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The model system C. elegans demonstrates the health benefits of legumes and the potential benefits of legume consumption

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THE MODEL SYSTEM *C. ELEGANS* DEMONSTRATES THE HEALTH BENEFITS OF LEGUMES AND THE POTENTIAL BENEFITS OF LEGUME CONSUMPTION

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Dedication

I would like to dedicate this thesis to the people I love most in the world: my family and my fiancé. My family's love and support encouraged my young interest in food science and supported me throughout my undergraduate study. Their encouragement helped me to realize at a young age my passion for food and later helped me realize my talent in academia. My wonderful fiancé, Sean, encouraged me to leave behind all that was familiar to me in Texas and encouraged me to pursue my dream of becoming a food scientist even at the cost of spending our engagement in two different states and many weekend car trips back and forth. This thesis is dedicated with much love to my family and my future husband.

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List of Abbreviations

(AI) Acceptable Intake

(*C. elegans*) *Caenorhabditis elegans*

(CHD) Coronary heart disease

(dH_2O) deionized water

(DRI) Daily Reference Intake

(E. coli OP50) *Escherichia coli* OP50

(HDL) High Density Lipoprotein

(LDL) Low Density Lipoprotein

(NGM) Nematode Growth Media

(NHANES III) National Health and Nutrition Examination Survey

(NSLP) National School Lunch Program

(PBS) Phosphate Buffered Saline

(RPI) Reticulocyte Production Index

(RPM) Revolutions per Minute

(STZ) Streptozotocin

(VLDL) Very Low Density Lipoprotein

(UV) Ultraviolet

Abstract

Legumes are high in protein and are a good source of fiber and folate. They contain beneficial oligosaccharides, plant sterols and phenolics. The purpose of this study was to demonstrate the health benefits of legumes using the model system *Caenorhabditis elegans* (*C. elegans*). Nine legumes (black beans, cranberry beans, dark red beans, great northern beans, large lima beans, lentils, light red kidney beans, navy beans and white kidney beans) were tested in the *C. elegans* model system. The various legume samples were ground using a centrifugal mill to 0.75mm, suspended in water (1% w/v) solution and autoclaved. *C. elegans* were prepared using an age synchronized design. The laboratory standard food source *Escherichia coli* OP50 (*E. coli* OP50) was used as a control diet after being UV treated. Within the first week of life, the *C. elegans* received the first legume treatment of 20%, 33.3% and 50% legume material with the remaining amount being *E. coli* OP50. Pharyngeal pumping rate, a surrogate marker of aging, was counted manually throughout the study. Travel distance data was collected using imaging software. Nile red fluorescence, a marker of fat deposition, was measured using fluorescence microscopy. Pharyngeal pumping rate in the 50% legume diet group was significantly higher than in the control group. The following legumes had a significant increase in pumping rate throughout the study compared to the control: great northern beans, cranberry, lentil and dark red kidney beans. Fat deposition was decreased in *C. elegans* when fed black and navy bean diets. Travel distance was not significantly different between treatment and control groups. The results suggested the benefits of sustained lifespan and decreased fat deposition in *C. elegans* when fed a legume diet.

The use of legumes in consumer products was evaluated in this thesis. A consumer study was conducted at a local middle school to determine the sensory attributes that are important to consumers.

It was determined that strong flavors and seasonings were important to students and hamburgers are acceptable to students that are not 100% beef.

Introduction

In the United States, it is projected that healthcare costs related to obesity will reach $860-$956 billion by the year 2030. It is also projected that 86.3% of adults will be classified as overweight and 51.1% will be classified as obese.[1] The most alarming and potentially devastating effects of obesity are seen in children; obesity in the 10-14y age category is the highest predictor of obesity in young adulthood.[2] As the nation's obesity rate rises, a search for strategies to control the obesity epidemic becomes more urgent. A number of obesity interventions in schools have been attempted including decreasing television viewing, decreasing high fat food consumption, increasing fruit and vegetable intake, increasing moderate and vigorous exercise, and classroom health education.[3-6] A more comprehensive intervention involving a broad redesign of foods offered in the National School Lunch Program (NSLP) as well as increased physical activity and health education is needed. The NSLP is an ideal target because children spend a majority of their time in school and many are being fed at least one meal a day by the NSLP which provided over 5 billion lunches served in 2009.[7]

The consumption of legumes has been dated back to as far as 5500BC and they are thought to be one of the first crops cultivated by man.[8] Legumes are good sources of protein, thiamin, iron, magnesium, and zinc; they are also high in dietary fiber and folate. They are low in fat and are a rich in complex carbohydrates.[9]

