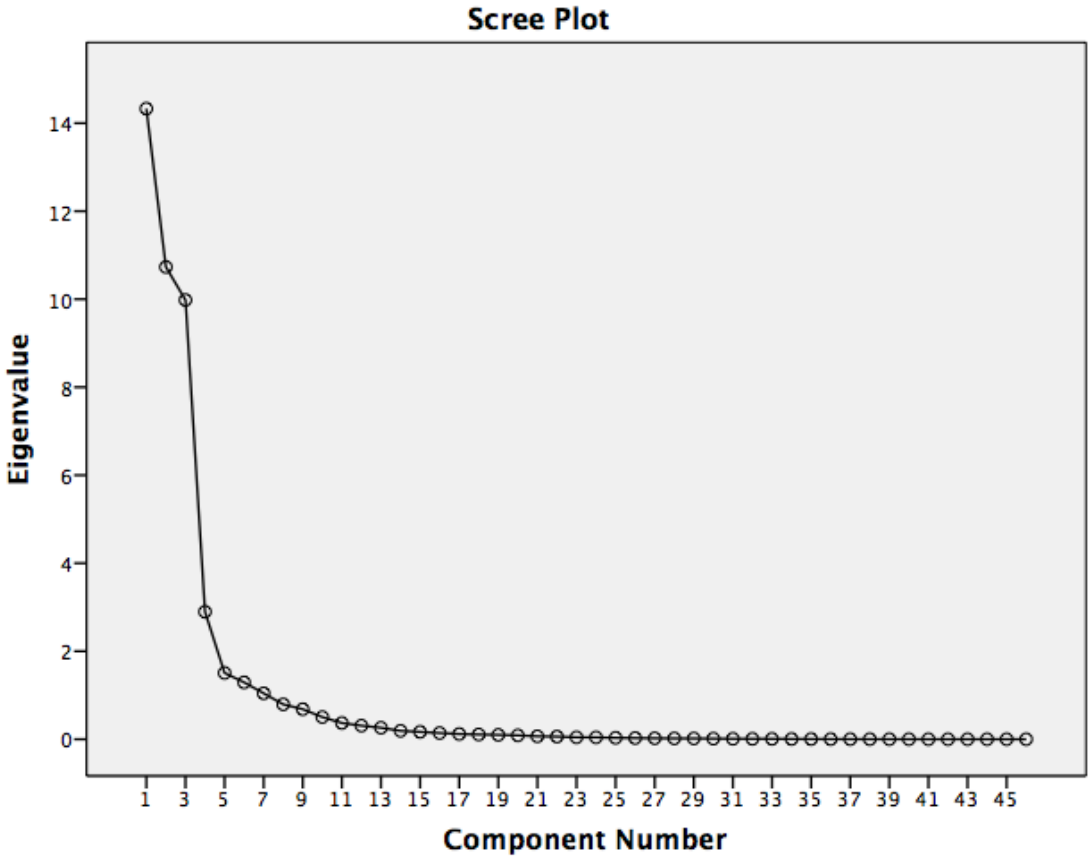


Figure 8. Scree plot for the metabolites principal component analysis.

Table 8

Eigenvalues of the correlation matrix for the metabolites principal component analysis

Component	Initial Eigenvalues		
	Total	% of Variance	Cumulative %
1	14.33	31.152	31.152
2	10.732	23.33	54.482
3	9.981	21.698	76.18
4	2.896	6.295	82.475
5	1.507	3.276	85.75
6	1.292	2.81	88.56
7	1.044	2.269	90.829
8	0.792	1.723	92.551
9	0.683	1.484	94.035
10	0.503	1.094	95.129
11	0.369	0.802	95.93
12	0.308	0.67	96.601
13	0.261	0.567	97.168
14	0.191	0.414	97.582
15	0.167	0.363	97.945
16	0.141	0.307	98.252
17	0.119	0.258	98.51
18	0.108	0.235	98.745
19	0.099	0.216	98.961
20	0.088	0.192	99.152
21	0.067	0.146	99.299
22	0.06	0.13	99.429
23	0.043	0.094	99.523
24	0.042	0.092	99.615
25	0.032	0.07	99.685
26	0.027	0.059	99.744
27	0.022	0.049	99.793
28	0.019	0.041	99.834
29	0.017	0.038	99.872
30	0.013	0.029	99.901
31	0.01	0.022	99.923
32	0.009	0.019	99.942

