AD_____

Award Number: W81XWH-09-1-0428

TITLE: Exploitation of Nontraditional Crop, Yacon, in Breast Cancer Prevention Using Preclinical Rat Model

PRINCIPAL INVESTIGATOR: Weiqin Jiang, M.D.

CONTRACTING ORGANIZATION: Colorado State University
Fort Collins, CO 80523

REPORT DATE: July 2011

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
01-07-2011	Final	1 JUL 2009 - 30 JUN 2011

4. TITLE AND SUBTITLE
Exploitation of Nontraditional Crop, Yacon, in Breast Cancer Prevention Using Preclinical Rat Model

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-09-1-0428

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Weiqin Jiang, M.D.

E-Mail: weiqin.jiang@colostate.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Colorado State University
Fort Collins, CO 80523

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Yacon has recently been introduced into farmer's markets and natural food stores in the US, but its preventive activity for breast cancer has rarely been evaluated. Objective are to determine the effect of dietary yacon on 1-methyl-1-nitrosourea (MNU) induced mammary carcinogenesis in rat; to evaluate the circulating factors and their association with the carcinogenesis; and to determine cellular signaling pathways – HDAC and downstream targets - AMPK/Akt-mTOR and ghrelin-IGF1 axis. Mammary carcinogenesis was initiated by injection of female rats with 50 mg MNU/kg body weight (i.p.) at 21 days of age. One week later, the rats were fed diets containing yacon powder at 0%, 15%, 30% or 60% (30 rats/group) for 8 weeks, respectively. Results showed that dietary yacon reduced the promotion and progression of MNU-induced mammary carcinogenesis in rat, which is associated with downregulation of IGF-1/HDAC/Akt/mTOR signaling pathway and anti-inflammation, i.e. reduction of plasma IL-6, TNFα, and C-reactive protein. More cytokines were analyzed during the extended period. The plasma IL-1a, IL-4, IL-12, IL-13, and IFNγ were decreased and plasma IL-5 was increased in the rats fed dietary yacon. These results indicate that anti-inflammatory effect of yacon accounts for, at least in part, the inhibitory effect of yacon on mammary carcinogenesis, which may be associated its effect on obesity that are risk factors for breast cancer. The study provided crucial biological information to complement the knowledge of natural functional foods rich in non-digestible, fermentable oligosaccharides.

15. SUBJECT TERMS
Yacon, mammary carcinogenesis and prevention, anti-inflammation

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	31	19b. TELEPHONE NUMBER *(include area code)*

Table of Contents

INTRUCTION

Yacon has recently been introduced into farmer's markets and natural food stores in the US, but its preventive activity for breast cancer has rarely been evaluated. ***Yacon*** contains a large amount of non-digestible oligosaccharide called inulin that belongs to a class of carbohydrates known as fructans [1]. Inulin-type fructans (ITF) decreases the rate of aberrant crypt foci (ACF), a pre-neoplastic lesion found in colon [2]. The mechanism by which ITF inhibits ACF is associated with ***butyrate*** produced by the anaerobic bacterial fermentation of ITF in the colon [2]. Butyrate can also be absorbed through the colonic epithelial cells into the portal blood and exert its effects within the body. Butyrate modulates gene transcription by inhibiting HDAC [2]. Cancer cells appear to be more sensitive than non-transformed cells to HDAC inhibitory compounds. In addition, the yacon ITF promotes satiety and retard the absorption of food-derived energy via reducing a gastrointestinal peptide-***ghrelin*** that is a growth hormone secretagogue [3;4]. Reduction of serum ghrelin results in decreases of GH and IGF-1 [5]. Subsequently, the PI3K/Akt-mTOR signaling pathway will be inactivated via increasing AMPK activity and its downstream events, inhibition of cell proliferation and induction of cell apoptosis will be observed. However, few studies have been shown the effect of the ITF on breast cancer. Understanding the inhibitory effect of **yacon** on the mammary carcinogenic response will provide crucial biological information to complement the knowledge of natural functional/whole food as the vehicle for delivery of health promoting chemicals in prevention of breast cancer, survivor recuration and chronic deseases. ***The hypotheses*** are that treatment with yacon will: 1) dose-dependently reduce the incidence and multiplicity of chemically-induced pre-malignant and malignant mammary tumors in rats, 2) increase butyrate and decrease ghrelin, growth hormone (GH) and insulin-like growth factor 1 (IGF-1) in the blood, 3) inhibit histone deacetylases (HDAC), and downregulate phosphatidylinositol 3-kinase (PI3K)/Akt-mammalian target of rapamycin (mTOR) signaling pathway through upregulated AMP activated kinase (AMPK) – a energy metabolic sensor, eventually decreasing cell proliperation and increasing apoptosis. **Objective are:** 1) To determine ***mammary carcinogenic responses to yacon*** in experimental animals. 2) To evaluate the association of the ***circulating mediator, gastrointestinal peptide, and growth factors*** with the carcinogenic responses in rats fed dietary **yacon**. 3) To determine the ***HDAC*** and identify AMPK/PI3K/Akt-mTOR ***cell signaling pathway*** that are downstream targets of HDAC and ghrelin-GH-IGF1 axis. Mammary carcinogenesis will be initiated by injection of female rats with 50 mg of 1-methyl-1-nitrosourea/kg body weight (i.p.) at 21 days of age. One week later, the rats will be fed diets containing yacon powder at 0%, 5%, 10% or 15% (30 rats/group) for 6 weeks, respectively. Rats will be palpated for mammary tumors and effects of **yacon** on the incidence, multiplicity and latency of mammary carcinomas will be evaluated. Plasma will be collected to determine blood glucose, insulin, leptin, butyrate, ghrelin, GH, and IGF-1. The tumor and liver tissues will be used for, but not limited to, histological evaluation and molecular biological assessments including HDAC and regulators that are associated with AMPK/PI3K/Akt-mTOR pathway in all mammary gland pathologies. Difference between diet groups will be statistically analyzed. **Yacon** can be readily introduced into the food supply since it has been in the market. The study will provide crucial biological information to complement the knowledge of natural functional foods that is relevant to a number of foods rich in non-digestible, fermentable oligosaccharides. Understanding the potential impact of such foods on the carcinogenic response could lead the way to the development a new type of functional whole food with a low calorie density and the ability to promote the intra-intestinal production of cancer inhibitory factors. This represents a novel strategy to preventing the development of breast cancer and has the potential to have a major impact on the occurrence of this disease. Consequently, the new type of functional food will significantly contribute to the goal of proventing initiation of breast cancer and reduce survivor recuration because it is natural, tolerantable and acceptable for the clinic patients and general population in aditional to benefit to obesity, diabetes II and cadiovascular diseases.

BODY

Task 1. To determine mammary carcinogenic responses to yacon in experimental animals.

a. Initiate Pre-clinical model for breast cancer in rat induced by 1-methyl-1-nitrosourea (MNU) and feed yacon in diet (Month 1-3).

One hundred twenty female Sprague Dawley rats were obtained at 20 days of age. They were injected with MNU (50 mg/kg body weight) at 21 days of age as previously described by us [6]. Following carcinogen administration, rats were randomized to one of four diet groups and 30 rats per group: AIN 93G (control) or AIN 93G supplemented with 150 g, 300 g or 600 g of yacon root powder/kg diet. Inulin concentration in the yacon is 4.66 g/100 g, which was analyzed by Warren Analytical Laboratory (Greeley, CO). The dose of 15% - 60% dietary yacon provided 0.7% - 2.8% of inulin-type fructans in diet) is about 0.5 – 2.0 g inulin-type fructans/kg body weight rat, which is the acceptable dose for human. Experimental diets were fed to the rats beginning one week post carcinogen administration in order to determine the inhibitory effect of yacon on promotion and progression of mammary carcinogenesis. All of rats were housed three per cage and weighed 1-2 times per week. At the end of study, no significant difference was observed in the final body among 4 dietary groups (ANOVA, P=0.165, see figure and table 1 in supporting data section). The average of body weight is 225, 222, 217 or 215 gram for groups of control, 15%, 30% or 60% yacon. From three weeks of post carcinogen until study termination, rats were palpated twice per week for detecting mammary tumors. A log was kept of all detected tumors and their locations. Experiment was terminated at 9 week of post carcinogen. At necropsy, the log was used to record all grossly visible tumors and to assist with confirmation of all palpable tumors. All tumors detected at necropsy were excised and weighed. One portion of excised tumors was processed for histological classification and the remainder part of each tumor was snap-frozen in liquid nitrogen and were available for the proposed mechanistic studies in Task 3. Abdominal-inguinal mammary glands from both sides of animal were excised. One side were spread out on a glass and fixed in 10% formalin and another side were spread out on a film, which was sealed in a bag and snap-frozen in liquid nitrogen for molecular analysis (in Aim 3). Liver was also excised and snap-frozen in liquid nitrogen. At the end of this experimental period, the samples for all of the Tasks were collected and stored at -80ºC for further analyses.

b. Process mammary glands and mammary tumors for the histological evaluations.