The consumption of legumes has been correlated with lower body weight in isocaloric diets. In a diet intervention study, participants were fed an isocaloric diet including 101g legumes per day for 21d. Weight was significantly decreased from 76.3kg to 75.3kg ($p<0.0125$).[10] In an epidemiological study on the correlation of legume consumption to health outcomes, it was demonstrated that legume

consumers had a lower body weight and waist circumference than those who did not consume legumes. Body weight in consumers was significantly less than non consumers 77.1±1.2kg and 80.4±0.3kg ($p<0.004$), respectively; legume consumers also had a significantly reduced waist size than non consumers 93.9±1.1cm and 96.0±0.3cm ($p=0.041$), respectively.[11]

Legumes are also correlated to lower rates of coronary heart disease[12-13] which is postulated to be a result of lowering low density lipoprotein (LDL) cholesterol and raising high density lipoprotein (HDL) cholesterol. Legumes have been shown to protect against metabolic syndrome and diabetes by reducing the glycemic load of a meal and providing slowly digestible carbohydrates.[14] The nutritional benefits of legumes in humans are based on the following physiological principles: slow digestion of legume proteins and fibers[15], gut fermentation of oligosaccharides and resistant starches[16-17], decreased absorption of dietary cholesterol due to plant sterols[18], lowered insulin response and increased insulin sensitivity.[19] Legumes are versatile food ingredients that are cost effective sources of protein and other components that are beneficial for health.[8]

Caenorhabditis elegans (C. elegans) are microscopic nematodes that are widely used as a model organism in the biological sciences. The organism is a useful model because of the short life span (approximately 18-21 days), the model is inexpensive, and the entire genome has been sequenced. Recently it has been used extensively for metabolic and nutrigenomic studies.[20] Key evolutionary pathways are conserved in *C. elegans*, and two thirds of genes involved in human diseases (such as Alzheimer's, Parkinson's and Huntington's disease) have homologues in *C. elegans*.[21] Most of the key regulatory pathways involved in energy homeostasis are similar to mammalian pathways. *C. elegans* have an insulin-like pathway that regulates glycogenesis, lipogenesis, and lipid homeostasis; a disruption in this pathway can result in a disturbance in longevity, reproductive development and

metabolism.[22] The release of serotonin in *C. elegans* controls lipid deposition and feeding behavior. The *C. elegans* model is a useful tool in understanding the basic biochemistry of nutrient interactions and obesity.[23]

There is limited research on the impact of whole foods or ingredients on longevity and fat deposition in *C. elegans*. The objectives of this study were to develop and implement a *C. elegans* based model to assess the influence of diets containing legumes on fat deposition, activity rate, and lifespan. These results in *C. elegans* have the potential to improve the understanding of the interactions between diet and health in humans.

The *C. elegans* model has the potential to be used as a screening tool to select food ingredients for use in nutraceuticals. In this thesis the *C. elegans* model for assessing food ingredients was developed and the system was demonstrated by evaluating 9 different legumes.

After selecting the legumes with the most health benefits in *C. elegans,* these legumes will be incorporated into different value and nutrition added products in future studies. It is the goal of the program to establish technology to produce extended ground meat products with at least 42.5% legume replacement of ground meat for application in the NSLP. The formulation of ground meat and bean patties will help address the question: "Can we improve the diets of fourth graders participating in the NSLP by introducing a nutritionally superior meat patty?" The food technology application will be demonstrated through sensory evaluation on the meat and bean patties in a middle school setting.

Literature Review

C. elegans

C. elegans have been used widely as a model organism since their initial use in research by Sydney Brenner in 1974; his work with *C. elegans* won him a Nobel Prize in 2002. The entire *C. elegans* genome has been sequenced[24] and the model has been used extensively. The model is favored for the organisms' short life span (18-21 days), the ability to grow the organisms in large populations, the low cost of maintaining and conducting research, and little concern of animal rights issues.

The *C. elegans* model was initially developed to study neural development and genetics.[25] Recently, the model has been used as a high throughput screening method for drug development and bioactive compunds[26], a model for the biology of obesity[23], as a model to study aging and age related diseases[21], and to assess the role of food compounds of lifespan.[27]

One of the most common applications of the *C. elegans* organism is the diet restriction model. Studies have shown that reducing the amount of nutrients available to the *C. elegans* organisms results in extended lifespan as well as slow development and reduced fecundity.[28-29]

The *C. elegans* model is ideal to study the role of food compounds on health because many of the key evolutionary pathways that regulate lipid and carbohydrate metabolism are conserved.[22-23]

