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COLLEGE OF AGRICULTURE
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Department of Nutrition and Food Science

Objective II: Human Bioavailability and anti-inflammatory properties of Mango Polyphenols (Comparison lean and obese over 24h, and 10 and 42 days)

Final Report

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Executive Summary

Significance: The molecular mechanisms of health benefits of mango polyphenols are not well investigated. *In vitro* studies cannot investigate the role of metabolites of mango compounds. To assess the role of mango metabolites in health benefits, we are investigating which metabolites are formed when we eat mango. Results from this study show that there are significant amounts of bioactive metabolites of mango tannins formed by the intestinal microbiome. There are significant differences between lean and obese individuals. This indicates that not everybody may benefit equally from consuming mangos and this may explain differences in the results that are observed in efficacy trials with mango. Findings from this study are highly significant to understanding how much mango individuals should consume and that prolonged intake is most beneficial to obtain benefits. This will help the National Mango Board towards the development of intake recommendations for fresh mango.

In this objective, the following studies were performed:

1. Pharmacokinetic study over 10-days
2. Pharmacokinetic study over 42-days comparing lean and obese subjects and continuous with interrupted consumption
    a. Pharmacokinetics of mango polyphenol metabolites in plasma and urine
    b. Biomarkers for microbial metabolism
    c. Biomarkers for inflammation in lean and obese subjects

Pharmacokinetic study over 10-days:

The absorption of mango polyphenols was investigated in a 10-day pharmacokinetic study in healthy, lean individuals. Results show that significant amounts of gallic acid derivatives and pyrogallol-derivatives (microbial metabolite) are present in urine. This indicates that metabolites were absorbed into the blood stream and this includes microbial metabolites that were generated in the intestines by the intestinal microbiome. After 10-days the excretion of mango polyphenol metabolites was increased significantly in all subjects. This indicates that a prolonged consumption increases the absorption of polyphenol metabolites and the resulting health benefit may be higher compared to a single consumption.

Pharmacokinetic study over 42-days comparing lean and obese subjects and continuous with interrupted consumption

a. Pharmacokinetics of mango polyphenol metabolites in plasma and urine

The absorption, metabolism, and excretion of gallic acid, galloyl glycosides, and gallotannins from mango fruit was assessed in lean and obese subjects who consumed mango continuously for 42 days and compared to a control cohort who consumed mango only on Days 1 and 42. Healthy volunteers (n=36; BMI 22-34) consumed 400 g of mango pulp daily for 42 days. Significant ($p<0.05$) increases were observed in their 24 h urinary excretion of 4-*O*-methylgallic acid-3-*O*-sulfate, methylpyrogallol-*O*-sulfate, pyrogallol-*O*-sulfate, and catechol-*O*-sulfate. Increases were also observed for plasma concentrations ($AUC_{0\text{-}8h}$) for individual metabolites. Overall, the concentrations of

metabolites were significantly lower in obese vs. lean individuals and this indicates that obese individuals may have difficulties in metabolizing and absorbing polyphenol metabolites. As expected, in the control cohort (n=11; BMI<25) who consumed mango only on two different days, non-continuously, Days 1 and 42, no increases were found in the 24 h urinary excretion or plasma comparing concentrations for any of the detected galloyl metabolites on days 1 and 42.

Continuous consumption of mango leads to increased metabolism and excretion of polyphenol metabolites and this is likely based on the adaptive increase in the metabolism of gallic acid, galloyl glycosides, and gallotannins by the intestinal microbiota. The increased generation of bioactive microbial metabolites may have significant implications for health-benefits derived from the consumption of mango. It seems that the continuous consumption of mango would yield the highest benefit based on increased exposure to mango polyphenol metabolites.

b. Biomarkers for microbial metabolism

After six weeks of mango consumption, plasma concentrations of galloyl metabolites increased in all subjects. Plasma in lean subjects contained 2.4 times higher levels of polyphenols compared to obese subjects, including the microbial pyrogallol derivatives. As shown in qPCR, the levels of pyrogallol-producing microbiota, *Aspergillus oryzae* and *Lactococcus lactis* were significantly lower in obese subjects compared to lean subjects on the baseline, but the levels were increased in obese subjects to match the lean subjects after six weeks. The levels of *Bifidobacterium Spp*. were not significantly changed but directly correlated with the amount of galloyl metabolites found in plasma ($p=0.0243$, $r=0.36$, Spearman correlation). The levels of *Bacteroides thetaiotaomicron* and *Clostridium leptum*, microbiota associated with obesity, were significantly higher in obese subjects compared to lean subjects on the baseline, but decreased after six weeks of mango intake to match the levels observed in lean subjects. In obese subjects, plasma concentrations of the inflammation markers, ($AUC_{0\text{-}8h}$) of IL-8 and MCP-1 were significantly decreased after six weeks, while as expected there were no changes in lean subjects. The plasma concentrations ($AUC_{0\text{-}8h}$) of IL-10 were significantly higher in lean subjects compared to obese subjects and correlated with the amount of metabolites found in plasma ($p=0.0218$, $r=0.38$, Spearman correlation). These results indicate that obese individuals do not exhibit the same level of adaptive absorption and metabolism as lean subjects but still benefit from a decrease of pro-inflammatory cytokines (IL-8 and MCP-1). The observed changes may in part be due to differences in gut microbiota composition and metabolism. It seems that continuous consumption of mangos might yield the higher benefit to obese and lean individuals.

c. Biomarkers for inflammation in lean and obese subjects

Inflammatory cytokines, metabolic hormones, and lipid profiles were examined in plasma of healthy lean (BMI 18-25kg/m^2) and obese (BMI>30kg/m^2) subjects at baseline and after 42 days of 400g mango pulp consumption. Results show that after 42-day systolic blood pressure was significantly decreased in lean subjects. The levels of hemoglobin A1c and PAI-1 were significantly improved in obese subjects. Plasma levels of inflammatory cytokines and lipid profiles were not significantly modulated as expected from the small subject number, but positive trends were noted. The potential of mango polyphenols to beneficially impact inflammation and lipid metabolism should

be confirmed in a larger clinical trial with lean and obese subjects where both groups are characterized by chronic inflammation. The increased generation of bioactive microbial metabolites may have significant implications for health-benefits derived from the consumption of mango. Galloyl derivatives from mango may possess therapeutic potential in the prevention and treatment of obesity and metabolic disorders. Lowered levels of inflammatory markers in obese individuals suggests that mango consumption may exert therapeutic effects in the treatment of chronic digestive disorders, however further research needs to investigate the differences between lean and obese subjects that indicate that the absorption of beneficial metabolites is lower in obese subjects.