All mammary glands whole mounts were stained for further analysis. All tumors excised at necropsy were processed for histological classification. Briefly, all lesions were put in cassettes individually. The cassettes were processed through toluene and molten paraffin. Then, the lesions were embedded in paraffin and cut at 4 um sections that were placed onto glass microscope slides. Sections were heat immobilized and then stained using an H&E protocol. At the end of this experimental period, all of slides were stained and ready for further evaluation.

c. Evaluate the mammary lesions histologically and summarize the mammary carcinogenic responses to dietary yacon.

All of the tumors were stained by H&E and were histologically diagnosed under microscope. The tumors that were detectable by palpation and that were histologically confirmed to be mammary adenocarcinoma (AC) were summarized to produce the cancer incidence, multiplicity, weight and latency curves. At the end of this period, we found that palpated cancer incidence was significantly reduced by dietary yacon (Chi-square test, p=0.05) while multiplicity (palpated cancer number/rat) was not significantly different among 4 groups (Kruskal-Walls Test, p=0.247). The cancer weight was significantly lower in group of 30% or 60% yacon compared to group of control or 15% yacon (Kruskal-Walls Test, overall p=0.0254). Latency of palpated carcinomas was delayed by dietary yacon (p=0.07, ANOVA, see figure 2 and table 1 in supporting data section).

d. Mammary gland density analysis.

During extended period, the mammary gland density was analyzed. The abdominal-inguinal mammary glands chains of rats were carefully excised with emphasis on retrieving the surrounding pleural membrane and prepared as whole mounts on 75 x 50 mm glass microscope slides and fixed in 10% neutral buffered formalin. The fixed whole mounts were processed and stained with 0.4% alum carmine and its digital images were captured for the analysis of mammary gland density using a semi-automated image acquisition system. The images were evaluated for total area of the mammary gland fat pad occupied by mammary epithelium as well as total area of the fat pad encompassed by the mammary ductal tree using Image-Pro® Plus software. Area occupied by mammary epithelium divided by total area encompassed by the mammary ductal tree was calculated as mammary gland density. The mammary gland density of rats fed dietary yacon at 30% and 60% was significantly lower compared control and 15% dietary yacon rats ($p<0.001$, ANOVA, see figure 3 and table 1 in supporting data section)

Conclusion of Task-1: Dietary yacon reduced the promotion and progression of mammary carcinogenesis in rat.

Task 2. To evaluate the association of circulating mediator, gastrointestinal peptide, and growth factors with the carcinogenic responses in rats fed dietary yacon (month 7-9 & extended period).

Blood samples were collected at necropsy for the evaluation of circulating butyrate, ghrelin, IGF-1, glucose, insulin, leptin, and inflammatory factors from the study described in Task 1.

a. Determine the circulating mediator – butyrate.

Butyrate will be determined by gas chromatography. There was 6% increase in plasma butyrate of 30% dietary yacon rats compared to the control rats though the difference was not significant ($p=0.658$, t test, see table 2 in supporting data section).

b. Determine plasma gastrointestinal peptide – ghrelin.

Plasma active ghrelin was determined and no significant difference was observed among 4 groups ($p=0.753$, ANOVA, see table 2 in supporting data section).

c. Determine plasma glucose, insulin, leptin, IGF-1, and inflammatory factors - C-reactive protein, IL-6 and TNFα as well as cytokines.

Plasma glucose, insulin, leptin, and insulin-like growth factor 1 (IGF-1) and inflammatory factors were determined. Compared to control and 15% yacon groups, plasma glucose in 60% yacon group was significantly higher ($p<0.03$, Bonferroni Test). No significant difference was observed in plasma insulin among 4 groups ($p=0.755$, ANOVA). Plasma leptin was lower in 30% yacon group. But no significant difference was observed in plasma leptin among 4 groups ($p=0.097$, ANOVA). Plasma IGF-1 were significantly different (ANOVA, $p=0.05$) among 4 groups. Regression analysis $p=0.005$. Compared to the control group, the plasma level of IGF-1 was significantly decreased in 60% yacon group ($p=0.05$, Bonferroni Test). In addition, plasma C-reactive protein (CRP) and IL-6 were significantly decreased by dietary yacon ($P<0.0001$, ANOVA). Compared to control group, plasma CRP and IL-6 in 30% and 60% yacon groups were significantly reduced ($p<0.001$, Bonferroni Test). Compared to 15% yacon group, CRP in 60% yacon group was significantly reduced ($p<0.001$, Bonferroni Test). Plasma TNFα was also significantly decreased by dietary yacon ($p=0.002$, Kruskal-Wallis test). Compared to control or 15% yacon group, plasma TNFα in 30% or 60% yacon group was significantly reduced ($p<0.05$, Dunn's Test). (see figure 4A and table 2 in supporting data section). In addition, plasma IL-2, IL-10, IL-13, and IFNγ were also significantly decreased in 30% and 60% yacon groups compared either control or 15% yacon group (Kruskal-Wallis with Dunn's Test, $p<0.05$). (See figure 4B and table 2 in supporting data section)

d. Summarize the data and identify the association of all circulating factors with the carcinogenic response to dietary yacon.

At the end of this period, the effect of dietary yacon on the levels of circulating glucose, insulin, leptin, ghrelin, IGF-1 and inflammatory factors were ascertained. In addition, the association between circulating factors (glucose, insulin, leptin, ghrelin, and IGF-1) and the carcinogenic responses (the incidence and multiplicity of adenomas) were identified. As expected, the incidence, multiplicity and weight of mammary carcinomas were significantly positive-correlated to each other. These carcinogenic responses were also positive-correlated to plasma IL-6. Moreover, the carcinogenic responses were significantly negative-correlated to the latency of the carcinomas as well as to plasma glucose, IL-6 and TNFα ($p<0.001$) while no significant correlation was observed between carcinogenic responses and other circulating factors. However, significant correlations were observed among the circulating factors, which were positive correlations between insulin and IGF-1, leptin, or glucose as well as between C-reactive protein and IGF-1 or leptin ($p<0.01$), also between IL-6 and C-reactive protein or IGF-1; and negative correlations between leptin and active ghrelin ($p<0.01$). (See table 3 in supporting data section)

Conclusion of Task-2: The inhibiton is associated with downregulation of IGF-1 and systemic anti-inflammation.

Task 3. To determine the HDAC and identify cell signaling pathway that were downstream targets of HDAC and ghrelin-IGF1 axis.

a. Determine the protein levels of HDAC, PI3K, Akt, mTOR and other related regulators in PI3K/Akt-mTOR pathway.
Frozen mammary gland, mammary tumors and liver of control and 30% yacon groups collected under Task 1 were homogenized for determining the levels of HDAC, Akt, mTOR, AMPK by western blotting. It has been observed that HDAC in all of three tissues were decreased in 30% yacon group compared the control group. Significant reduction was observed in both mammary and mammary carcinomas ($p<0.05$). There was no significant difference in Akt and AMPK of three tissues between control and yacon group. However, phosphorylated mTOR and mTOR were significantly decreased in mammary carcinomas of 30% yacon group to compared with the control group ($p<0.0001$) while only mTOR was significantly reduced in mammary gland of 30% yacon group. (See figure 5 and table 4 in supporting data section)

b. Summarize the data and identify the regulations of HDAC and Akt-mTOR/AMPK pathway by HADC and ghrelin-IGF1 axis.
The current data suggest that dietary yacon inhibits HDAC and downregulates mTOR signaling pathway in mammary gland and mammary carcinomas. The changes were more obvious in the mammary tumors than in the mammary gland. In addition, the reduction of plasma IL-6, TNFα and C-reactive protein as well as IL-2, IL-10, IL-13 and IFNγ by dietary yacon suggests that yacon has impact on inflammation. Further investigation will focus on yacon anti-inflammation effect and its association with the inhibition of breast cancer in high inflammatory status, such as metabolic syndrome in women and in pre-clinical animal model.

Taken together, our data indicate that yacon shows inhibitory effect on the promotion and progression of chemically-induced mammary carcinogenesis in rat. The inhibition is associated with down regulation of IGF-1/HDAC/Akt/mTOR signaling pathway and with anti-inflammation.

Additional Task completed during extended period. To determine the effect of dietary yacon on high-fat diet induced obesity in mice and on associated Metabolomics.

a. Determine the dietary yacon on high-fat diet induced obesity in mice.

Twenty-four C57BL/6J mice, fed an obesogenic high fat diet from birth were obtained from Jackson Laboratory (Bar Harbor, Maine) at nine months of age. Mice were randomized to one of three groups and assigned to the high fat diet containing: 0% (n=8), 30% (w/w) yacon. Eight mice were assigned to a low fat diet (Research Diets formulation D12329). The experimental feeding was seven weeks. Body weight was collected and body weight curve was drawn. The data were analyzed using repeated measures ANOVA and the overall $p<0.001$. The statistically significant difference was observed among high-fat diet, low-fat dite and 30% yacon high-fat diet groups. The area under curve (AUC) was calculated. It is shown (See Figure 6 in supporting data section) that the AUC in 30% yacon group was the lowest, even lower than the low-fat diet group. It suggests that dietary yacon results in the reversal of diet-induced obesity.

b. Determine the dietary yacon on metabolomics in plasma and liver of mice.