Legumes in Clinical Trials

Clinical trials have demonstrated that legumes consumption reduces LDL cholesterol, reduces weight and reduces total cholesterol.[13, 30-35] (Table 1) In a 1997 publication, 9 male subjects 41-78y with BMI values of 22.1 to 38.9 were held in a metabolic ward for two six-week periods. A crossover study

design was used and LDL, VLDL, HDL and total cholesterol values were obtained throughout the study. LDL cholesterol was significantly reduced ($p=0.003$) with legume consumption of 120g/d. Other macronutrients were kept constant.[30] In a study with free living men and women 20-70y with fasting total cholesterol levels between 200-260mg/dL, subjects were fed 1/2c navy beans/d for eight weeks and total cholesterol was reduced by 5.6±1.5%, and LDL levels were decreased by 5.4±2.3%.[31] In another study, 13 normo-cholesterolemic men 18-26y ate 450g baked beans daily for 14 days as part of their normal diet. During bean treatment, total cholesterol was significantly reduced from 5.08±0.22mmol/L at baseline to 4.49±0.28mmol/L ($p<0.02$).[32]

In a clinical trial 130g/d of pinto beans were fed to 10 free living men and women with pre-metabolic syndrome for 12 weeks (Pre-metabolic syndrome: waist circumference≥96.5cm for men and ≥88.9cm for women and at least one of the following other symptoms: serum HDL <55mg/dL, serum triglycerides between 150-199mg/dL, fasting blood glucose between 100-125mg/dL or blood pressure between 120/80 to 140/85mm Hg). Total cholesterol was significantly reduced in participants consuming legumes by approximately 4% ($p<0.014$).[13] In a nine month trial, 62 obese and overweight subjects (BMI≥25) were given a white kidney bean (150mg) and mung bean (25mg) extract. At 3 and 12 mo. total serum cholesterol was significantly reduced. LDL and HDL were significantly improved (decreased and increased, respectively) at 12 months.[33] Sixteen mildly insulin resistant adults (blood glucose ≥15μU/mL and < 50μU/mL) consumed 1/2c. of pinto beans, black-eyed peas or carrots (control) each day for eight weeks. The pinto bean diet resulted in a decrease in total cholesterol of -19 ±5mg/dL and LDL had a mean change of -14±4mg/dL [34]

Anderson *et al.* (1984) fed 20 hypercholesterolemic participants isocaloric diets of either oat-bran (98±0.5g/d) or pinto and navy beans (101±4g/d) for three weeks. LDL was significantly reduced

($p<0.0005$) and weight was significantly decreased ($p<0.0125$).[10] Anderson *et al.* (1990) fed 28 male subjects with hyperlipoproteinemia (without secondary causes of hyperlipoproteinemia such as diabetes) 120-162g of beans in the form of pork and beans. In this study cholesterol and triglycerides were also significantly reduced by 8.4±2.8%, and 10.8±3.9%, respectively and body weight was significantly reduced by 1.7±0.3%.[35]

Table 1. Summary of legume clinical trials.

Study	**Intervention**	**LDL**	**Total Cholesterol**
Duane (1997)[30]	120g/d mixed legumes for 6 weeks	↓ from approx 135mg/dL to 125 mg/dL p=0.003	194mg/dL in control vs. 204mg/dL in treatment, p=0.06
Winham (2007)[31]	½ c baked beans (navy beans) for 8 wk	↓5.4±2.3%	↓ 5.6±1.5%
Shutler (1988)[32]	450g/d baked beans for 14 days	Not reported	↓ from 5.08±0.22 to 4.49±0.28, p<0.02
Finley (2007)[13]	130g/d pinto beans for 12 wk	↓ by approx 7mg/dL p<0.05	↓ by approx 12mg/dL p<0.014
Birketvedt (2002)[33]	Bean extract (175mg) for 3 months	4.4mMol/L, unchanged	↓ from 6.6±1.3 to 6.2±1.3, p<0.05
Winham (2007)[34]	101±4g pinto and navy beans for 8 wk	↓ p<0.0005	↓19 ±5, p<0.003
Anderson (1990)[35]	120-162g/d pork and beans for 21 days	↓ -8.4%, p<0.01	↓ 16.3±1.8% p<0.01

In an animal study, black beans were fed to Swiss male mice at an equivalent of 3.8g/kg, 38g/kg and 76g/kg of body weight. The black beans protected against cyclophosphamine initiated DNA damage

with a 34%, 22% and 33% reduction DNA lesions in leukocytes with diets supplemented with 3.8, 38 and 76g of black beans per kg of body weight, respectively.[36] In another study, a common bean extract (200mg/kg body weight) was fed to 30 streptozotocin (STZ) diabetic rats and the bean extract had a hypoglycemic and hypolipidemic effect.[37]

Plant Sterols

Legumes are a natural source of plant sterols and stanol esters which have the potential to help lower cholesterol. Legumes contain between 86mg/100g of β-sitosterol in kidney beans and 160mg/100g in chickpeas or garbanzo beans.[38] The amount of sterols in a serving (1/2 cup) of beans is equal to approximately 75 to 140mg.