Benefits to the Mango Industry:

The potential health benefits of gallic acid and pyrogallol are well documented, but there is little information on the relative benefits of their corresponding phase II metabolites. The prevalence of phase II polyphenolic metabolites in urine and the lack of non-conjugated forms following mango consumption suggests that gallic acid and pyrogallol metabolites should be incorporated in future absorption and efficacy studies with polyphenols from mango, as these may significantly contribute to derived health benefits of consuming fresh mango pulp.

The increased generation of bioactive microbial metabolites may have significant implications for health-benefits derived from the consumption of mango. Galloyl derivatives from mango may possess therapeutic potential in the prevention and treatment of obesity and metabolic disorders. Lowered levels of inflammatory markers in obese individuals suggests that mango consumption may exert therapeutic effects in the treatment of chronic digestive disorders, however further research needs to investigate the differences between lean and obese subjects that indicate that the absorption of beneficial metabolites is lower in obese subjects.

The performed research resulted in the following research communications:

Abstracts:

R Barnes, K Krenek, S Talcott, S Talcott. Profile of Gallic Acid Metabolites in Urine After the Intake of Mango (Mangifera indica, L.) cv. Keitt in Humans. Experimental Biology 2015, Boston

H Kim, R Barnes, C Fang, S Talcott, S Mertens-Talcott. Intestinal Microbiota and Host Metabolism Respond Differentially in Lean and Obese Individuals Following Six-Week Consumption of Galloyl Derivatives from Mango (Mangifera Indica L.) Pulp. Experimental Biology 2017, Chicago

C Fang, H Kim, R Barnes, S Talcott, S Mertens-Talcott. Daily Mango (Mangifera Indica L.) Consumption For 42 Days Differentially Modulates Metabolism And Inflammation In Lean And Obese Individuals. Experimental Biology 2017, Chicago

SU Mertens-Talcott, H Kim, S Talcott, R Barnes. Adaptation of Gallic Acid, Galloyl Glycoside, and Gallotannin Metabolism and Excretion after 42 Days of Mango (Mangifera Indica L.) Consumption. Experimental Biology 2017, Chicago

Chuo Fang, Hyemee Kim, Ryan C Barnes, Stephen T Talcott, Susanne U Mertens-Talcott. Daily Mango (Mangifera Indica L.) Consumption for 42 Days Differentially Modulates Metabolism and Inflammation in Lean and Obese Subjects. Interdisciplinary Regional NORC meeting, Texas A&M University, May 2017.

Manuscripts/peer reviewed publications:

R Barnes, K Krenek, S Talcott, S Talcott. Urinary metabolites from mango (Mangifera indica L. cv. Keitt) galloyl derivatives and in vitro hydrolysis of gallotannins in physiological conditions. Mol. Nutr. Food Res. 2016, 60, 542–550

In preparation:

R Barnes, H Kim, S Talcott, SU Mertens-Talcott. Adaptation of Gallic Acid, Galloyl Glycoside, and Gallotannin Metabolism and Excretion after 42 Days of Mango (Mangifera Indica L.) Consumption.

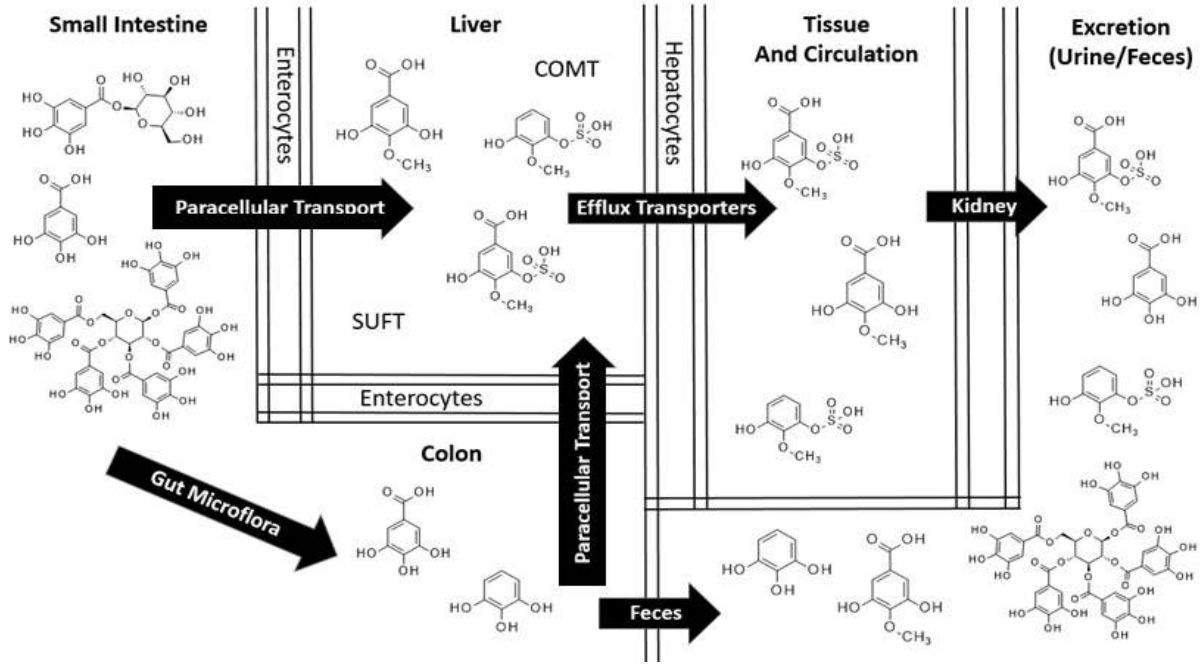
H Kim, R Barnes, S Talcott, SU Mertens-Talcott. Differential Microbial Gallotannin Metabolism and Excretion in Lean and Obese Subjects after 42 Days of Mango (Mangifera Indica L.) Consumption.

Chuo Fang, Hyemee Kim, Ryan C Barnes, Stephen T Talcott, Susanne U Mertens-Talcott. Daily Mango (Mangifera Indica L.) Consumption for 42 Days Differentially Modulates Metabolism and Inflammation in Lean and Obese Subjects.

Introduction

Our ultimate goal is to identify and demonstrate chemical and biochemical properties of mangos that will allow to better market fresh mangos. This research will also lay vital groundwork towards obtaining legal and marketing claims relating to the composition and health benefits of mangos. We recognize that these foundations are critical to build for a strong working case for these future goals.