Component	Initial Eigenvalues		
	Total	% of Variance	Cumulative %
33	0.008	0.018	99.96
34	0.005	0.011	99.971
35	0.004	0.009	99.98
36	0.003	0.006	99.986
37	0.002	0.004	99.99
38	0.002	0.003	99.993
39	0.001	0.002	99.995
40	0.001	0.002	99.997
41	0	0.001	99.998
42	0	0.001	99.999
43	0	0.001	99.999
44	0	0	100
45	4.84E-05	0	100
46	9.04E-06	1.97E-05	100

Using 0.3 as a cut off for the eigenvectors (Table 9), the result indicated CML2 replicates were clustered on the positive side of component 2 and were clearly separated by component 2. CML0 and CML1 replicates were separated by both component 1 and component 3. Most of DIO and AIN93 replicates were located on the positive side of component 3, which means component 3 contributed most to separate DIO and CML1 from other diets (Figure 9).

Table 9

Eigenvectors of principal component 1, principal component 2, and principal component 3 for the metabolites principal component analysis

Component	Prin1	Prin2	Prin3
DIO_1	0.989	-0.022	-0.064
CML0_6	0.974	-0.006	-0.156
CML0_2	0.973	-0.037	-0.19
CML1_2	0.97	-0.028	-0.225
CML1_5	0.969	-0.002	0.08
AIN93_7	0.964	-0.038	0.025
AIN93_8	0.964	-0.043	-0.117
CML1_6	0.959	0.003	0.101
CML0_7	0.947	0.011	0.08
DIO_4	0.935	0.07	-0.06
AIN93_2	0.934	-0.029	-0.289
CML0_3	0.919	-0.066	0.087
CML1_7	0.898	-0.092	0.274
AIN93_4	0.718	-0.062	0.595
CML0_1	0.66	-0.111	0.574
DIO_9	0.456	0.016	0.035
CML1_10	-0.011	0.996	0.002
CML2_4	-0.017	0.992	0.009
CML2_5	-0.011	0.99	-0.007
CML1_8	-0.026	0.987	-0.006
CML2_6	-0.027	0.985	-0.011
CML2_2	-0.016	0.985	0.009
CML2_1	-0.034	0.982	0.005
CML2_7	-0.032	0.981	-0.029
CML1_9	-0.024	0.981	0.02
CML2_8	-0.024	0.977	0.002
CML2_3	-0.037	0.943	-0.023
DIO_8	0.113	-0.013	0.958
CML0_10	-0.043	-0.062	0.955
CML0_9	-0.193	-0.098	0.921
CML0_4	-0.022	-0.052	0.912
CML0_8	-0.181	-0.04	0.891

Component	Prin1	Prin2	Prin3
CML1_4	0.168	-0.064	0.795
DIO_6	-0.073	-0.042	0.789
DIO_3	-0.093	-0.058	0.786
AIN93_3	-0.242	-0.036	0.761
CML1_3	0.565	-0.07	0.668
AIN93_6	-0.02	-0.045	0.648
CML1_1	0.606	0.016	0.641
DIO_5	0.193	-0.011	0.575
AIN93_9	-0.095	0.131	0.514
DIO_7	0.262	0.043	0.477
AIN93_5	0.026	0.078	0.4
CML0_5	-0.014	-0.08	-0.374
DIO_2	0.025	-0.023	0.305
AIN93_1	0.176	-0.065	-0.233