Plant sterols have been demonstrated to help reduce total cholesterol.[39] Lower cholesterol levels are achieved by blocking cholesterol absorption (both endogenous and exogenous). Plant sterols replace cholesterol in the bile acid micelles and cause less cholesterol to be reabsorbed. Sterols have a higher micellular solubilization than cholesterol because sterols are hydrophobic molecules with a higher affinity to micelles than cholesterol.[40] Assuming this mechanism of action, a more marked reduction in cholesterol can be expected in hypercholesterolemic patients when high cholesterol diets are replaced with components such as legumes that contain phytosterols. If plant sterols are consumed with foods containing cholesterol, the plant sterols may restrict the amount of exogenous cholesterol being absorbed.

A study (Table 2) conducted in Germany reveals an association between serum plant sterol concentration and decreased risk for vascular disease. Men and women (n=1,242) were recruited who were 65-89y and who were healthy or who had a diagnosis of coronary heart disease (CHD), peripheral artery disease (PAD), or cerebrovascular disease. Presence or absence of vascular diseases

was compared to serum plant sterol levels. It was shown that plant sterols are a negative risk factor for vascular disease. A two-fold increase in sitosterol would mean a 22% reduction in the risk for coronary heart disease. This data (Table 2) shows an association between serum sitosterol, which equates to plant sterols in the diet, and a decreased risk for vascular disease.[41]

Table 2. Odds ratios for association between serum plant sterols and disease risk factors .

Risk Factor	**Odds Ratio**
Sitosterol	0.78
Male sex	1.06
Female sex	0.24
Cholesterol	1.09
Diabetes mellitus	2.67
Current smoking	1.13
Hypertension	0.94

De Jong *et al.* (2008) fed 45 subjects margarine that was not supplemented with sterols for 4 weeks. One of the selection criteria for subjects was drug treatment with statins. The participants were divided into three groups (n=15/group): one control group, and two groups which consumed 2.5g/d of either plant sterol or plant stanol enriched margarine. The study found that both plant sterols and stanols significantly lowered LDL cholesterol (p=0.027). There was a decrease in LDL cholesterol from the run in period of -0.27±0.39mmol/L in the plant sterol group and a change of -0.42 ±0.53mmol/l in the plant stanol group. Markers for enzymatic and non-enzymatic antioxidants and markers of oxidative stress and low grade inflammation were also tested although none of these results were significant.[42]

Varady *et al.* (2005) fed 84 subjects margarine supplemented with plant sterols or stanols for eight weeks to assess the effects of endurance exercise, consumption of plant sterols and a combination of exercise and plant sterols. The combination of exercise and 1.8g/d supplementation with plant sterol produced an effective regimen to improve cardiovascular health markers. Total cholesterol was

reduced from 5.44±0.33mmol/L to 5.02±0.31mmol/L, LDL cholesterol was reduced from 3.60±0.33mmol/L to 3.30±0.31mmol/L, HDL cholesterol was increased from a baseline of 1.09±0.06mmol/L to 1.18±0.06mmol/L after treatment and triacylglycerols were reduced from baseline of 1.34±0.14mmol/L to 1.19±0.12mmol/L after treatment.[43] Because plant sterols have a proven track record for lowering cholesterol they have been suggested for use as an adjunct to therapeutic lifestyle change (TLC) type diet plan that involves both diet and exercise.[44]

Plant sterols also influence carotenoid and fat soluble vitamin absorption. With supplementation of 1-4g/d free sterols, plant sterol esters or plant stanol esters, absorption of fat soluble vitamins and/or carotenoids was decreased between 7.8% and 26.1%.[39] However beans and lentils contain less than 200mg of β-Sitosterol, camposterol, and stigmasterol for beans, lentils and peas.[38] One serving of a popular margarine spread containing plant sterols contains 0.85g plant stanol esters. The amount of sterols present in beans will not influence carotenoid and fat soluble vitamin absorption when consumed as part of a normal diet. However, at the concentration normally consumed, legumes may offer the advantage of cholesterol reduction.