Background: Mango is a rich source of polyphenolics which have been shown to exert anti-inflammatory and anti-carcinogenic properties (*1*). Mango's polyphenolic content is composed primarily of gallic acid, gallic acid glycosides, and gallotannins (*2*). Gallic acid was previously reported to be absorbed from the small intestine and metabolized in the liver where the endogenous phase II enzymes catechol-*O*-methyltransferase and sulfotransferases conjugate it to predominantly produce 4-O-methylgallic acid and 4-*O*-methylgallic acid-3-*O*-sulfate (*3*). Gallic acid not absorbed will transit to the colon where it is catabolized by host bacteria to produce smaller phenols, such as pyrogallol and catechol, which are further absorbed and metabolized by the host (*4*). Oligomeric galloyl glucose and gallotannins, however, are too large to be absorbed intact in the small intestine and must be depolymerized by hydrolytic esterases residing in the gastrointestinal tract or through non-enzymatic hydrolysis to generate bioaccessible gallic acid (*5*). Thus, mango pulp is a source of several pro-gallic acid compounds with the potential to produce galloyl and pyrogallol metabolites. *Lactobacillus plantarum*, *Lactobacillus reuteri*, and *Lactococcus lacti* are well-known tannase producers present in human gut microbiota (*6, 7*). As a result, these are some of the main metabolizers of mango polyphenols. Thus, the modulation of these gut microbiota may play a critical role in the absorption of mango polyphenol derivatives. Certain chronic conditions, such as obesity and Inflammatory Bowel Diseases (IBD), are well-known to be linked to changes in gut microbiota composition that may significantly reduce the bioavailability and pharmacokinetics of polyphenols and their derivatives in obese individuals (*8*). Therefore, it is crucial to investigate the absorption of mango polyphenols and their metabolism in a human digestive system to determine the overall health benefits that mango can exert.



Overview of Intestinal Tannin Metabolism

Objectives: The objectives of this research were to evaluate **a)** the urinary metabolites produced after the consumption of Keitt mango pulp in an effort to characterize the bioavailability of the major polyphenolics in mango over a 10-day period, as well as **b)** the metabolism and excretion gallic acid and galloyl glycosides and **c)** the modulation of gut microbiota and anti-inflammatory markers in lean and obese individuals following 42-day consumption of Ataulfo mango pulp.

Hypotheses: We hypothesize that the consumption of fresh mango will lead to the absorption, metabolism, and excretion of gallic acid and galloyl glycosides, and that repetitive consumption of mango will lead to an increase in the concentration of gallic acid metabolites due to adaptions in colon and xenobiotic metabolism. Additionally, we hypothesize that the response to mango intake will differ significantly between lean and obese individuals.

Significance: Currently, the molecular mechanisms of health benefits of mango polyphenols are not well investigated. *In vitro* studies cannot investigate the role of metabolites of mango compounds. In order to assess the role of mango metabolites in health benefits, we are investigating which metabolites are formed when we eat mango. If the health mechanism of mango polyphenols is clearly understood, it would pave the way towards substantiating health claims regarding mango consumption.

Study Approach:

Mango (cv. Keitt) 10 Day Polyphenol Absorption: Eleven healthy volunteers participated in the 10-day mango consumption pilot study. After an overnight fast of at least 12 hours, urine collections were made (baseline) from each participant. Each participant was given 400g of mango to consume and urine collections were made at 0-3, 3-6, 6-8, and 8-12 hours. The volume of urine was recorded and an aliquot immediately frozen at

-80°C until analysis. Participants were given 400g of mango to consume daily for the next eight days and the study design was repeated again on the 10th day. The study protocol was approved by the Institutional Review Board (IRB) of Texas A&M University. All analysis was performed via HPLC-MS.

Daily Consumption of Mango (cv. Ataulfo) Pulp for 42 Days: Healthy volunteers (n = 32, aged 18 -50) with no medical history of digestive disorders participated in a 42-day mango consumption study divided into three cohorts. The Control cohort (n=11) consumed 400 g of mango pulp only on Days 1 and 42. The remaining two cohorts consumed 400 g mango pulp daily for 42 days and were divided into Lean and Obese groups. The Lean cohort (n=12) had a BMI from 18-25 and the Obese cohort (n= 9) had a BMI > 30. Three days prior to when the study began subjects refrained from consuming foods known to contain gallic acid or pro-gallic acid polyphenolics including mango, grape products, tea, chocolate, and berries. Baseline urine samples were collected prior to mango consumption and post-prandial from 0-3, 3-6, 6-9, 9-12, and 12-24 h and on study Days 1 and 42. The volume of urine output was measured for each collection, and 12 mL acidified with 0.1 mL of 88% formic acid and passed through a 0.45 µm filter for metabolite analysis by LC-MS. Baseline blood samples were collected prior to mango consumption, and post-prandial at 0.5, 1, 1.5, 2, 3, 4, 6, and 8 h through a blood-draw catheter on Days 1 and 42. Blood samples were centrifuged at 3,000 x g for 5 min, and plasma acidified with 25 µL of formic acid. Plasma samples were stored at -80 °C until processed. Briefly, 50 µL of an internal standard, ethyl gallate, was incubated with 500 µL of acidified plasma and 75 µL of 10% SDS was then added to denature proteins followed by addition of 400 µL of 0.1% formic acid methanol. Plasma was sonicated for 10 min, centrifuged for 5 min at 10,000 x g, and spiked with 50 µL of saturated KCl to remove residual SDS. Samples were held at 4 ºC for 1 h prior to being filtered through a 0.45 µm filter prior to LC-MS analysis. The study protocol was approved by the Institutional Review Board (IRB) of Texas A&M University.

LC-MS Analyses: Samples were analyzed on a Thermo Finnigan Surveyor LCQ Deca XP Max MS^n ion trap mass spectrometer equipped with an ESI source. Separations were in reversed-phase using a Thermo Finnigan Surveyor HPLC coupled to a Surveyor PDA detector and gradient separations were performed using a Phenomenex Kinetex™ (Bannockburn, Ill) C18 column (150 x 4.6 mm, 2.6 µm) at room temperature. Injections were made into the column by use of a 50 µL sample loop. For separation of urine metabolites mobile phase A: 0.1% formic acid in water, and mobile phase B: 0.1% formic acid in methanol were run at 0.4 mL/min. A gradient was run of 0% Phase B for 2 min and changed to 10% Phase B in 4 min, 10 to 15% Phase B in 8.5 min, 15% to 27% Phase B in 11 min, 27% to 90% Phase B in 15 min, 90% was held to 0.5 min before returning to initial conditions. For separation of plasma metabolites mobile phase A was 0.1% formic acid in water and mobile phase B was 0.1% formic acid in methanol run at 0.45 mL/min. A gradient was run of 0% Phase B for 2 min and changed to 10% Phase B in 4 min, 10% Phase B was held to 10 min, 10 to 40% Phase B in 25 min, and 40% to 65% Phase B in 35 min, 65% to 85% Phase B in 41 min, 85% was held to 49 min before returning to initial conditions. The electrospray interface worked in negative ionization mode. Source and capillary temperatures were set at 325°C, source voltage

was 4.0 kV, capillary voltage was set at -47 V, and collision energy for MS/MS analysis was set at 35 eV. The instrument operated with sheath gas and auxiliary gas (N_2) flow rates set at 10 units/min and 5 units/min, respectively. The instrument was tuned for 4-*O*-methylgallic acid and metabolites quantified using extracted ion chromatograms from their respective parent compounds as standards.

qPCR: Bacterial DNA was extracted from 100mg of fecal sample using a bead-beating phenol-chloroform method (*9*). Further steps of DNA extraction and purification were performed using a QIAamp DNA stool mini kit (Qiagen, Germany) according to the manufacturer's instructions. The following qPCR assays for selected bacterial groups were performed to determine levels of tannase producers and obesity-related gut microbiota. The decision of targeting only a subset of bacterial groups was based on the author's previous work (*9*). Real-time PCR conditions, sequences of primers, and annealing temperature were followed as described in (*10*). The qPCR data was expressed as log amount of DNA normalized to the log amount of total bacterial DNA (Universal F341 and R518) (*10*).