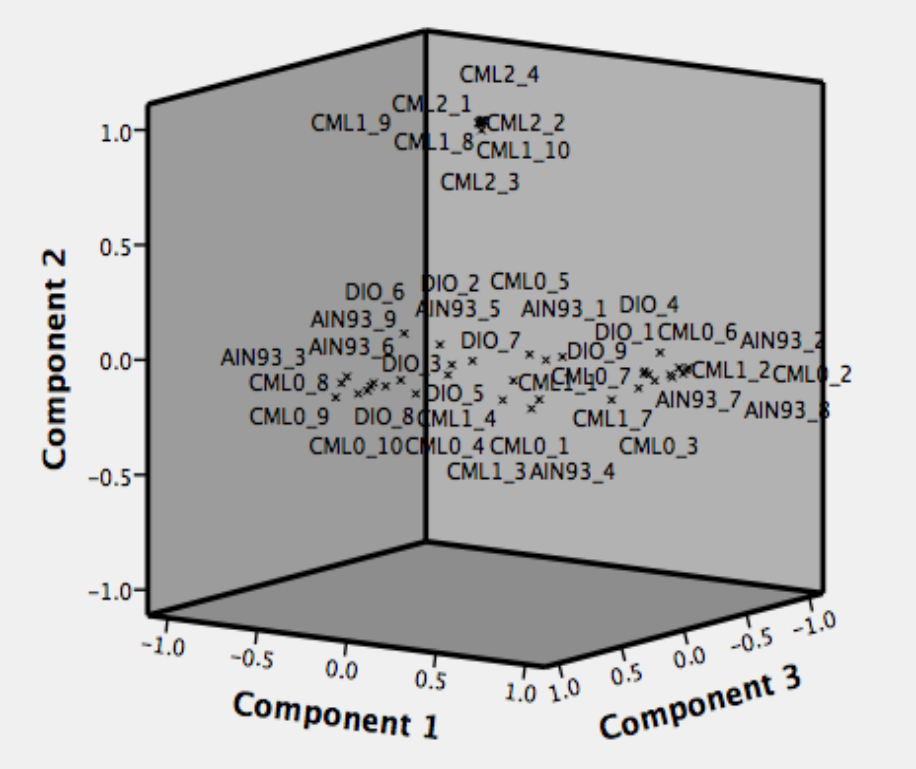


Figure 9. Three-dimension score plot for metabolites principal component analysis (PC1, PC2, and PC3).

Glucose content in plasma

Glucose content was quantified using internal standard and a standard curve based on the GC-MS results. According to result (Figure 10), CML diets had no effect on the glucose level in plasma (CML0: 107.91 mg/dL, CML1: 108.46 mg/dL, and CML2: 105.61 mg/dL) compared to DIO (105.31 mg/dL) and AIN93 (104.85 mg/dL). However, statistical analysis using one-way ANOVA was performed and the result showed no significant difference in all five diets as well as only in three CML diets (Figure 10 & 11).

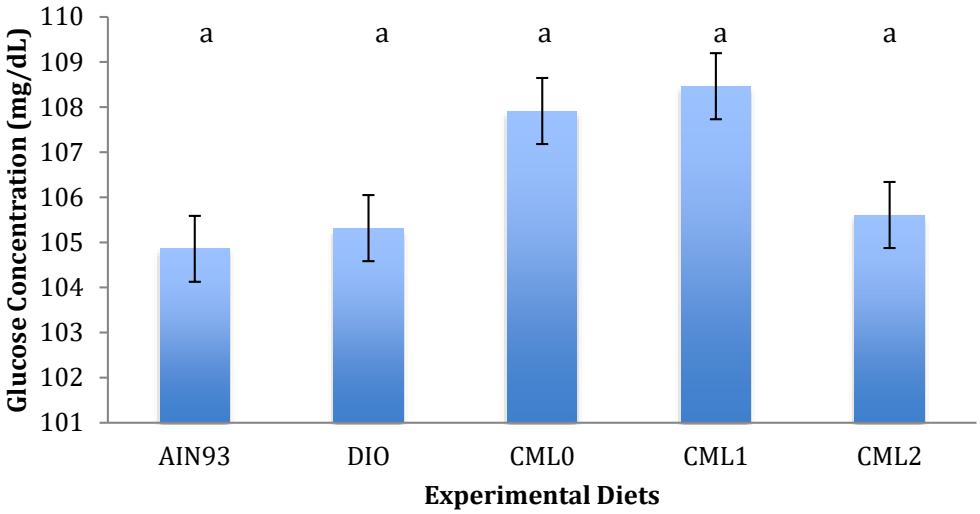


Figure 10. Mean plasma glucose content in AIN93, DIO, CML0, CML1, and CML2 ± standard error. Different lettered columns indicate a significant difference between diets ($p < .05$), which were determined by one-way ANOVA and Tukey post hoc analysis.