Plasma and liver were collected from the C57BL/6J mice described above, eight samples for each group per tissue type and 48 samples were sent to Metabolon for the analysis of metabolomics. The samples were extracted and prepared for analysis using Metabolon's standard solvent extraction method. The extracted samples were split into equal parts for analysis on the GC/MS and LC/MS/MS platforms. A total of 332 named biochemicals in liver and a total of 310 named biochemicals in plasma were detected. Following log transformation and imputation with minimum observed values for each compound, Welch's two-sample *t*-tests were used to identify biochemicals that differed significantly between experimental groups. A summary of the numbers of biochemicals that achieved statistical significance ($p\leq0.05$) and those approaching significance ($0.05<p<0.1$), as well as associated Principle Component Analysis is shown in Table 5 and Figure 7 in supporting data section.

Biochemical Summary

In an age with increasing appreciation of the long-term health impacts of diet composition, this study, which explores the effects of dietary high fat intake with or without yacon as well as low fat intake on global metabolism in a mouse model, has the potential to provide significant insight into the role of diet in a variety of human diseases including metabolic syndrome, diabetes, cardiovascular disease, and cancer. Comparison of global biochemical profiles in plasma and liver under high fat (HF), low fat (LF), and 30% yacon high-fat (YCN) diet treatment regimens revealed several key metabolic differences as highlighted below.

- **Inositols and diet**: Although significantly elevated levels in HF and/or YCN were observed for some small sugars and sugar alcohols, including ribose, ribitol, and mannose, a marked, large fold of change increase was observed for scyllo-inositol in both liver and plasma in the Ycn group compared to both LF and HF groups. This inositol isomer is one of several isoforms found in most animal tissues with the glucose-derived myo-inositol isomer typically present at highest levels. Inositols, particularly as inositol phosphates, have many roles in cellular signaling pathways. It may be notable that a role for inositols in glycemic control and the normal metabolic response to insulin is supported in the literature, including myo-inositol, and an additional inositol isomer, D-chiro-inositol (DCI) as well as its metabolite pinitol (3-O-methyl-DCI), both below the limit of detection in this study. The relatively stable levels for the biosynthetic precursors to scyllo-inositol, including glucose-6-phosphate, inositol 1-phosphate, and myo-inositol, suggest that the special diet may include supplementation of one or more inositol isomers and/or facilitates improved uptake of certain small sugars including scyllo-inositol. The idea of increased uptake is supported by the significantly elevated levels in liver of methyl-alpha-glucopyranoside (methyl-D-glucoside, α-MG), a compound frequently used experimentally as a glucose analog to measure transport at the renal proximal tubule and intestine. In support of a hypothesis of a direct supply of scyllo-inositol in the special diet, is the known high content of this inositol (synonym: cocositol) in the coconut palm in conjunction with the observation in this study of relatively high levels in liver and plasma of 12-carbon and 14-carbon fatty acids, which also are singularly prevalent in

coconut palm oil, together indicating that these differences in YCN from LF and HF may support a coconut palm-derivation in the special diet formulation.

- **Lipid metabolism under distinct fat-content diet conditions**: Global biochemical profiles in plasma and liver under three diets with distinct fat content produced the somewhat surprising outcome that the HF group did not show dramatic elevations compared to the LF in either circulating or liver-specific free fatty acid levels for the common diet-supplied fats including long-chain fatty acids (LCFAs) and the essential fatty acids. Animal fat-derived dietary fats typically are most abundant in palmitate (16:0), stearate (18:0), oleate (18:1), and linoleate (18:2n6). The HF condition produced similar or lower levels for these typical dietary fats in both plasma and liver compared to the LF condition. Likewise, for the YCN group, circulating LCFA levels generally were lower, often significantly, than those observed in either the LF or HF groups. By contrast, for both HF and YCN groups, elevated levels were observed for circulating medium-chain fatty acids (MCFAs) and the shorter LCFAs, ranging from the 10-carbon caprate to the 15-carbon pentadecanoate, which frequently achieved statistical significance.

  Elevated circulating levels for MCFAs and shorter LCFAs may arise directly by dietary supply or by altered metabolism. Fatty acids of carbon chain length less than C16 are relatively rare as a direct source of dietary fats yet are abundant in some plant sources including coconut palm oil, for example, or may arise as a result of increased dietary supply of very long-chain fatty acids (VLCFAs), which are metabolized in the peroxisomes to MCFAs and short-chain fatty acids that are then transported as their carnitine-conjugates for further beta-oxidation in the mitochondria. Alternatively, elevated levels of circulating MCFAs and shorter LCFAs may be derived metabolically under conditions of perturbed mitochondrial beta-oxidation.

  Under the YCN condition in liver, MCFAs also were elevated compared to both HF and LF conditions, and in addition, several LCFAs including the essential omega-3 and omega-6 fatty acids, linolenate and linoleate, were elevated. Levels of free fatty acids in liver reflect the combined impacts of uptake from the circulation, rates of release from/incorporation into triacylglycerides and membrane phospholipids, and rates of biosynthesis and beta-oxidation. The pattern of increased liver free fatty acids, in conjunction with the decreased circulating LCFAs but elevated <C16 fatty acids with YCN, suggests that this diet condition is associated with increased uptake.

  The liver produces ketone bodies, beta-hydroxybutyrate (BHBA) and acetoacetate, from acetyl-CoA for release to the circulation. In general, ketogenesis is greatlyincreased when the rate of liver fatty acid beta-oxidation exceeds the capacity of the TCA cycle to use acetyl-CoA, frequently under conditions of nutrient deprivation and high circulating free fatty acids. In this study, both HF and YCN were associated with significantly decreased levels of circulating BHBA compared to LF. This result suggests that the subtle elevation in circulating LCFA in the LF condition relative to HF and YCN, is sufficient to drive fatty acid beta-oxidation and produce excess acetyl-CoA compared to either HF or YCN.

- **Glucose metabolism**: The liver controls circulating glucose levels in response to dietary supply and hormonal signals by regulated uptake, storage and release from glycogen, as well as biosynthesis via gluconeogenesis and release to the circulation when required. Recent work, particularly with respect to metabolic syndrome and diabetes, has supported the idea that high circulating fatty acid levels, as typically encountered with high dietary fat intake, is associated with negative impacts on glucose metabolism. Moreover, the capacity of liver and muscle to respond to insulin signals for glucose regulation and subsequent effects on circulating glucose levels is known to decrease with long-term exposure to high fat diet. Although the influence of fatty acid chain length in producing these effects is unclear, there is some evidence that dietary MCFA supplementation may not influence glucose metabolism in the same manner as LCFAs.

  In this study, HF and YCN plasma glucose levels were elevated over LF with HF achieving statistical significance. Likewise, plasma levels for 1,5-anhydroglucitol, a negatively-correlating glycemic marker corresponding to glycemic control over a time period of days to weeks, was significantly lower in both

HF and YCN, compared to LF. Together, these changes support elevated circulating glucose levels with the high fat and YCN diets.

Although liver glucose levels differed little between diet groups, elevated levels for lactate and pyruvate, as well as several intermediates shared by the glycolytic/gluconeogenic pathways, particularly 3-phosphoglycerate and 2-phosphoglycerate also were elevated significantly, or approaching significance, with HF and showed a similar trend with YCN. In liver, active gluconeogenesis despite high circulating glucose levels is one sign of insulin resistance; however, elevated levels for these metabolites also are consistent with increased rates of glucose disposal in liver via glycolysis.

Excess glucose also can be shunted in liver to the pentose phosphate pathway (PPP), sorbitol pathway, and hexosamine pathway, each of which shows increased levels in HF or YCN, or both. The PPP, which supports anabolic metabolism by the production of reducing equivalents in the form of NADPH, and ribose 5-phosphate for nucleic acid production, showed significantly higher levels for the intermediates 6-phosphogluconate and sedoheptulose-7-phosphate in YCN compared to LF, while sorbitol was elevated in both HF and YCN, compared to LF. These diet-based differences in glucose metabolic pathways suggest that in the YCN condition but not the HF condition, elevated glucose may support an increase in biosynthetic metabolism. In addition, both N-acetylglucosamine and N-acetylmannosamine, produced via hexosamine pathway metabolism, showed elevation in HF compared to LF approaching statistical significance, and significant elevation, with notably large fold of change differences, in YCN compared to both HF and LF. Hexosamine products are key to protein N-glycosylation reactions suggesting potential protein functional impacts of this heightened activity. Thus, a shared feature of both HF and YCN diets appears to be elevated circulating glucose, with excess liver glucose disposal evident along sorbitol, PPP, and hexosamine pathways.

- **Gut microbiome**: A significant impact of diet on levels of biochemicals reflecting metabolism of the gut microbiome was evident in plasma and liver and observed for several classes of metabolites. Biochemicals showing a diet-related change in liver and/or plasma levels included bile acids modified by gut bacteria as well as several amino acid derivatives.