Multiplex bead assay: Protein extract (50μg) was loaded to determine the relative abundance of cytokines (TNFα, IL-1β, IL-6) using the multiplex kits (Millipore, Billerica, MA). The multiplex analysis was performed by the Luminex L200 instrument (Luminex, Austin, TX), and data were analyzed using the Luminex xPONENT software (*11*).

Statistical Analyses: Significant differences for metabolites in urine were calculated from the sum of their excretions over 24 h using students t-test in Sigma Plot software (SPSS, Chicago, IL). Significant differences between cohorts were calculated by using ANOVA. Non-compartmental pharmacokinetic analysis was performed by use of the PkSolver Microsoft Excel Add-In. Results are reported at the mean ± standard error of the mean.

Results

Mango (cv. Keitt) 10 Day Polyphenol Absorption

This study investigated the presence of mango polyphenol metabolites in urine because any mango polyphenol that has been absorbed will eventually be excreted through the urine. For the first time, seven urinary metabolites sourced from gallic acid and pyrogallol were characterized and quantified following mango consumption (Figure 1).

Of the seven metabolites found in the urine compounds, **M2** and **M3** are thought to have been absorbed in the small intestine. This is because both still have the acid or (COOH) still attached to them. Compounds **M1** and **M4-M7** are missing this group. This acid group is thought to be used as a carbon source by microflora in the colon. These compounds are different from the parent compounds that were found in the fruit. After 10 days of mango consumption, no significant difference ($p<0.05$) in excretion for urinary metabolites **M2** and **M3** were found between days 1 and 10 (**Figure 2**). Overall, urine analysis revealed that 43 to 54% of orally administered from gallic acid was absorbed, metabolized, and eliminated as **M2** and **M3**.

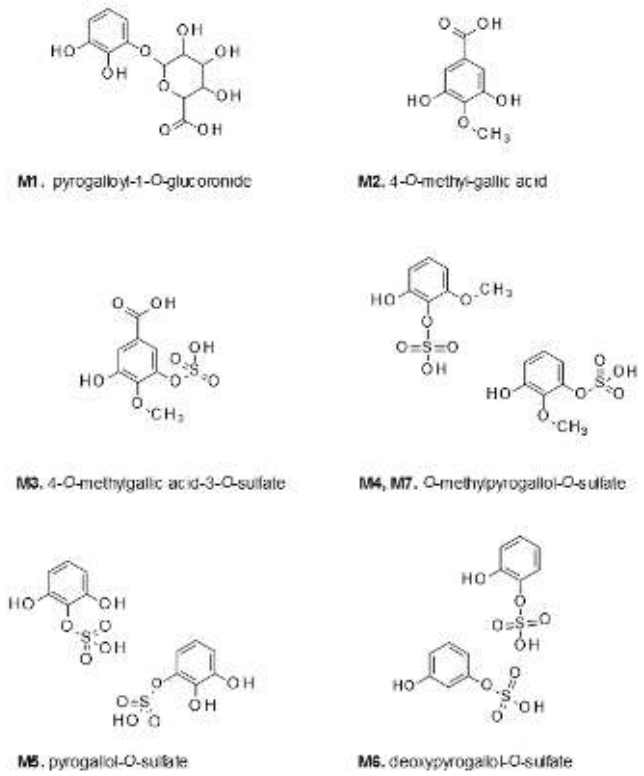


Figure 1. Tentative chemical structures for the seven-characterized urinary gallic acid metabolites.

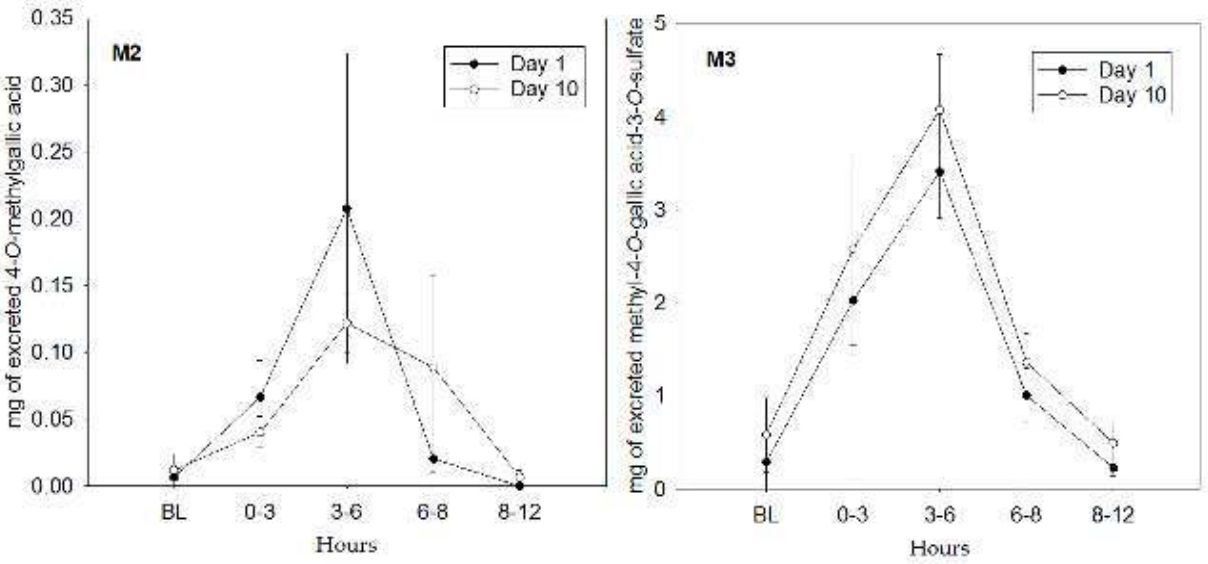


Figure 2. Excretion in mg for metabolites 4-*O*-methylgallic acid (M2) and 4-*O*-methylgallic acid-3-*O*-sulfate (M3) at baseline (BL), 0-3, 3-6 , 6-8, and 8-12 h after consumption of 400 g mango cv. Keitt for days 1 and 10. Values are reported as mean ± standard error of the mean.