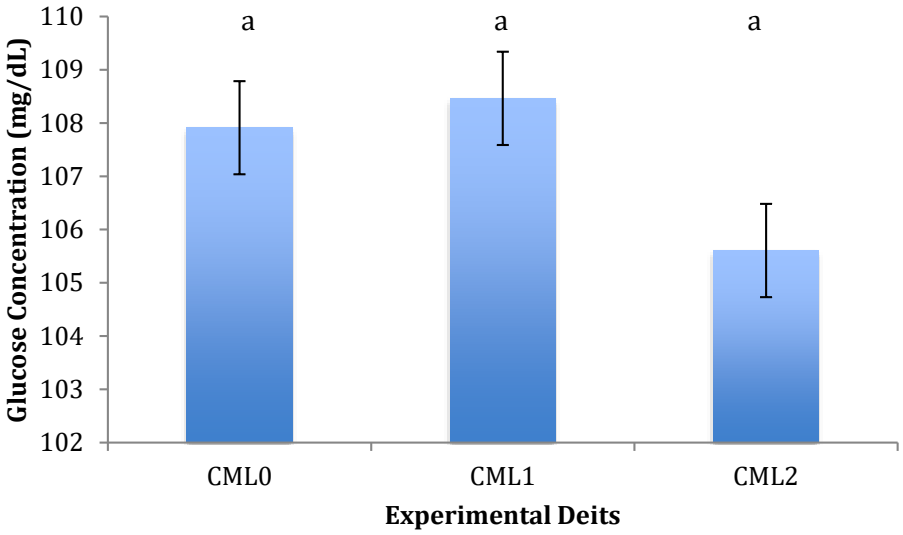


Figure 11. Mean plasma glucose content in CML0, CML1, and CML2 ± standard error. Different lettered columns indicate a significant difference between diets ($p < .05$), which were determined by one-way ANOVA and Tukey post hoc analysis.

Discussion

There were no significant differences in glucose content between five experimental diets ($p > .05$). The result is not in accordance with previous studies that consumption of diet high in CML increased the glucose concentration and would lead to the development of diabetes compared to diet low in CML or CML-restricted diet. Since some SCFAs were higher in the cecal contents of the mice fed the CML2 diet, we hypothesized a higher CML content in diet would increase the SCFA level. However, we did not see any significant difference in SCFAs detected by GC-MS, which were acetic acid and butanoic acid.

According to plasma metabolomics analysis, we found there was a significant difference in lactic acid ($p < .05$). The result of Tukey HSD post hoc indicated that there was a significant difference between AIN93 and CML2 diets. And the lactic acid content was increased with the increasing CML levels in diets. We can conclude that dietary CML did play a role in increasing the plasma lactic acid concentration. Studies have been conducted to investigate the correlation between plasma lactate concentration and T2D. The results of these studies demonstrated that plasma lactate was strongly associated with T2D and higher fasting glucose among nondiabetics (Crawford et al., 2010). From the OGTT data in our study, we saw that mice fed CML2 diet had the highest fasting glucose compared to the other four diets. Furthermore, the statistical comparison applied limited to the three CML diets indicated that high CML diet, CML2, was associated with a higher

fasting glucose. In another study, plasma lactate was higher in non-diabetics as well as in obese diabetics compared to lean subjects (Chen, Varasteh, & Reaven, 1992). In our study, the result of mice body weight was similar to Chen's study. Mice fed the CML diets and DIO diet were higher in weight compared to AIN93 control diet (Figure 12). Overall, plasma lactic acid content lead to higher body weight, the development of T2D and is associated with obesity and higher fasting glucose.