  Bile acid metabolism: Bile acids are synthesized in the liver from cholesterol, most frequently conjugated to taurine or glycine to increase water solubility, and excreted to the intestinal lumen as bile constituents essential for proper dietary fat absorption. In the intestines, bile acids are further metabolized by resident bacteria in dehydroxylation and deconjugation reactions to produce the secondary bile acids. The liver efficiently extracts both primary and secondary bile acids from the hepatoportal circulation with only a small fraction of these metabolites escaping to the systemic circulation under normal conditions. In this study, the YCN condition was associated with a distinct plasma bile acid signature that likely arose by a combination of taurine depletion and altered gut bacterial activity. Thus, circulating levels of all taurine-conjugated bile acids, including the primary bile acids taurocholate and tauro-beta-muricholate, and the secondary bile acids tauroursodeoxycholate and taurodeoxycholate were observed at lower levels in YCN plasma compared to LF and frequently also compared to HF. By contrast, the remaining bile acids which likely are all representative of bacterial activity, were observed at significantly higher levels in YCN plasma compared to both LF and HF. The lone exception to this pattern was chenodeoxycholate, which was observed at significantly lower levels in YCN compared to LF and HF.

  Amino acid metabolites: Multiple amino acid metabolites reflecting activity of the gut microbiome contribute to host metabolic pathways and/or must be metabolized further by the liver, sulfation for example, for renal excretion. In this study, evidence for altered gut microbial activity with diet was provided by metabolites for several amino acids, which could reflect increased dietary access to these amino acids. Among gut bacterial metabolites with highest levels observed under the YCN condition were bacteria-specific metabolites of phenylalanine and tyrosine, including phenylacetylglycine and phenol sulfate, the tryptophan metabolite 3-indoxyl-sulfate, the lysine metabolite pipecolate, and the carnitine metabolite 3-dehydrocarnitine. Overall the changes in the gut microbiome could be either

altered metabolism within a relatively static population or could reflect changes in the species distributions.

In summary, plasma and liver from mice under 3 dietary regimens differing showed distinct global metabolic profiles. Key differences were observed for lipid metabolism, with strongest differences observed for levels of medium-chain fatty acids which could arise either by dietary supply or metabolic differences, for glucose metabolism, and for markers of metabolic activity of the gut microbiome. For several of the impacts observed in the current study and for future studies as well, alternative explanations could be resolved by metadata concerning the diet content. Future studies also may benefit from analysis of changes over distinct time-frames of exposure to the different diets.

KEY RESEARCH ACCOMPLISHMENTS

- Dietary yacon reduced the promotion and progression of mammary carcinogenesis in rat.
- The inhibiton is associated with downregulation of IGF1/HDAC/Akt/mTOR signaling pathway and with systemic anti-inflammation, as well as reversal of obesisy.
- Yacon diet associated metabolomic changes may account for, in part, its effects on cancer inhibition and anti-inflammation, as well as reversal of obesisy. The precise mechanisma need to be investigated in future.

REPORTABLE OUTCOMES

- Presentation-1: Some of the current results have been presentated at 9th Annual AACR International Conference on Frontiers in Cancer Prevention Research (November 7-10, 2010, Pennsylvania Convention Center, Philadelpha, PA).
- Presentation-2: All of main results will be presentated at "The sixth Era of Hope Conference" held at the Orlando World Center Marriott in Orlando, Florida, on August 2–5, 2011.
- Paper-1: A manuscript titled "Effect of Dietary Yacon on Promotion and Progression of Cheminally-induced Mammary Carcinogenesis in Rat" is going to submit to Cancer Prevention Research.
- Paper-2: A manuscript titled "Effect of Dietary Yacon on Metabolimic Profile in Obese Mice" will be submitted to British Journal of Nutrition.

CONCLUSION

Results showed that dietary Yacon can potentially reduce the promotion and progression of chemically-induced mammary carcinogenesis in rat, which may be associated with downregulation of IGF1/HDAC/Akt/mTOR signaling pathway and with systemic anti-inflammation, as well as reversal of obesisy. Unique metabolomic profiles in liver and plasma of diet-induced obesity mice fed 30% yacon high fat diet provided a valuable field to investigate in the future.

REFERENCES

1. Roberfroid,M.B. (2005) Introducing inulin-type fructans. *Br.J.Nutr.*, **93 Suppl 1**, S13-S25.
2. Pool-Zobel,B., van,L.J., Rowland,I., and Roberfroid,M.B. (2002) Experimental evidences on the potential of prebiotic fructans to reduce the risk of colon cancer. *Br.J.Nutr.*, **87 Suppl 2**, S273-S281.
3. Gale,S.M., Castracane,V.D., and Mantzoros,C.S. (2004) Energy homeostasis, obesity and eating disorders: recent advances in endocrinology. *J.Nutr.*, **134**, 295-298.
4. Geyer,M., Manrique,I., Degen,L., and Beglinger,C. (2008) Effect of yacon (Smallanthus sonchifolius) on colonic transit time in healthy volunteers. *Digestion*, **78**, 30-33.
5. Kojima,M., Hosoda,H., and Kangawa,K. (2001) Purification and distribution of ghrelin: the natural endogenous ligand for the growth hormone secretagogue receptor. *Horm.Res.*, **56 Suppl 1**, 93-97.
6. Zhu, Z., Haegele, AD, Thompson, HJ. (1997) Effect of caloric restriction on pre-malignant and malignant stages of mammary carcinogenesis. Carcinogenesis, 18: 1007-1012.

APPENDICES: N/A

SUPPORTING DATA

Figure 1. Final body weight (BW). The values are mean and error bars are SEM. Dietary yacon had no effect on the final body weight.

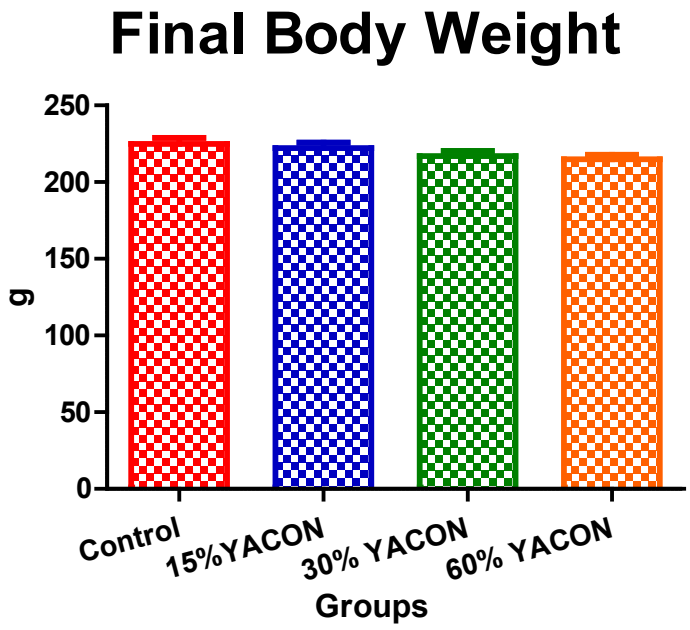
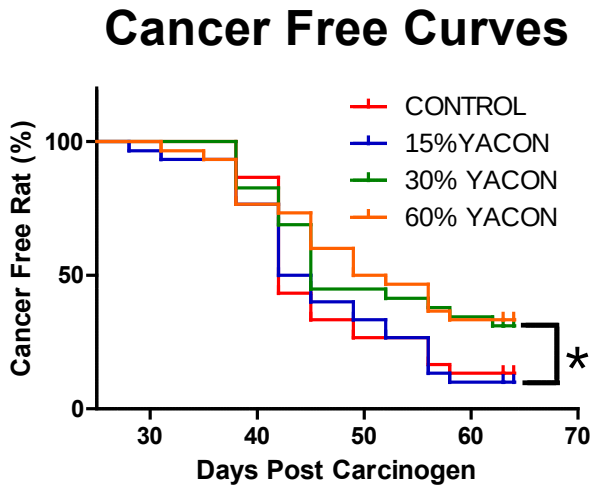
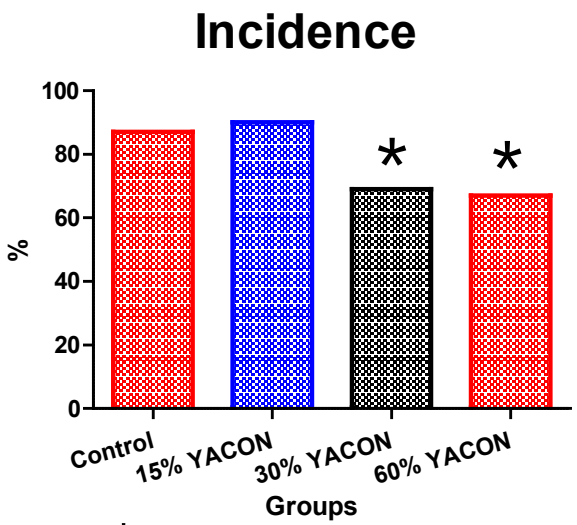


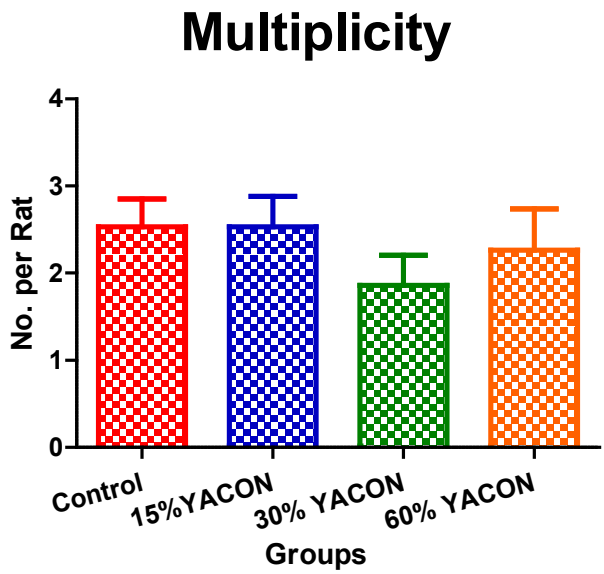
Figure 2. Carcinogenic Responses. Dietary yacon (>=30%) significantly inhibited mammary cancers in rats.