Phenolics

Legumes contain a mixture of phenolic compounds which have antioxidant properties and may exert some health benefits such as reducing inflammation. Polyphenolic compounds are a large class of compounds which include phenolic acids, phenylpropanoids, flavonoids, lignans and lignins. These compounds are produced in plants to protect them from chemical, bacterial, fungal or viral attacks.[45]

Phenolic acids are derived from either benzoic acid (ex. gallic, syringic, vanillic acid) or cinnamic acid (ex. caffeic, ferulic, sinapic, *p*-coumaric acid). Phenylpropanoids are a class of compounds such as

coumarins and hydroxycinnamic acids (ex. caffeic, ferulic, sinapic). Flavanoids are a large class of more than 3000 compounds such as catechin and epicatechin; this class includes anthocyans, isoflavonoides, flavones and flavanols. The group of tannins can be divided into two groups, hydrolysable and condensed tannins. Lignans have a structure similar to phytoestrogens. The main phenolic compounds in legumes are phenolic acids, phenyl propanoids, flavonoids, lignans, neolignans and lignins.[45]

Twenty four beans were analyzed and found to have the same hydroxycinnaminic acids but were found to have distinct differences in the flavanoid compounds. The twenty four beans were assigned six classes based on the concentration of flavanoids: black beans, pinto beans, light red kidney, small red, pink and dark red kidney, and then the group of beans that contained no detectable flavanoids. The hull of the beans is the main source of flavanoid compounds.[46] Total phenolic content of the hull extract of selected beans are as follows: red bean hulls 223.5mg catechin equivalent/gm, brown bean hulls 253.2 mg/gm, black bean hulls 270.0 mg/gm, and white bean hulls 6.7mg/gm.[47] Whole beans have between 3.3-16.6mg catechin equivalents per gm bean.[48] Phenolics are known to have antioxidant properties.

Xu, Chang (2009) [49] found total phenolic acids and total flavanols to have a significant correlation with antioxidant activities (DPPH, 2,2-diphenyl-1-picrylhydrazyl, used to determine anti-radical activity[50]; FRAP, Ferric Reducing Ability of Plasma, used to assess "antioxidant power"[51]; ORAC, Oxygen Radical Absorbance Capacity, which is also used to measure "total antioxidant potential".[52]) Although there is no evidence that phenolics act as antioxidants *in vivo*, studies have shown that polyphenolics in beans and other plants have anti-inflammatory activity *in vitro*[53-55].

Polyphenols have the potential to reduce cellular oxidation. Antioxidants including phenols have also been shown to reduce inflammation, a symptom which has been linked with many chronic diseases including cancer, diabetes, and obesity.[56-57] The amount of phenols in a serving (1/2 cup) of beans is equal to approximately 290mg -1.5g catechin equivalents.[48]

Fiber

Dietary fiber promotes normal bowel movements by adding bulk to the stool. It also helps prevent gastrointestinal disorders such as diverticulitis, diarrhea, irritable bowel syndrome, Crohn's disease and colorectal cancer.[58] It is also predicted to prevent and treat obesity and reduce serum cholesterol. In NHANES III, children aged 6-12y old had an average fiber intake of 13.4g/d.[59] Food intake surveys of children 7-10y old reported that those who met the suggested intake of fiber also had higher intakes of vitamin A, vitamin E, folate, magnesium and iron.[60] Foods with high fiber contents are generally more micronutrient dense.

There are many different opinions and methods to determine the amount of fiber that is appropriate for children. The equation (child's age + 5-10g fiber/d) to estimate the amount of dietary fiber required has been widely used and was developed by Williams, *et al.*[61] The acceptable Intake (AI) for fiber for the particular age group of interest can also be referenced to determine an appropriate amount of dietary fiber. According to the Daily Reference Intake (DRI)'s published in 2005, for 4-8y the AI for dietary fiber is 25g/d, at 9-13y boys should consume 31g/day and girls should consume 26g/day. (These values were developed by using the adult value of 14g/1000kcal which was shown to reduce the risk of coronary heart disease (CHD). This data was then extrapolated to children and adolescents. However, in the planning committee for Dietary Reference Intakes Review Workshop in 2008 it was discussed that the AI's in 1-13y olds (19-31g/day) are far from being met based on epidemiological

data (about 12g/day in preadolescents). Therefore the meeting concluded that the current AI's may be unrealistic or physiologically inappropriate.) In 1-13 year olds using the equation (age +5-10g/d) by Williams, *et al.*[61] the fiber recommendations would range from 6g-18g/day. The Williams *et al.* method was used in Hampl, *et al.*[60] which showed that only 32% of 7-10 year olds consumed adequate amounts of fiber.

Ten year-old children in Louisiana had a mean dietary fiber intake of 12.27±7.57g/d or about 5g/1000kcal. The fiber came mostly from vegetables and soups at 28% of intake, breads and grains comprised 24% of fiber intake, 15% from milk, and fruit and fruit juices made up 12% of fiber in the diets.[62]

Based on the fiber recommendations of (age +5-10g/d) by Williams, *et al.*[61] and the less reliable DRI's (2005), American children and adolescents aren't consuming the recommended amount of dietary fiber. Children who are at nutritional risk for mineral deficiencies require extra caution when consuming a high fiber diet because a high fiber diet may decrease bioavailability of minerals. Phytate (inositol hexaphosphate) binds with minerals making them less bio-available and the oxalic acid in some foods containing fiber can interfere with iron absorption[59] which can be especially a concern with adolescent females due to the onset of menarche.