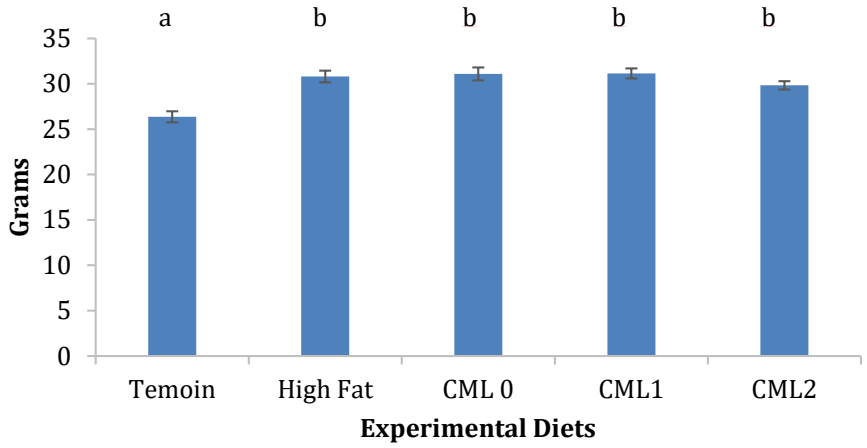


Figure 12. Mice final body weight after 8 weeks feeding for Temoin (AIN93), Hight fat (DIO), CML0, CML1, and CML2 diet groups. Different lettered columns indicate a significant difference between diets ($p < .05$), which were determined by one-way ANOVA and Tukey post hoc analysis.

2-Pyrrolidone-5-carboxylic acid was detected to have significant difference between diets ($p < .05$), and the Tukey HSD post hoc showed the CML2 diet had a significantly higher level of 2-Pyrrolidone-5-carboxylic acid compared to AIN93 control diet. Another study found that in the cereborospinal fluid (CSF) the concentration of 2-Pyrrolidone-5-carboxylic acid correlated with hepatic encephalopathy and liver disease. And 2-Pyrrolidone-5-carboxylic acid concentration in CSF reported in this study was comparable to that reported previously for normal plasma (Cooper, Dhar, Kutt, & Duffy, 1980). Further research can be performed to investigate whether a higher consumption of CML would affect the concentration of 2-Pyrrolidone-5-carboxylic acid in plasma and have association to liver disease. Oleic acid was the other metabolites that showed a significant difference between treatments. The oleic acid content was significantly higher in CML0 diet compared to AIN93. The reason that CML0 had the highest oleic acid among the three CML diets was unclear since they are identical with the exception that the CML1 and CML2 diets had increased the level of CML compared to the CML0 diet.

In summary, mice fed diets with a higher CML content diet did affect the metabolites profile to some extent, and an increased intake of dietary CML may lead to a higher body weight as well as the development of T2D. Further research is needed to elucidate the mechanisms involved in the metabolomics profile changes.

Conclusion

To investigate the effect of CML, we had hypothesized the diets with different CML levels would lead to changes in the metabolite profile. From the experiment results, lactic acid, phosphoric acid, and 10 unknown metabolites showed significant differences between the five experimental diets. When only CML diets were compared, lactic acid was the only metabolite that was significant difference. From the study we can conclude that higher CML consumption can affect the metabolite profile, but further studies can be performed to investigate specific mechanism involved in such metabolic profile changes.

Glucose content in plasma was not significantly different according to the one-way ANOVA analysis.

The metabolites PCA provided a separation of CML2 diet on PC2. Moreover, DIO and AIN93 diets were mostly separated by PC3. In addition, both PC1 and PC 3 affected CML0 and CML2. The further studies may focus on how these three principal components affect the diet and metabolites.

References

Ames, J. M. (2007). Evidence against dietary advanced glycation endproducts being a risk to human health. *Molecular nutrition & food research, 51*(9), 1085-1090.

Assar, S. H., Moloney, C., Lima, M., Magee, R., & Ames, J. M. (2009). Determination of N ε-(carboxymethyl) lysine in food systems by ultra performance liquid chromatography-mass spectrometry. *Amino acids, 36*(2), 317-326.

Birlouez-Aragon, I., Saavedra, G., Tessier, F. J., Galinier, A., Ait-Ameur, L., Lacoste, F., . . . Lecerf, J.-M. (2010). A diet based on high-heat-treated foods promotes risk factors for diabetes mellitus and cardiovascular diseases. *The American journal of clinical nutrition, 91*(5), 1220-1226.

Bosch, L., Sanz, M. L., Montilla, A., Alegría, A., Farré, R., & del Castillo, M. D. (2007). Simultaneous analysis of lysine, Nε-carboxymethyllysine and lysinoalanine from proteins. *Journal of Chromatography B, 860*(1), 69-77.