The remaining five metabolites (**M1** and **M4-M7**) were characterized as conjugates of pyrogallol. Pyrogallol is not found naturally in mango, but is known to be produced from gallic acid reacting with enzymes expressed by the gut microbiota where it can be absorbed and metabolized.(*12, 13*) Within 12 h after intake of mango pulp, **M5** and **M6** constituted the majority of excreted urinary metabolites and together averaging to 64.3% and 65.5% for day 1 and 10 respectively. The urinary excretion of all five metabolites tended to increase on day 10 compared to day 1 with significant increases ($p<0.05$) for metabolites **M5** and **M6** (**Figure 3**). The total amount of excreted **M5** increased from 28.5 ± 2.84 mg to 55.4 ± 7.10 mg, on days 1 and 10 respectively, and the total amount of excreted **M6** was found to increase from 23.6 ± 1.56 mg to 47.7 ± 3.39 mg.

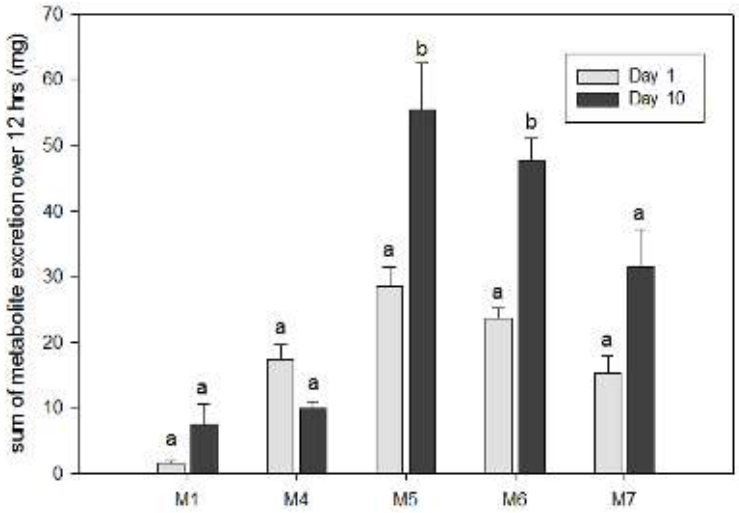


Figure 3. The sum of metabolite excretion in mg for metabolites pyrogallol-1-*O*-glucuronide (M1), *O*-methylpyrogallol-*O*-sulfate (M4, M7), pyrogallol-*O*-sulfate (M5), deoxypyrogallol-*O*-sulfate (M6) after consumption of 400 g mango cv. Keitt for days 1 and 10. Metabolites with different letters designate a significant difference for the day's total excretion between days 1 and 10 ($p < 0.05$).

Daily Consumption of Mango (cv. Ataulfo) Pulp for 42 Days

The urinary excretion of phase II metabolites derived from galloyl derivatives following consumption of 400 g of Ataulfo mango pulp was evaluated in Lean and Obese subjects who consumed mango daily for 42 days and compared to a Control cohort that only consumed mango on Days 1 and 42. Seven gallic acid and pyrogallol based phase II metabolites were tentatively identified in the urine from m/z previously reported (**Figure 1**). The 400 g of pulp fed to subjects contained a total of 259 mg of galloyl derivatives of which 95.4 mg were non-tannin, including gallic acid (3.64 mg) and monogalloyl glucose (91.7 mg), and the remaining 164 mg were gallotannins ranging in degree of polymerization from 5-10 galloyl groups. Mango pulp contains a majority of pro-gallic acid polyphenolics and they can comprise over 50% of the total phytochemicals present.(*14*)

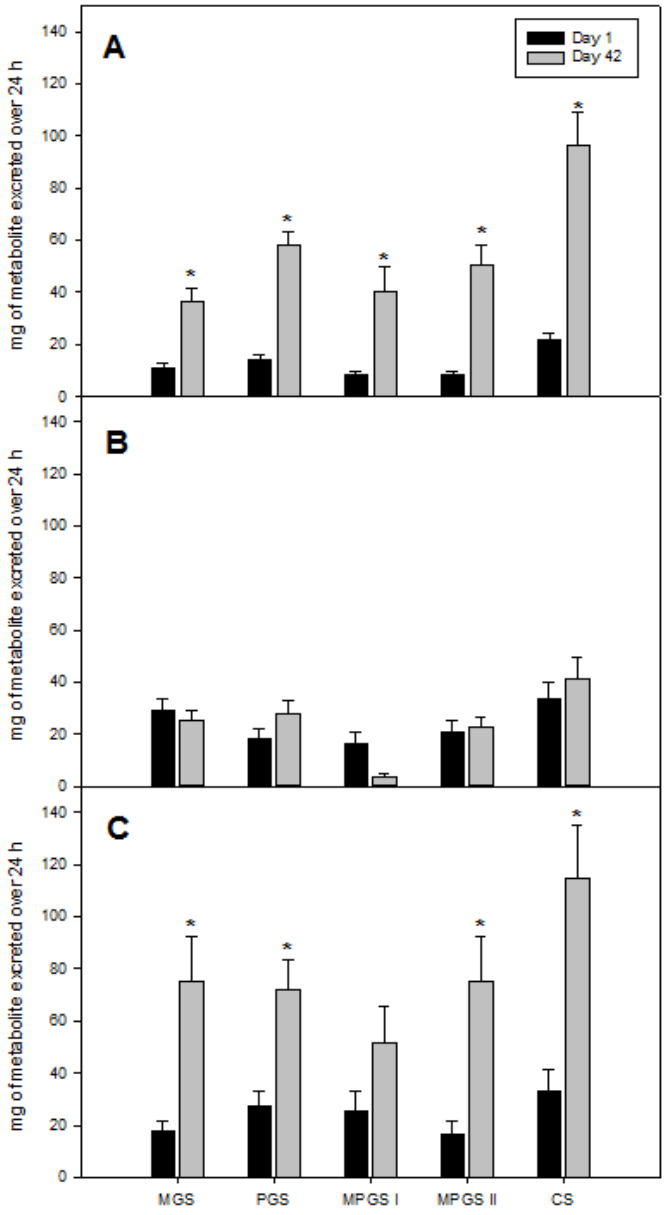
Five of the seven metabolites, 4-*O*-methylgallic acid-3-*O*-sulfate (**M3**), pyrogallol-*O*-sulfate (**M5)**, catechol-*O*-sulfate (**M6)**, and two isomers of methylpyrogallol-*O*-sulfate (I

and II) (**M4, M7**) were measured in the urine up to 24 h post-prandial. In the Lean cohort, a significant ($p<0.05$) increase was found in the excretion of all quantifiable metabolites (Error! Reference source not found. **4**). Specifically, the cumulative 24 h excretion of **M3** increased from 10.6 ± 2.38 to 36.13 ± 5.53 mg, **M5** from 14.3 ± 1.50 to 57.8 ± 5.37 mg, **M4** from 8.58 ± 1.16 to 40.3 ± 9.51 mg, **M7** from 8.40 ± 1.43 to 50.3 ± 8.00, and **M6** from 21.7 ± 2.90 to 96.3 ± 12.8 mg between Days 1 and 42 respectively. The same increase in 24 h urinary excretion was observed in the Obese cohort for all quantifiable metabolites except **M4**, which was found to have an insignificant increase from 22.1 ± 5.70 to 45.7 ± 11.1. In the Control cohort, no difference in urinary excretion was observed for the galloyl metabolites with the exception of **M4**, which was less excreted at Day 42 compared to Day 1 (Error! Reference source not found. **4**; $p< 0.05$).