We are proposing the addition of legumes to meat patties that would increase the fiber by 0.5-2g in a meat patty serving. The amount of fiber in the legumes used in this study ranges from 0.89g/100g to 4.1g/100g. This amount of fiber in a food product which does not traditionally contain fiber will help children to meet the daily fiber recommendations. Half a gram to two grams of fiber will not will not contain enough phytate to bind enough minerals in the diet for the child to become deficient even if they are at nutritional risk.

Fiber Aided Weight Reduction

Anderson *et al*. reviewed fifteen studies evaluating the influence of fiber on weight loss weight loss by measuring weight loss at weeks four and eight. Nine of these studies also reported weight loss data at 12-weeks. Studies showed a mean weight change of -3kg at 4 weeks with fiber supplementation, while the placebo or control group had a mean weight change of -1.7kg. At eight weeks the fiber supplemented groups had a mean weight loss of 4.9kg and the placebo group had a mean weight loss of 2.7kg. At twelve weeks the fiber supplemented group had lost 4.9kg and the placebo group lost 2.7kg. These data all have a confidence interval of 95%. The fiber-supplemented diet facilitated a greater percent weight change.[63]

A population of 82 overweight/obese (as measured by waist circumference ≥87cm for women and ≥90cm for men) age 18-65y were instructed to restrict kcal by 500-800 a day while following a reduced-glycemic-load (RGL) diet or a low fat diet (control). [64] Weight loss and blood lipids were measured at 12, 24 and 36 weeks. At 12 weeks participants had a weight loss of -4.9±0.5 kg in the RGL group and-2.5±0.5kg in the control (adjusted p=0.002), at 36w a weight change of 4.5± 0.7 kg in the RGL group and-2.6±0.9 kg in the control group (adjusted p= 0.085). The reduced-glycemic load group lost more weight initially and at 36 weeks had lost more weight overall than the control group. The heart health parameters showed that the RGL diet produced no adverse effects and although none of the values were statistically significant between groups, HDL increased 3.8±1.4mg/dL by week 36 in the RGL group and HDL in the control group increased 1.9±0.8mg/dL. LDL decreased 2.8±3.2mg/dL in the RGL group at 36wk while it decreased 1.9±2.9mg/dL in the control group at 36wk.[64] It can be concluded from this study that reduced-glycemic-load/high fiber diet can accentuate weight loss and cause a modest reduction in elevated blood lipids.

It is clear that increasing fiber and reducing dietary fat intake can improve blood lipid profiles, help control weight and improve insulin sensitivity. NSLP meals tend to have excess fat and calories and too little fiber. One potential solution is to dilute ground meat meals with legumes, which will increase the fiber. The extended meat products in such a program must deliver the expectation of the current ground meat products in terms of convenience and taste.

Introducing a meat and bean patty in the NSLP would lead to reduced calorie, fat and cholesterol and increased fiber. In addition to patties the extended meat products can be delivered in the form of meat sauces, tacos, lasagna, Salisbury steak, meatloaf and similar products. The reduction in caloric content, total fat, cholesterol and increased fiber may have the potential to accentuate weight loss or weight management. The products should also help improve blood lipid profiles and help improve carbohydrate metabolism.

Obesity in Children

Current data from the Third National Health and Nutrition Examination Survey (NHANES III) confirms that 37.2±1.9% of 6-11y olds are overweight and 18.8±1.3% of 6-11y olds are obese.[65] In the United States "at risk for overweight" is defined as Body Mass Index (BMI) ≥85th percentile and "overweight" is defined as BMI≥95th percentile; the data for Center for Disease Control growth charts are based on weights of children and adolescents 2-20y from 1963-1980, well before the obesity epidemic was clearly documented. The International Obesity Task Force has defined overweight in children and adolescents 2-18y as BMI≥25 and obesity as BMI≥30.[66] Although the definition of obesity in children varies widely, for the purpose of this thesis, overweight will be defined as BMI, waist circumference or waist-to-hip ratio of ≥85th percentile or simply BMI≥25. Obesity in children will be defined as BMI, waist circumference, or waist-to-hip ratio ≥95th percentile or simply BMI≥30.