Bruce, S. J., Tavazzi, I., Parisod, V., Rezzi, S., Kochhar, S., & Guy, P. A. (2009). Investigation of human blood plasma sample preparation for performing metabolomics using ultrahigh performance liquid chromatography/mass spectrometry. *Analytical Chemistry, 81*(9), 3285-3296.

Chen, Y., Varasteh, B., & Reaven, G. (1992). Plasma lactate concentration in obesity and type 2 diabetes. *Diabete & metabolisme, 19*(4), 348-354.

Cooper, A. J., Dhar, A. K., Kutt, H., & Duffy, T. E. (1980). Determination of 2-pyrrolidone-5-carboxylic and α-ketoglutaramic acids in human cerebrospinal fluid by gas chromatography. Analytical biochemistry, 103(1), 118-126.

Crawford, S. O., Hoogeveen, R. C., Brancati, F. L., Astor, B. C., Ballantyne, C. M., Schmidt, M. I., & Young, J. H. (2010). Association of blood lactate with type 2 diabetes: the Atherosclerosis Risk in Communities Carotid MRI Study. *International journal of epidemiology, 39*(6), 1647-1655.

Deng, P., Jones, J. C., & Swanson, K. S. (2014). Effects of dietary macronutrient composition on the fasted plasma metabolome of healthy adult cats. *Metabolomics, 10*(4), 638-650.

Dunn, W. B., Broadhurst, D., Begley, P., Zelena, E., Francis-McIntyre, S., Anderson, N., . . . Haselden, J. N. (2011). Procedures for large-scale metabolic profiling of serum and plasma using gas chromatography and liquid chromatography coupled to mass spectrometry. *Nature protocols, 6*(7), 1060-1083.

Esmaillzadeh, A., Kimiagar, M., Mehrabi, Y., Azadbakht, L., Hu, F. B., & Willett, W. C. (2007). Dietary patterns, insulin resistance, and prevalence of the metabolic syndrome in women. *The American journal of clinical nutrition, 85*(3), 910-918.

Foerster, A., & Henle, T. (2003). Glycation in food and metabolic transit of dietary AGEs (advanced glycation end-products): studies on the urinary excretion of pyrraline. *Biochemical Society Transactions, 31*(6), 1383-1385.

Furniss, D., Vuichoud, J., Finot, P., & Hurrell, R. (1989). The effect of Maillard reaction products on zinc metabolism in the rat. *British journal of nutrition, 62*(03), 739-749.

Hintze, K. J., Benninghoff, A. D., & Ward, R. E. (2012). Formulation of the Total Western Diet (TWD) as a basal diet for rodent cancer studies. *Journal of Agricultural and Food Chemistry, 60*(27), 6736-6742.

Hull, G. L., Woodside, J. V., Ames, J. M., & Cuskelly, G. J. (2012). Nε-(carboxymethyl) lysine content of foods commonly consumed in a Western style diet. *Food Chemistry, 131*(1), 170-174.

Kanner, J. (2007). Dietary advanced lipid oxidation endproducts are risk factors to human health. *Molecular nutrition & food research, 51*(9), 1094-1101.

Kellow, N. J., Coughlan, M. T., Savige, G. S., & Reid, C. M. (2014). Effect of dietary prebiotic supplementation on advanced glycation, insulin resistance and inflammatory biomarkers in adults with pre-diabetes: a study protocol for a double-blind placebo-controlled randomised crossover clinical trial. *BMC endocrine disorders, 14*(1), 55.

Lawton, K. A., Berger, A., Mitchell, M., Milgram, K. E., Evans, A. M., Guo, L., . . . Milburn, M. V. (2008). Analysis of the adult human plasma metabolome.

Mills, D., Tuohy, K., Booth, J., Buck, M., Crabbe, M. J. C., Gibson, G., & Ames, J. (2008). Dietary glycated protein modulates the colonic microbiota towards a more detrimental composition in ulcerative colitis patients and non-ulcerative colitis subjects. *Journal of applied microbiology, 105*(3), 706-714.

Nursten, H. E. (2005). *The Maillard reaction: chemistry, biochemistry, and implications*: Royal Society of Chemistry.

Oste, R. E., Dahlqvist, A., Sjoestroem, H., Noren, O., & Miller, R. (1986). Effect of Maillard reaction products on protein digestion. In vitro studies. *Journal of Agricultural and Food Chemistry, 34*(2), 355-358.