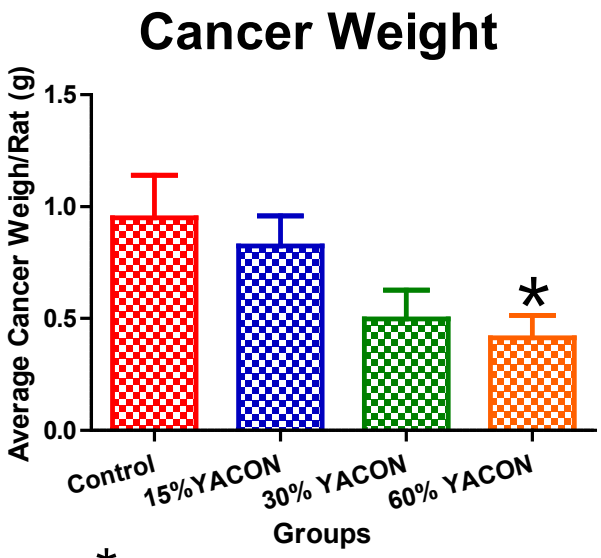


*Log-rank trend, p=0.025



* Comparison to control, p<0.05





* Comparison to control, p<0.05

Figure 3. Effects of Dietary on Mammary Gland Density. Dietary yacon at 30% or 60% significanly reduced mammary gland density of rats compared to the control and 15% dietary yacon.

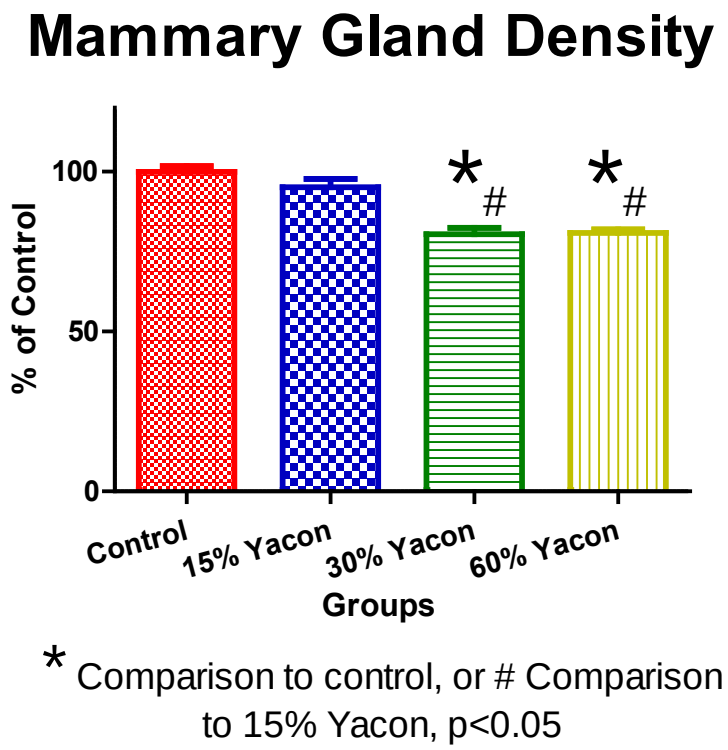
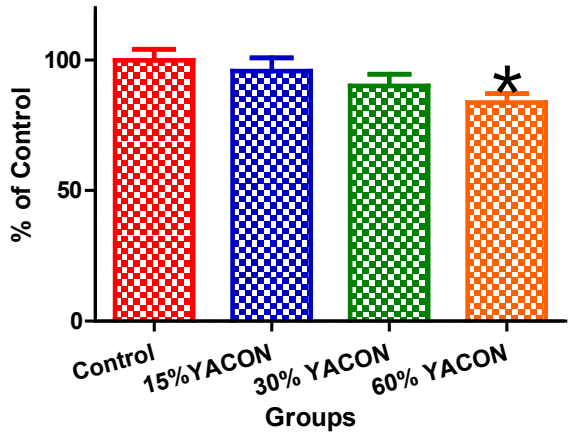
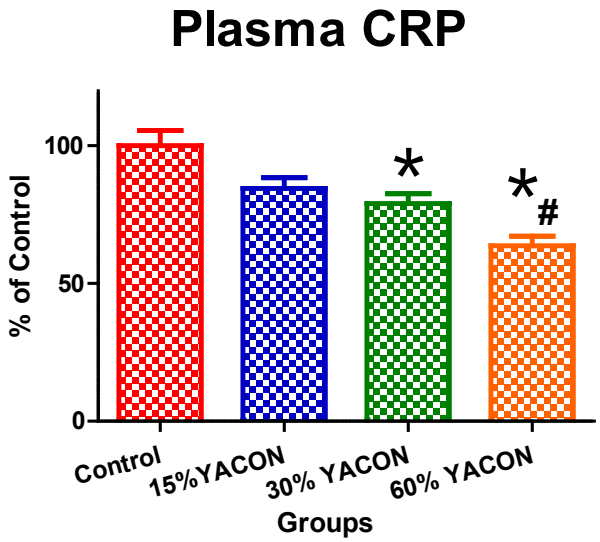


Figure 4. Circulating Analytes. Dietary yacon (>=30%) significantly reduced plasma IGF-1, C-reactive protein (CRP), IL-6 and TNFα.

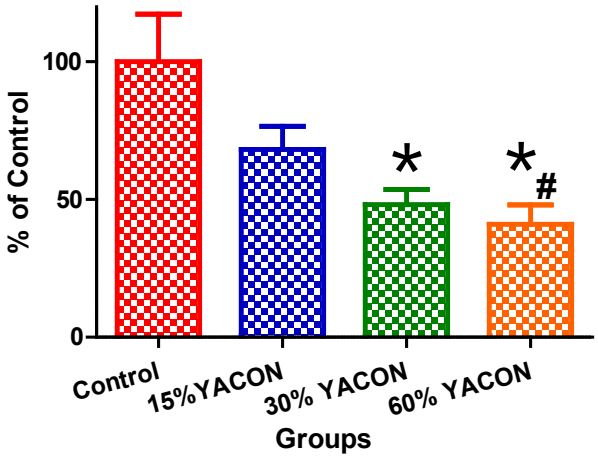
Pannel A



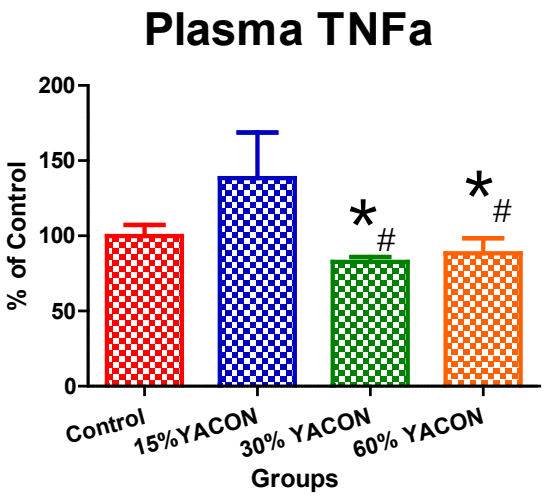
ANOVA, p=0.05
* Comparison to control, p<0.05