From 1988-2004, abdominal obesity in children 6-11y increased 42% in boys and 83.4% in girls.[67] Abdominal obesity was measured by taking the waist circumference and waist-to-height ratio. Waist circumference can be interpreted as abdominal subcutaneous and visceral fat combined. The abdominal subcutaneous to abdominal visceral fat ratio cannot be obtained using the waist circumference and waist-to-height measures. Regardless of this shortcoming, it is a useful indicator for the risk of complications from obesity. The measure of central adiposity using waist circumference is a better indicator of visceral adiposity than BMI and can indicate a greater risk for cardiovascular disease and diabetes later in life.[68] Visceral fat has shown to be a risk factor for complications such as metabolic syndrome, diabetes mellitus, and heart disease.[68]

Metabolic Syndrome in Children

Metabolic syndrome is known in adults to be a group of symptoms including dyslipidemia, hyperglycemia, obesity and hypertension.[69] In children the criteria for diagnosis of metabolic syndrome is >90th percentile in peripheral insulin resistance, obesity, hypertension and hypertriglyceridaemia. The signs and symptoms of metabolic syndrome in children can be different from those in adults including acantosis nigricans, early puberty, stretch marks in skin, elevated height, abnormal lab values including (low reticulocyte production index (RPI), insulin-like growth factor binding protein, and high levels of cortisol, testosterone and plasmogen activator inhibitor). Waist circumference may be a less invasive way of predicting metabolic syndrome in children and the other signs and symptoms can help to determine if further medical testing is warranted.[69]

Interventions in Overweight/Obese Children

An extensive study with overweight children was conducted attempting to reduce the adverse effects of excess weight.[70] A 16-week exercise and dietary intervention was conducted with 236 overweight

Polish children age 3-15y. The dietary intervention reduced energy consumption to 1507±489.1 kcal in 7-9y group and 1396.4±396.9 kcal in 10-12y group, and added 21.0±6.8g dietary fiber. At baseline in thirty-eight of the participants, evidence of lipid metabolism abnormalities (elevated total cholesterol, elevated LDL, elevated triglycerides and low HDL levels) was observed. No evidence of carbohydrate metabolism abnormalities (such as elevated glucose) was observed before or after treatment in any of the groups. The treatment had a significant impact on weight loss; in 7-12y children a mean weight loss of 2kg over the 16 week period. In participants with hyperlipidemia, normalization of elevated blood lipids was as follows: total cholesterol before treatment was 222.6±22.1mg/dL and after the 16 week treatment was 195.4±29.3mg/dL; mean LDL cholesterol before treatment was 155.7±21.8mg/dL and after treatment was 128.1±17.9mg/dL; mean HDL cholesterol was 43.8±10.8mg/dL before treatment and after treatment was 54.4±29.7mg/dL; mean triglycerides were 161.7±35.0 before treatment and after treatment were 113.7±28.9mg/dL. Weight loss was associated with improved blood lipid profiles in overweight/obese children. The results of this study suggest that lipid metabolism abnormalities related to overweight/obesity may be reversible in children and adolescents.[70]

School based obesity interventions have common methods of targeting the diet, reducing sedentary behavior, increasing activity and making behavior modifications. Dietary modifications included decreasing fatty foods[71], increasing fruit and vegetable consumption[72], decreasing sweetened and unsweetened carbonated beverages[73], preparing healthy snacks[74] and reducing fat and sodium in NSLP menus[75]. School based obesity interventions that are shorter than one year were effective in reducing the prevalence of obesity. Interventions that lasted longer than one year were more successful at reducing obesity with an odds ratio of -0.62.[76]

Nutritional Quality of Diets in School Children

The NSLP was evaluated in the Third School Nutrition Dietary Assessment Study from 2004-2005. The study compared the diets NSLP participants and non-participants. Participation in the NSLP in elementary school children decreased deficiencies in both macro and micro nutrients compared to children who did not participate in NSLP; however participants consumed excessive amounts of kcal, fat and sodium and inadequate amounts of fiber. Average NSLP participants consumed 2,131± 14.2kcal while non-participants consumed 2,003±22.7kcal, a significant amount less ($p<0.01$). The estimated energy requirement (EER) for this group is approximately 1760kcal. Although both groups consumed well over the EER, the NSLP participants consumed more energy. NSLP participants were 76.8% within the acceptable macronutrient distribution range for total fat while 94.2% of non-participants were within acceptable range. Participants in the NSLP had a mean percent adequate intake (AI) of fiber of 50.5±0.91% and non-participants had a percent AI of 45.0±1.45% which is significantly less ($p<0.01$) than participants. The tolerable upper intake level for sodium was exceeded by 95.0± 2.01% in participants and non-participants exceeded the upper intake level by 88.1±4.86%. Elementary school students who participate in NSLP compared to non-participants consumed 105% of the kcal, 89% of the fat, 125% of the saturated fat, 110% of the fiber and 101% of the sodium of non-participants.[77]