Sandu, O., Song, K., Cai, W., Zheng, F., Uribarri, J., & Vlassara, H. (2005). Insulin resistance and type 2 diabetes in high-fat–fed mice are linked to high glycotoxin intake. *Diabetes, 54*(8), 2314-2319.

Scheppach, W. (1994). Effects of short chain fatty acids on gut morphology and function. *Gut, 35*(1 Suppl), S35-S38.

Šebeková, K., & Somoza, V. (2007). Dietary advanced glycation endproducts (AGEs) and their health effects–PRO. *Molecular nutrition & food research, 51*(9), 1079-1084.

Seiquer, I., Díaz-Alguacil, J., Delgado-Andrade, C., López-Frías, M., Hoyos, A. M., Galdó, G., & Navarro, M. P. (2006). Diets rich in Maillard reaction products affect protein digestibility in adolescent males aged 11–14 y. *The American journal of clinical nutrition, 83*(5), 1082-1088.

Somoza, V., Lindenmeier, M., Hofmann, T., Frank, O., Erbersdobler, H. F., Baynes, J. W., . . . Bek, S. (2005). Dietary Bread Crust Advanced Glycation End Products Bind to the Receptor for AGEs in HEK-293 Kidney Cells but Are Rapidly Excreted after Oral Administration to Healthy and Subtotally Nephrectomized Rats. *Annals of the New York Academy of Sciences, 1043*(1), 492-500.

Somoza, V., Wenzel, E., Weiß, C., Clawin-Rädecker, I., Grübel, N., & Erbersdobler, H. F. (2006). Dose-dependent utilisation of casein-linked lysinoalanine, N (epsilon)-fructoselysine and N (epsilon)-carboxymethyllysine in rats. *Molecular nutrition & food research, 50*(9), 833-841.

Styczynski, M. P., Moxley, J. F., Tong, L. V., Walther, J. L., Jensen, K. L., & Stephanopoulos, G. N. (2007). Systematic identification of conserved metabolites in GC/MS data for metabolomics and biomarker discovery. *Analytical Chemistry, 79*(3), 966-973.

Uribarri, J., Cai, W., Sandu, O., Peppa, M., Goldberg, T., & Vlassara, H. (2005). Diet-Derived Advanced Glycation End Products Are Major Contributors to the Body's AGE Pool and Induce Inflammation in Healthy Subjects. *Annals of the New York Academy of Sciences, 1043*(1), 461-466.

Uribarri, J., Woodruff, S., Goodman, S., Cai, W., Chen, X., Pyzik, R., . . . Vlassara, H. (2010). Advanced glycation end products in foods and a practical guide to their reduction in the diet. *Journal of the American Dietetic Association, 110*(6), 911-916. e912.

Vlassara H, B. R., Striker L. (1994). Pathogenic effects of advanced glycosylation: biochemical, biologic, and clinical implications for diabetes and aging. *Lab Invest, 70*(2), 138-151.

Vlassara, H., Cai, W., Crandall, J., Goldberg, T., Oberstein, R., Dardaine, V., . . . Rayfield, E. J. (2002). Inflammatory mediators are induced by dietary glycotoxins, a major risk factor for diabetic angiopathy. *Proceedings of the National Academy of Sciences, 99*(24), 15596-15601.

Waller, G. R., & Feather, M. S. (1983). *The Maillard reaction in foods and nutrition.* Paper presented at the ACS symposium series (USA).

Wang, T. J., Larson, M. G., Vasan, R. S., Cheng, S., Rhee, E. P., McCabe, E., . . . Fernandez, C. (2011). Metabolite profiles and the risk of developing diabetes. *Nature medicine, 17*(4), 448-453.

Wong, J. M., de Souza, R., Kendall, C. W., Emam, A., & Jenkins, D. J. (2006). Colonic health: fermentation and short chain fatty acids. *Journal of clinical gastroenterology, 40*(3), 235-243.

Yamagishi, S.-i., Ueda, S., & Okuda, S. (2007). Food-derived advanced glycation end products (AGEs): a novel therapeutic target for various disorders. *Current pharmaceutical design, 13*(27), 2832-2836.