* Comparison to control, or # comparison to 15% YACON, p<0.05

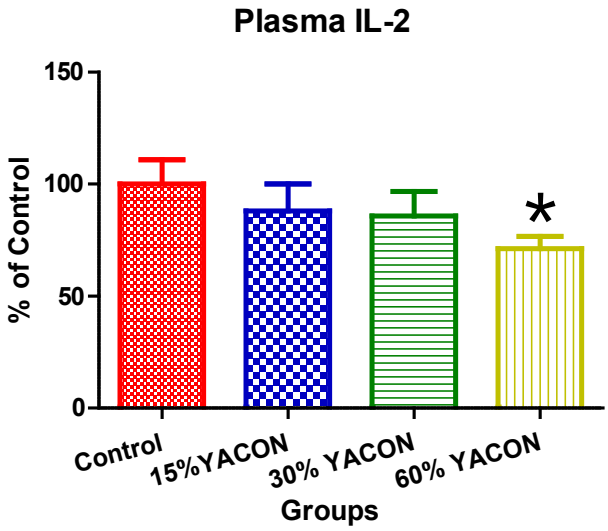


* Comparison to control, or # comparison to 15% YACON, p<0.05

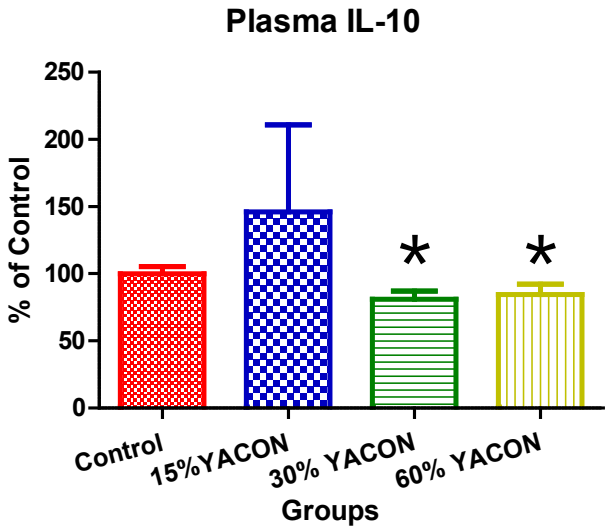


* Comparison to control, or # comparison to 15% YACON, p<0.05

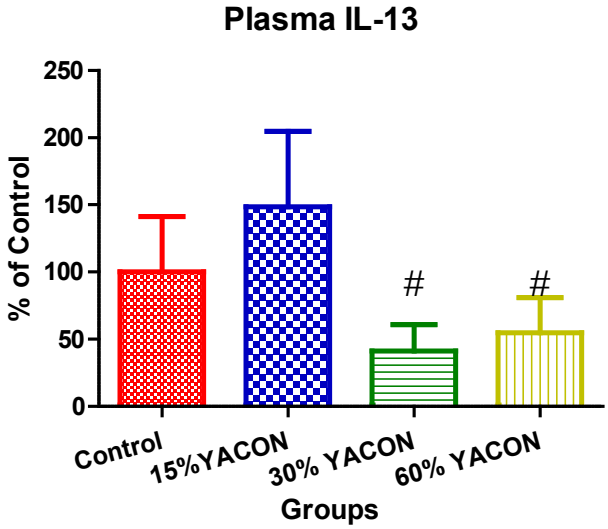
Pannel B



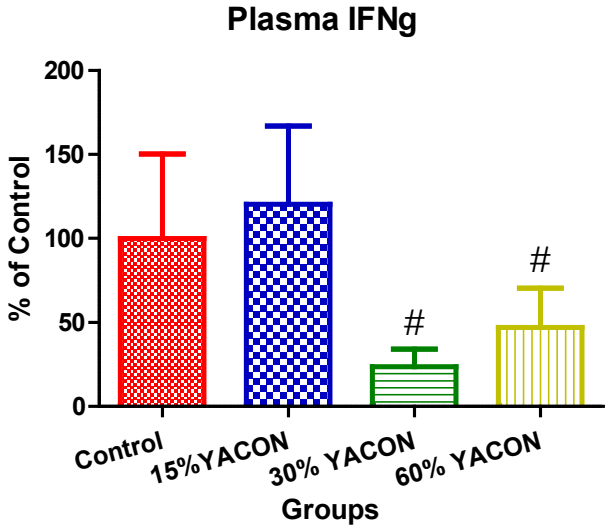
* Comparison to control, p<0.05



* Comparison to control, p<0.05

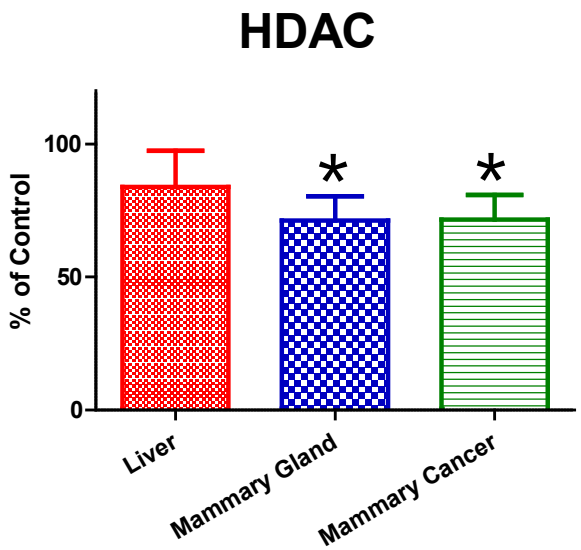


#Comparison to 15% YACON, p<0.05

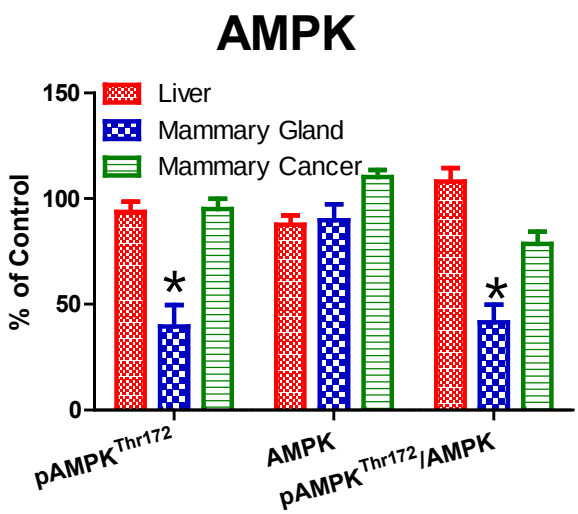


#Comparison to 15% YACON, p<0.05

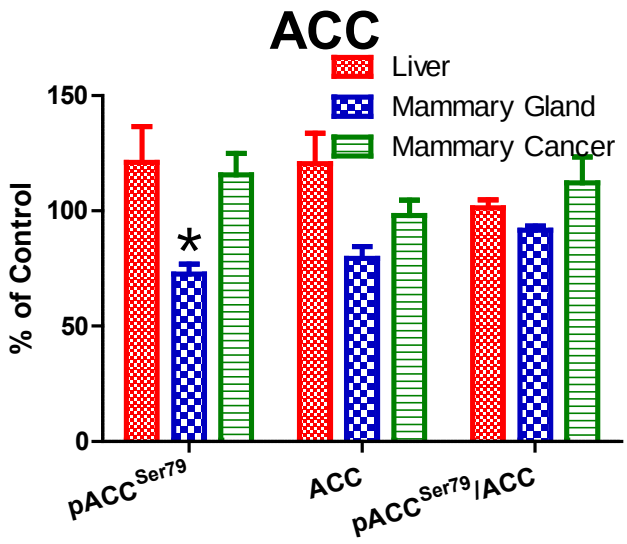
Figure 5. Western Blotting. Dietary yacon (>=30%) significantly reduced histone deacetylase (HDAC) in mammary gland and carcinomas and mTOR in mammary carcinomas.