The increase in galloyl metabolism and excretion has been hypothesized to be due to changes in the composition of the subjects' gut microbiota. It has previously been demonstrated that repetitive polyphenolic intake will affect the composition of a hosts' gut microbiota such as an increase in the population sizes of the probiotic bacteria species of *Bifidobacterium* and *Lactobacillus*.(*15, 16*) On Day 1 of this study, total urinary excretion of all galloyl metabolites corresponded to 24.3% of the 259 mg of galloyl derivatives consumed in the lean cohort and 45.2% in the obese cohort. Gallotannins are poorly absorbed from the small intestine in comparison to lower molecular weight polyphenolics such as gallic acid, and are hypothesized to transit to the colon and be hydrolyzed by the microbiota to create gallic acid, pyrogallol, and catechol by organisms such as *Lactobacillus plantarum* and *Streptococcus galloylitcus*.(*17*) We hypothesized that on Day 1 the intestinal microbiome would not be fully used to hydrolyse gallotannins, but over time microbiota selectively acclimated to utilize gallotannins as an energy source and produced higher concentrations of absorbable galloyl metabolites. The significant increases in total urinary excretion in the Lean and Obese cohorts from Day 1 to Day 42 support the hypothesis as the 24 h urinary excretion of galloyl derivatives increased to 107 and 130% of the dose of galloyl derivatives fed.

Five phases II galloyl metabolites, 4-*O*-methylgallic acid (**M2**), 4-*O*-methylgallic acid-3-*O*-sulfate (**M3**), pyrogallol-*O*-sulfate (**M5**), methylpyrogallol-*O*-sulfate (**M4**), and catechol-*O*-sulfate (**M6**), were characterized and semi-quantified in plasma up to 8 h post-prandial following consumption of Ataulfo mango (**Error! Reference source not found.**). In contrast to the higher urinary excretion observed in the Lean and Obese cohorts after 42 days of consuming 400 g of mango no significant increase was observed based on the $AUC_{0\text{-}8h}$ for individual plasma metabolites (**Table 1**). However, a trend of insignificant increases was observed for all individual metabolites in the Lean cohort along with non-significant increases for catechol-*O*-sulfate and 4-*O*-methylgallic acid sulfate in the Obese cohort where high coefficients of variation between subjects (ranging from 32 – 118%) kept comparisons from being significant. High coefficients of variation are frequently reported for polyphenolic metabolomics investigations, and are hypothesized to be due to differences in gut microbiota compositions per subject.(*18*) The lack of meaningful accumulation of gallic acid or its metabolites in blood observed both in this study and in others is likely the result of their rapid metabolism and excretion. It is unknown exactly how rapid excretion of these metabolites affects the potential health benefits from consumption of gallic acid glycosides and gallotannins.

Though studies which have investigated the bioefficacy of habitual consumption of pro-gallic acid and pro-pyrogallol foods such as strawberries, raspberries, grapes, and tea have reported benefits such as reduction in pro-inflammatory biomarkers, however the exact mechanisms remain unknown.(*19, 20*)



Error! Reference source not found. **4.** The sum of urinary excretion in mg for the metabolites 4-*O*-methylgallic acid-3-*O*-sulfate (MGS), pyrogallol-*O*-sulfate (PGS), two isomers of methylpyrogallol-*O*-sulfate (MPGS), and catechol-*O*-sulfate (CS) after consumption of 400 g of mango cv. Ataulfo for Days 1 and 42 in the (A) Lean and (C) Obese Cohorts who consumed mango daily for 42 days and the (B) Control Cohort who consumed mango only on Days 1 and 42. A (*) designates a significant difference between Days 1 and 42 for the respective metabolite.

Table 1. Area Under the Curve (AUC) from 0 to 8 h of phase II metabolites sourced from gallic acid and galloyl glycosides following daily consumption of mango cv. Ataulfo pulp for 42 days in Lean and Obese Cohorts, and compared against subjects who consumed mango only on days 1 and 42, Control Cohort.

	Control Cohort $AUC_{0\text{-}8h}$ (µg/L · h)		Lean Cohort $AUC_{0\text{-}8h}$ (µg/L · h)		Obese Cohort $AUC_{0\text{-}8h}$ (µg/L · h)	
Metabolite	**Day 1**	**Day 42**	**Day 1**	**Day 42**	**Day 1**	**Day 42**
4-*O*-methylgallic acid (**M2**)	527 ± 108	515 ± 300	431 ± 233	1,430 ± 760	585 ± 227	504 ± 96.3
4-*O*-methylgallic acid-3-*O*-sulfate (**M3**)	4,440 ± 1,140	5,110 ± 1,450	3,390 ± 487	4,410 ± 728	1,120 ± 299	1,729 ± 457
pyrogallol-*O*-sulfate (**M5**)	5,010 ± 1,580	2,290 ± 317	5,120 ± 1,430	6,830 ± 1,880	1,380 ± 496	1,010 ± 397
methylpyrogallol-*O*-sulfate (**M4**)	4,680 ± 1,380	2,303 ± 298	2,710 ± 562	4,920 ± 1,260	3,550 ± 894	3,440 ± 1,130
catechol-*O*-sulfate (**M6**)	9,420 ± 3,350	7,790 ± 2,200	8,790 ± 1,920	11,800 ± 3,060	7,318 ± 1,980	10,720 ± 1,790

Six weeks of mango consumption decreased systolic blood pressure (mean ± SD: 119.83±13.16 vs. 115.42±12.33; $p<0.05$) in lean subjects, but had no significant effect in obese subjects **(Table 2)**.

Table 2. Baseline and 6-week characteristics of anthropometrics and blood pressure in subjects supplemented with mangoes.

Variable	Group	Lean (n=12)			Obese (n=9)		
		Week 0	Week 6	p value	Week 0	Week 6	p value
Gender	Female Male	25% 75%			55.56% 44.44%		
Age (years)	Mean SD	25.58 (4.23)			27.78 (8.30)		
Height (cm)	Mean SD	172.58 (5.96)			168.34 (12.06)		
Weight (kg)	Mean SD	68.19 (7.24)	68.57 (7.29)	0.70	98.47 (17.57)	98.08 (17.40)	0.31
BMI (kg/m^2)	Mean SD	22.87 (2.22)	22.94 (2.39)	0.49	34.60 (4.89)	34.49 (4.58)	0.33
Pulse (BPM)	Mean SD	71.33 (14.35)	66 (11.17)	0.08	77.78 (10.22)	75.67 (9.68)	0.39
Body temperature (°C)	Mean SD	36.72 (0.32)	36.66 (0.39)	0.29	36.46 (0.32)	36.44 (0.27)	0.40
Oxygen saturation (%)	Mean SD	98.09 (0.94)	98.08 (1.08)	0.50	97.25 (1.16)	98 (1.22)	0.05
Systolic blood pressure (mmHg)	Mean SD	119.83 (13.16)	115.42 (12.33)	0.05	119.22 (16.93)	123 (13.36)	0.78
Diastolic blood pressure (mmHg)	Mean SD	74.08 (12.75)	76.67 (7.80)	0.73	83.89 (11.05)	80.78 (6.06)	0.20

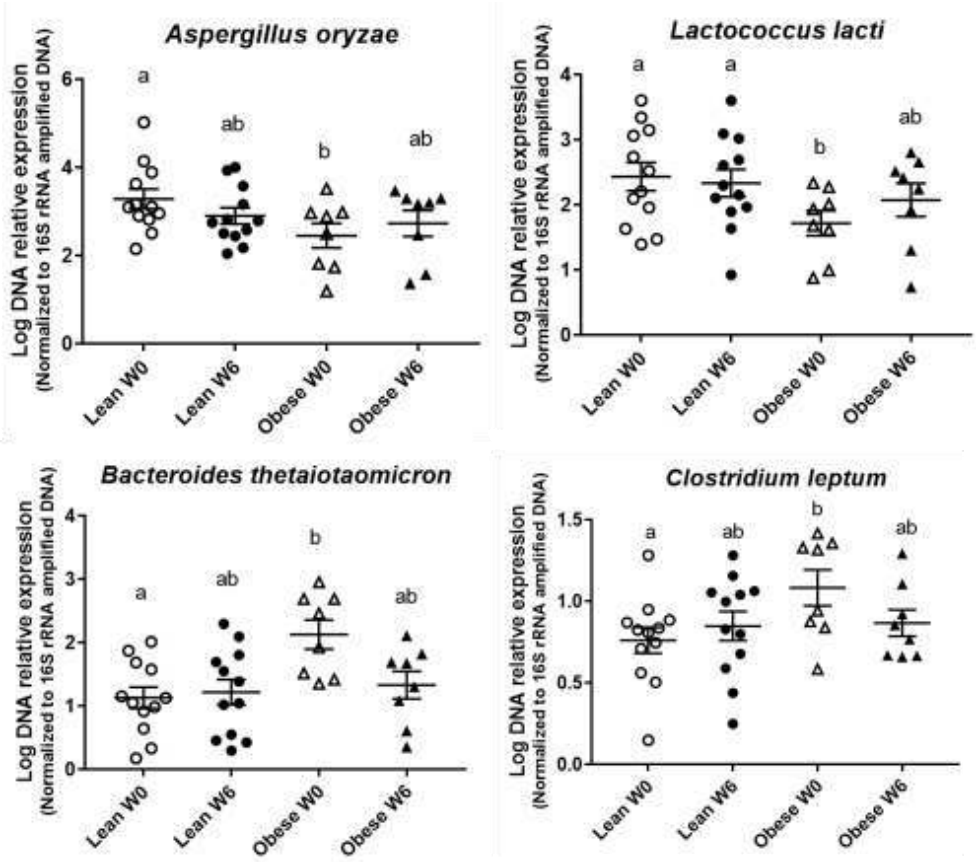


Figure 5. The effect of mango intake on microbiota composition in lean and obese individuals.

The levels of pyrogallol-producing microbiota, Aspergillus oryzae and Lactococcus lactis were significantly lower in obese subjects compared to lean subjects on the baseline, but the levels were increased in obese subjects to match the lean subjects after six weeks. The levels of Bacteroides thetaiotaomicron and Clostridium leptum, microbiota associated with obesity, were significantly higher in obese subjects compared to lean subjects on the baseline, but decreased after six weeks of mango intake to match the levels observed in lean subjects (**Figure 5**).

The level of hemoglobin A1c was slightly improved in obese but not lean subjects. Reduced expression of PAI-1, associated with decreased risk of atherosclerosis and thrombosis, was observed in both groups (In lean group, mean ± SD: 30.93±18.12 vs. 23.69±17.58; $p<0.1$; In obese group, mean ± SD: 31.34±8.09 vs. 24.93±12.20; $p<0.05$). There was a non-significant trend towards lowered levels of C-reactive protein, and elevated levels of IL-10 in both groups **(Table 3).**