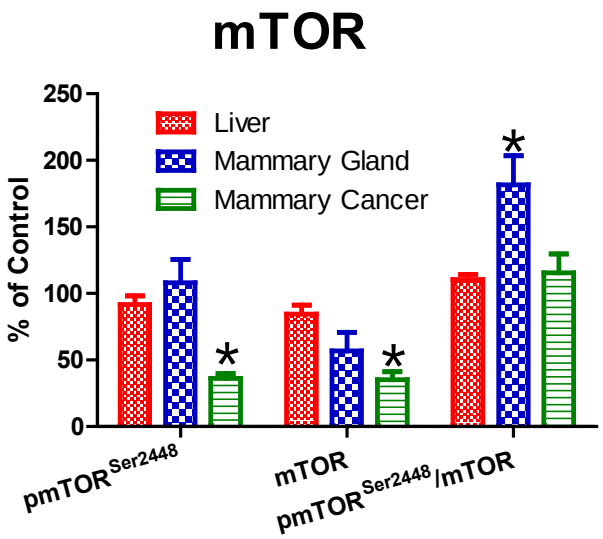
* Comparison to control, p<0.05



* Comparison to control, p<0.05

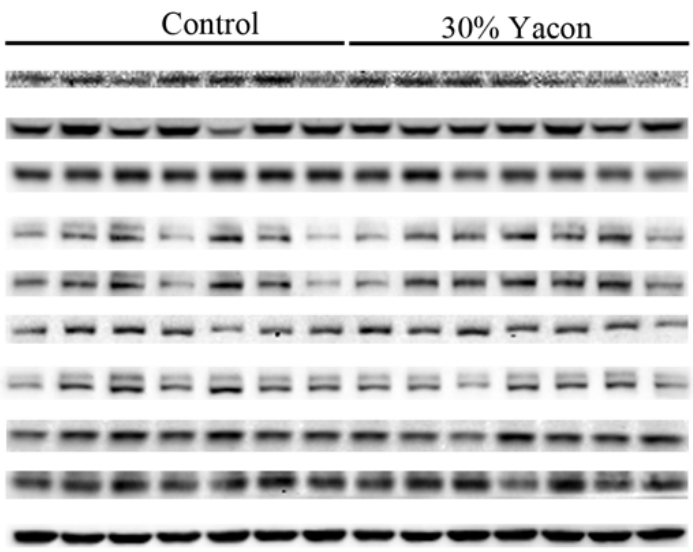


* Comparison to control, p<0.05

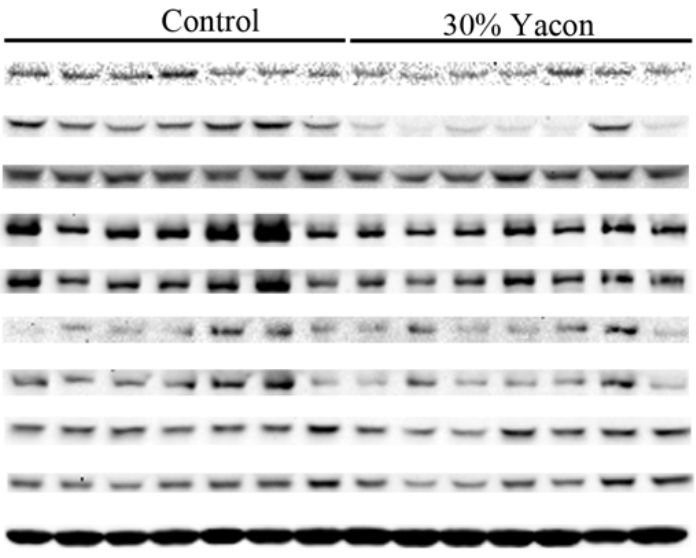


* Comparison to control, p<0.05

Apnel A. Liver



Apnel B. Mammary Gland



Apnel C. Mammary Carcinomas

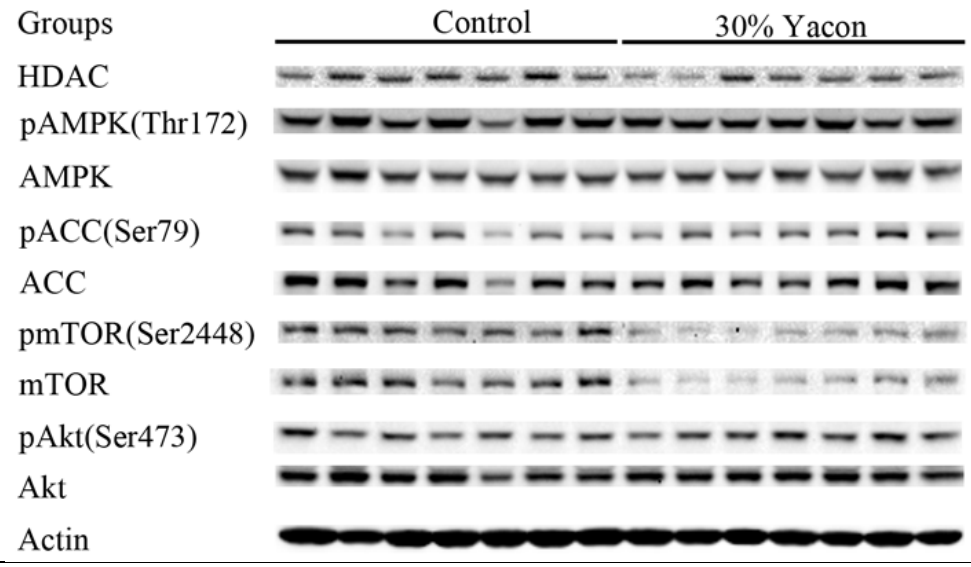


Figure 6. Effect of Dietary Yacon on Diet-Induced Obesity in Mice. C57BL/6J mice (Jackson Labs) were fed either higher fat diet, high fat diet containing 30% yacon, or low fat diet for 7 weeks. Body weight was collected and body weight curve was drawn. The area under curve (AUC) was calculated. It is shown that the AUC in 30% yacon group was reduced similar to the low fat diet group. It suggests that dietary yacon results in the reversal of diet-induced obesity.

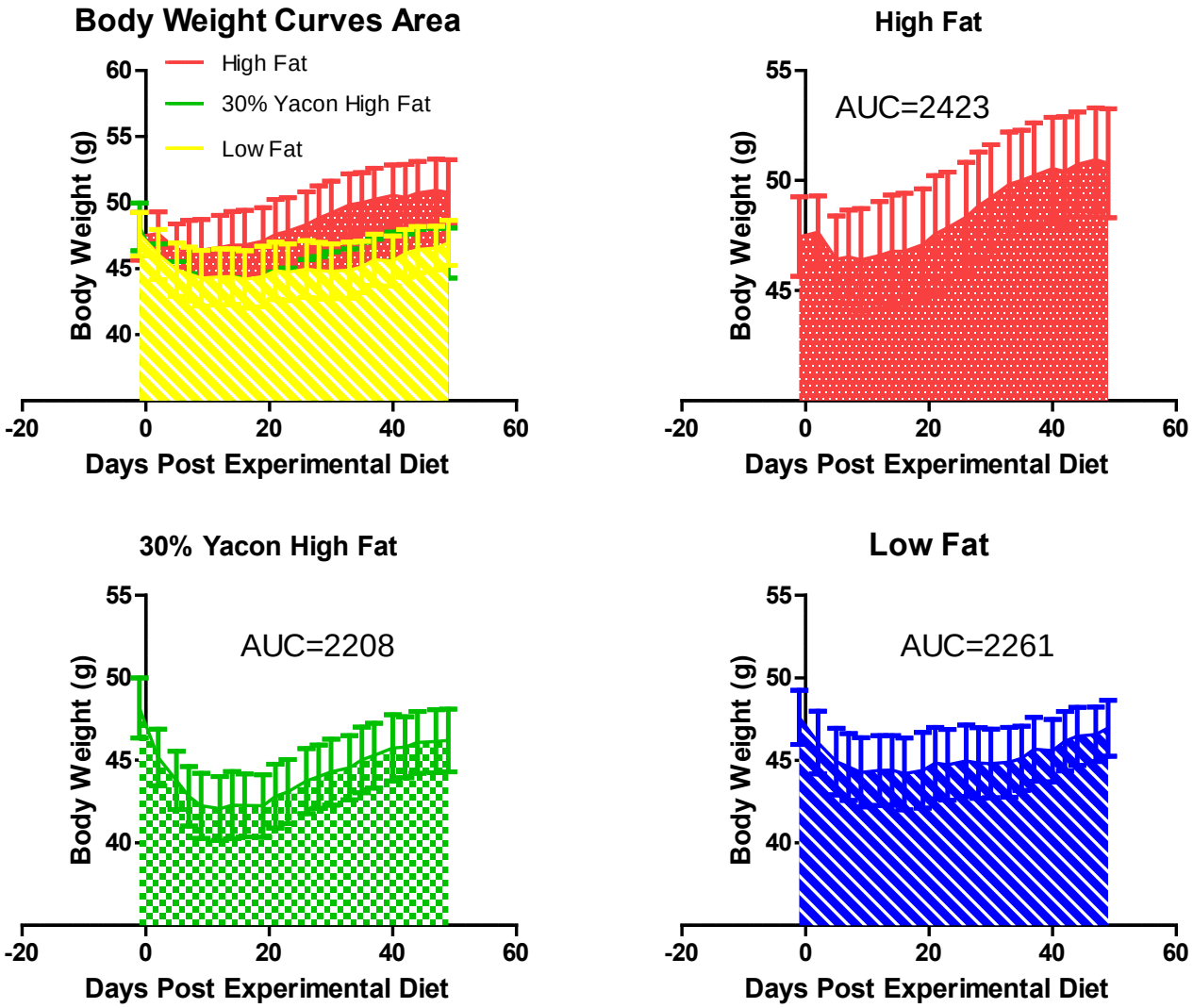


Figure 7. Effect of Dietary Yacon on metabolomics in liver and plasma of Diet-Induced Obesity Mice. C57BL/6J mice (Jackson Labs) were fed either higher fat diet, high fat diet containing 30% yacon, or low fat diet for 7 weeks. Principle Component Analysis (PCA) showed a seperation of metabolomic profile in either liver or plasma of mice fed three types of diet, especially between the yacon diet and high/low fat diet.