Table 3. Plasma levels of inflammatory cytokines and metabolic hormones

Variable	Group	Lean			Obese			Δ		
		Week 0	Week 6	P value	Week 0	Week 6	P value	Lean	Obese	p value
Inflammatory biomarkers										
TNF-α (pg/mL)	Mean	1.28	1.17	0.40	1.47	1.22	0.25	-0.11	-0.25	0.72
	SD	0.58	0.40		0.59	0.52		0.65	0.75	
IL-1β (pg/mL)	Mean	0.01	0.08	0.06	0.02	0.07	0.13	0.06	0.06	0.91
	SD	0.03	0.11		0.03	1.47		0.12	0.12	
IL-6 (pg/mL)	Mean	0.80	0.94	0.18	0.63	0.65	0.60	0.13	0.02	0.75
	SD	0.63	0.50		0.24	0.23		0.48	0.38	
IL-8 (pg/mL)	Mean	2.81	2.49	0.15	1.60	1.80	0.85	-0.33	0.20	0.10
	SD	1.43	1.52		1.42	1.39		1.30	0.69	
IL-10 (pg/mL)	Mean	3.43	4.25	0.32	1.83	2.02	0.20	0.81	0.19	0.78
	SD	1.72	2.98		0.92	0.49		2.49	0.81	
IFNγ (pg/mL)	Mean	0.40	0.60	0.06	0.20	0.32	0.10	0.21	0.12	0.89
	SD	0.22	0.41		0.24	0.35		0.49	0.37	
MCP-1 (pg/mL)	Mean	105.00	112.20	0.77	68.10	61.34	0.28	7.20	-6.76	0.39
	SD	52.47	36.23		49.13	56.06		58.77	32.71	
Cardiovascular biomarkers										
C-Reactive Protein (mg/L)	Mean	2.96	1.43	0.23	17.73	14.96	0.37	-1.53	-2.77	0.92
	SD	4.32	1.16		19.37	15.82		3.79	14.39	
Hormone biomarkers										
Ghrelin (pg/mL)	Mean	8.94	9.70	0.77	7.43	7.86	0.46	0.76	0.43	0.50
	SD	4.39	6.94		1.84	2.15		5.42	2.22	
Adiponectin (μg/mL)	Mean	19.06	21.15	0.81	15.59	12.79	0.02	2.10	-2.80	0.04
	SD	11.40	13.17		10.57	9.08		7.27	2.82	
Leptin (ng/mL)	Mean	3.31	4.42	0.01	15.97	17.75	0.82	1.12	1.77	0.14
	SD	2.58	2.94		6.24	7.32		1.66	8.98	
Resistin (ng/mL)	Mean	20.59	19.69	0.35	32.68	33.75	0.63	-0.91	1.07	0.53
	SD	5.01	1.37		15.22	21.88		4.64	11.37	
PYY (pg/mL)	Mean	23.56	24.64	0.55	15.32	22.11	0.90	1.07	6.79	0.36
	SD	15.58	12.38		10.46	9.05		17.86	13.86	
PAI-1 (ng/mL)	Mean	30.93	23.69	0.09	31.34	24.93	0.05	-7.24	-6.41	0.93
	SD	18.12	17.58		8.09	12.20		14.82	8.19	
C-Peptide (ng/mL)	Mean	0.46	0.51	0.63	0.81	1.04	0.04	0.05	0.24	0.48
	SD	0.39	0.23		0.32	0.48		0.41	0.37	
GIP (pg/mL)	Mean	4.99	9.46	0.04	10.82	9.87	0.42	4.47	-0.95	0.16
	SD	4.11	6.37		9.54	7.12		7.70	7.64	
Glucose (pg/mL)	Mean	73.16	72.06	0.26	77.57	80.51	0.73	-1.10	2.93	0.57
	SD	8.64	8.83		9.60	13.88		5.40	11.81	
Insulin (pg/mL)	Mean	318.93	303.33	0.58	485.94	506.29	0.50	-15.60	20.35	1.00
	SD	239.24	172.95		165.92	316.20		182.73	242.38	
HbA1c (mmol/mol)	Mean	12.28	11.65	0.62	17.53	14.35	0.06	-0.63	-3.17	0.26
	SD	4.49	5.07		6.09	7.04		5.29	6.23	

A Wilcoxon signed rank test for paired differences comparisons is used to compare week 0 to week 6 measurements within each group. For each variable, the difference week6-week0 is obtained for each subject. An Exact Wilcoxon Mann Whitney test is used to compare the lean group with the obese group. $p<0.05$ shows a significant difference within/between groups.

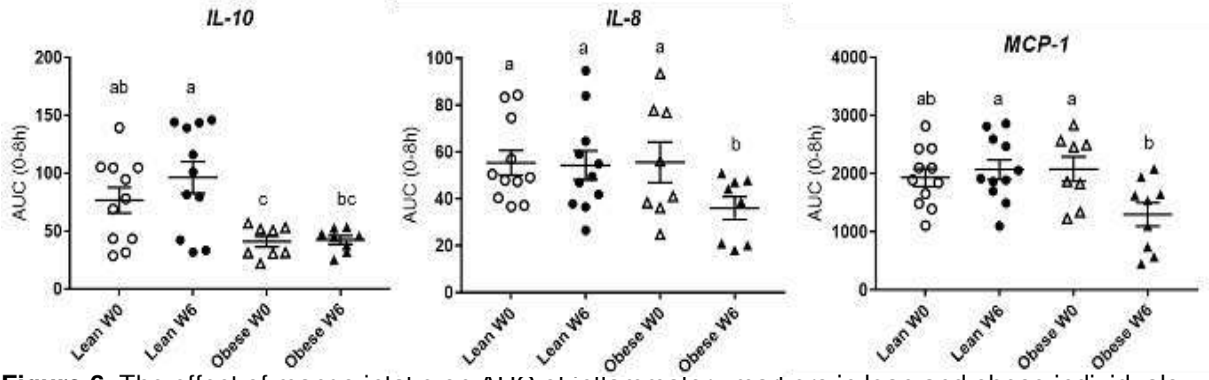


Figure 6. The effect of mango intake on AUC of inflammatory markers in lean and obese individuals.

In obese subjects, plasma concentrations (AUC0-8h) of IL-8 and MCP-1 were significantly decreased after six weeks, while there were no changes in lean subjects. The plasma concentrations (AUC0-8h) of IL-10 were significantly higher in lean subjects compared to obese subjects and correlated with the amount of metabolites found in plasma ($p=0.0218$, $r=0.38$, Spearman correlation) (**Figure 6**).

Summary:

Both gallic acid and monogalloyl glucose can produce similar amounts of the phase II metabolites, 4-*O*-methylgallic acid and 4-*O*-methylgallic acid-3-*O*-sulfate. The presence of gallic acid and pyrogallol metabolites in human urine following the consumption of 400g of Keitt mango indicates the absorption, metabolism, and excretion of mango galloyl derivatives. Colon metabolism produced five additional GA metabolites with pyrogallol-*O*-sulfate and catechol-*O*-sulfate identified as the most prevalent with significantly more excretion following 10 days of mango consumption.

The urinary excretion and plasma concentrations of phase II metabolites sourced from mango gallic acid, galloyl glycosides, and gallotannins was assessed in lean and obese subjects who consumed mango daily for 42 days, and compared against subjects who consumed mango only on Days 1 and 42. In the Lean and Obese cohorts, significant increases were found in the cumulative 24 h urinary excretion of 4-*O*-methylgallic acid-3-*O*-sulfate, methylpyrogallol-*O*-sulfate, pyrogallol-*O*-sulfate, and catechol-*O*-sulfate and were hypothesized to be due to adaptations in the subjects gut microflora. No significant differences were found in the individual plasma concentrations of these metabolites up to 8 h post-prandial. It was hypothesized that the galloyl and pyrogallol metabolites are rapidly excreted and will not accumulate in plasma. Studies investigating the mechanisms of action for galloyl phase II metabolites need to be performed to see if their bioefficacy is limited to a specific site of action or through regulation of cell signaling pathways.

Obese individuals possessed significantly lower levels of tannase-producing gut microbiota, as well as higher levels of obesity-related bacteria at the baseline than lean individuals. After 6 weeks of daily mango consumption, the levels of tannase-producers were not significantly different in obese individuals, but there was no longer a significant difference between lean and obese individuals. Additionally, inflammatory markers were

not significantly different after 6 weeks of mango consumption compared to the baseline in lean individuals. However, obese individuals showed significant decreases in hemoglobin A1c and PAI-1, associated with decreased risk of atherosclerosis and thrombosis, and inflammatory markers IL-8 and MCP-1 after 6 weeks of mango consumption compared to baseline.

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