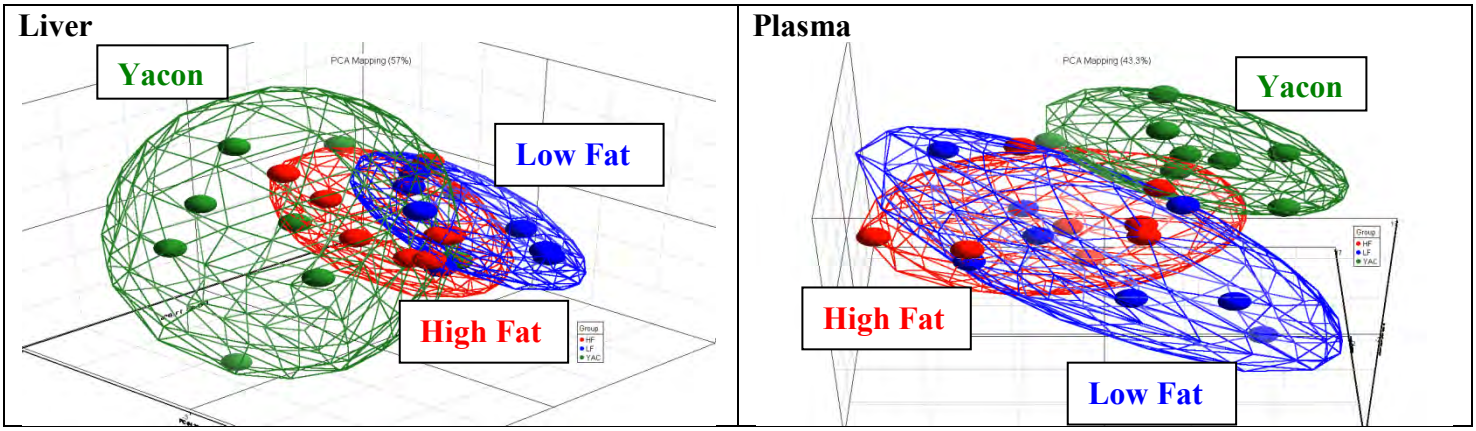


Table 1. Effect of Dietary Yacon on Final Body Weight and Mammary Carcinogenic Responses to Chemical Carcinogen[1]

	Control	15% Yacon	30% Yacon	60% Yacon	Overall P Value
Body Weight (g)	**225 ± 4**	**222 ± 4**	**217 ± 3**	**215 ± 3**	**>0.05**
Cancer Incidence (%)	**87%[a]**	**90%[a]**	**69%[b]**	**67%[b]**	**0.05**
Cancer Multiplicity (No. carcinomas/Rat)	**2.53 ± 0.32**	**2.53 ± 0.35**	**1.86 ± 0.34**	**2.27 ± 0.47**	**>0.05**
Cancer Burden (Avg. cancer mass/rat (g))	**3.58 ± 0.74[a]**	**3.31 ± 0.65[a]**	**1.55 ± 0.37[b]**	**2.12 ± 0.64[b]**	**0.025**
Cancer Latency (day)	**47 ± 2**	**46 ± 2**	**51 ± 2**	**52 ± 2**	**0.07**
Mammary Gland Density (%)	**38.5 ± 0.7[a]**	**36.6 ± 1.0[a]**	**31.0 ± 0.7[b]**	**31.1 ± 0.4[b]**	**<0.001**

[1]Values are mean ± SEM except cancer incidence. Differences among four groups were analyzed by Chi-square test (cancer incidence), ANOVA with post hoc comparisons by the method of Bonferroni (body weight, cancer latency and square root cancers counts per rat) or Kruskal-Wallis test with post hoc comparisons by the method of Dunn's test (cancer burden). Significant difference among groups is indicated by different superscript (a or b).

Table 2. Circulating Analytes[1]

	Control	15% Yacon	30% Yacon	60% Yacon	Overall P Value
Glucose (mg/dl)	85 ± 4[a]	86 ± 4[a]	90 ± 3[a,b]	101 ± 4[b]	0.009
IGF-1 (ng/ml)	205 ± 9[a]	196 ± 10[a,b]	185 ± 9[a,b]	171 ± 7[b]	0.05
Insulin (pg/ml)	603 ± 48	598 ± 73	595 ± 60	526 ± 44	0.755
Leptin (pg/ml)	485 ± 71	524 ± 113	298 ± 49	347 ± 63	0.097
Active Ghrelin (pg/ml)	240 ± 46	247 ± 44	259 ± 49	311 ± 60	0.753
C-reactive Protein (ug/ml)	497 ± 28[a]	420 ± 19[a,b]	393 ± 18[b]	317 ± 17[c]	<0.0001
IL-6 (pg/ml)	185 ± 32[a]	126 ± 16[a,b]	89 ± 10[b,c]	76 ± 13[c]	<0.0001
TNFa (pg/ml)	9.2 ± 0.8[a]	13 ± 3[a]	7.6 ± 0.3[b]	8.2 ± 0.9[b]	0.001
GM-CSF (pg/ml)	198 ± 13	175 ± 12	178 ± 14	166 ± 14	0.3499
IL-1a (pg/ml)	1919 ± 608	1602 ± 452	877 ± 291	1023 ± 410	0.0912
IL-1b (pg/ml)	8.8 ± 0.6	8.3 ± 0.8	7.4 ± 0.5	7.7 ± 0.4	0.4037
IL-2 (pg/ml)	121 ± 13[a]	107 ± 15[a,b]	104 ± 13[a,b]	86 ± 7[b]	0.2166
IL-4 (pg/ml)	198 ± 99	120 ± 35	62 ± 23	72 ± 36	0.1427
IL-5 (pg/ml)	14 ± 2	13 ± 1	26 ± 12	29 ± 9	0.6294
IL-10 (pg/ml)	121 ± 6[a]	176 ± 78[a,b]	98 ± 7[b]	102 ± 10[b]	0.0306
IL-12 (pg/ml)	708 ± 296	245 ± 54	160 ± 48	257 ± 159	0.0591
IL-13 (pg/ml)	946 ± 389[a,b]	1404 ± 531[a]	389 ± 188[b]	517 ± 250[b]	0.0375
IL-17 (pg/ml)	17 ± 2	17 ± 3	14 ± 1	13 ± 1	0.5794
IL-18 (pg/ml)	65 ± 9[a]	87 ± 32[a,b]	55 ± 9[a,b]	43 ± 4[b]	0.1968
IFNγ (pg/ml)	3188 ± 1640[a,b]	3842 ± 1482[a]	750 ± 338[b]	1503 ± 743[b]	0.0851
VEGF (pg/ml)	51 ± 2	53 ± 5	51 ± 2	53 ± 3	0.919
Butyrate (umol/l)	1.10 ± 0.09	N/A	1.17 ± 0.11	N/A	0.658

[1]Values are mean ± SEM except cancer incidence. Differences among four groups were analyzed by ANOVA with post hoc comparisons by the method of Bonferroni (glucose, IGF-1, insulin, leptin, ghrelin, C-reactive protein IL-6, butyrate and VEGF) or Kruskal-Wallis test with post hoc comparisons by the

method of Dunn's test (TNFα, IL-1a, IL-1b, IL-2, IL-4, IL-5, IL-10, IL-12, IL-13, IL-17, IL-18, and IFNγ). Significant difference among groups is indicated by different superscript (a, b or c).

Table 3. Correlations among Mammary Carcinogenic Responses and Circulating Factors[1]

	Cancer Incidence	Cancer No./Rat	Cancer Weight	Cancer Lantency	Glucose	IGF-1	Leptin	IL-6	TNFα	CRP
Cancer Incidence	1.00									
Cancer No./Rat	0.73*	1.00								
Cancer Weight	0.72*	0.84*	1.00							
Cancer Lantency	-0.72*	-0.74*	-0.83*	1.00						
Glucose	-0.21*	-0.10	-0.08	0.19*	1.00					
IGF-1	-0.05	-0.18	-0.20*	0.19*	0.12	1.00				
Leptin	0.07	-0.03	-0.01	-0.12	0.14	0.13	1.00			
IL-6	0.24*	0.34*	0.27*	-0.27*	-0.12	-0.01	0.16	1.00		
TNFα	0.15	0.12	0.28*	-0.23*	-0.15	-0.02	-0.04	0.23*	1.00	
CRP	0.003	0.04	0.05	-0.01	-0.16	0.24*	0.22*	0.14	0.04	1.00

[1]Values are correlations. *$p<0.05$.

Table 4. Effects of Dietary Yacon on Cellular Signaling Pathways[1]

Tissues	Liver		Mammary Gland		Mammary Carcinomas	
Groups	Control	30% Yacon	Control	30% Yacon	Control	30% Yacon
HDAC	100	92	100	80*	100	74*
$pAMPK^{Thr172}$	100	113	100	39*	100	114
AMPK	100	88*	100	91	100	128
pAMPK/AMPK	100	133	100	44*	100	96
$pACC^{Ser79}$	100	146	100	76*	100	130
ACC	100	139	100	84	100	110
pACC/ACC	100	103	100	92*	100	135
$pmTOR^{Ser2448}$	100	96	100	140	100	37*
mTOR	100	89	100	63	100	36*
pmTOR/mTOR	100	110	100	330*	100	118
$pAkt^{Ser473}$	100	101	100	101	100	130
Akt	100	102	100	94	100	112
pAkt/Akt	100	106	100	105	100	120

[1]Values are the percent of control. Differences among four groups were analyzed by ANOVA with post hoc comparisons by the method of Bonferroni. Compared to the control group, *$p<0.05$.

Table 5. Effects of Dietary Yacon on Metabolomics in Plasma and Liver of Mice*

Welch's Two Sample t-Tests	Plasma			Liver		
	HF/LF	Ycn/HF	Ycn/LF	HF/LF	Ycn/HF	Ycn/LF
Total number of biochemicals with $p \leq 0.05$	**32**	**55**	**65**	**38**	**62**	**104**
Biochemicals (↑|↓)	**23|9**	**30|25**	**45|20**	**29|9**	**50|12**	**88|16**
Total number of biochemicals with $0.05 < p < 0.10$	17	27	23	27	51	32
Biochemicals (↑|↓)	11|6	14|13	11|12	25|2	34|17	31|1

*HF: high-fat diet; LF: low-fat diet; Ycn: 30% yacon diet.