Ninety-two Canadian school children grades 2-4 participated in a 24h dietary recall (self-reported) revealing the number of servings of carbohydrates, fruits and vegetables, milk products, meat and other protein sources as well as "other foods" which usually consisted of sugary or fatty snack foods. It was determined that 76.4% of study participants consumed fewer than the minimum amount of servings of fruits and vegetables than recommended by Canada's Food Guide (which is similar to the

US Food Pyramid, Canada's food guide recommends 5 servings of fruits and vegetables a day while the US Food Pyramid recommends 3.5 cups or approximately 7 servings) and 58.4% of study participants consumed less than the minimum amount of servings of milk. The school did not offer a lunch service and all lunches were brought by the students.[78] A baseline of what students eat for lunch with no school lunch program was established which correlates to NSLP non-participants who are also having trouble meeting the requirements for a healthy diet. It is evident that children are not meeting the requirements for fruit and vegetable servings. Adding products into the NSLP which provide a serving of vegetables may help participating children to consume more vegetable servings.

State of School Lunches

A cross-sectional study based on the data collected in the School Nutrition Dietary Assessment Study (2004-2005) correlated that the NSLP, although didn't contribute to obesity, did not contribute to lower student weight. This conclusion was based on an association with NSLP participation and obesity. The prevalence of overweight and obesity in elementary students is 38.8±2.2%. A large percentage of students are consuming NSLP meals; 51% of students eat NSLP five days a week and 86.5% of students eat NSLP at least one day a week. The correlation co-efficient for obesity to participation was -0.003 (not statistically significant) which indicates that participation and obesity rate are almost 100% unrelated.[79]

Another review of the NSLP found that although 95% of elementary public schools offered vegetables during a representative week, only 8% offered legumes; this data is based surveys sent to 298 representative NSLP schools. Based on 24h dietary recalls, 51% of NSLP participants consumed vegetables while only 3% consumed legumes. Conversely, 37% of schools offer snacks and desserts while 51% of participants consumed snacks and desserts based on a 24 hour dietary recall.[80] Even

though vegetables are being offered more frequently than desserts, more children are choosing snacks and desserts. Legumes are not offered consistently in most NSLP menus.

A two year intervention study in Somerville, MA was conducted to improve the nutrient quality of school lunches offered to elementary school students. Three key areas of need for improvement were identified: improvement in meal offerings, staff and facility development and communication with students and their families. The intervention included recipe development, altering the foods offered, a new criterion for snacks available for purchase, $34,351 worth of necessary kitchen equipment, staff trainings, meetings with the food service director, monthly tasting events and student advisory groups. Fruit and vegetable offerings were increased from two times a week to five and the fat and sugar content of snacks available for purchase was decreased.[81] This study shows that improving the NSLP is possible although it requires an investment of money and a willingness to change. Further research is needed to determine if these successful changes towards a more healthy lunch program contribute to a lower incidence of obesity/overweight in elementary students.

The purpose of this thesis study was to develop and implement a *C. elegans* based model to assess the influence of 9 legumes on fat deposition, and lifespan in *C. elegans*. The *C. elegans* model also has the potential to be used as a screening tool to select the legumes that are most beneficial for health. Prospectively, these legumes will be added to foods for use in the NSLP. The food technology application was further investigated by conducting sensory evaluation on the important attributes of hamburgers to middle school students.

Materials and Methods

C. elegans and their food source *Escherichia coli* (*E. coli*) OP50 were obtained from the Caenorhabditis Genetics Center (CGC, University of Minnesota). Unless otherwise specified, all chemicals were purchased from Sigma-Aldrich Chemical Co., (St. Louis, MO).

Legume Nutrient Composition

The nutrient composition of the legumes was analyzed by first grinding the beans to 0.75mm using a centrifugal mill (Retsch ZM 200; Haan Germany). The crude protein content was analyzed using the Kjeldahl method for protein determination. The crude fat content was analyzed using Soxhlet gravimetric analysis by acid hydrolysis. The crude fiber content of the legumes was analyzed by using the filter bag technique with the ANKOM 2000 Fiber Analyzer (Macedon, NY). Carbohydrates were calculated by difference.

Sample Preparation

Black beans, cranberry beans, dark red beans, great northern beans, large lima beans, lentils, light red kidney beans, navy beans and white kidney beans (obtained from Archer Daniel Midland, Decatur; IL and a local produce stand, Baton Rouge; LA) were prepared by grinding though a centrifugal mill (Retsch ZM 200; Haan, Germany) with a 0.75mm screen. The legume powder was then suspended as a dispersion in deionized water (1% w/v solution). The mixture was homogenized with a Brinkman homogenizer (Riverview, FL) for forty minutes. The mixture was then autoclaved at 120ºC with a cycle time of 50 minutes (Brinkman, Riverview, FL). The legume samples were divided into 1.5mL aliquots and frozen at -20ºC until required. Samples were not stored more than three weeks.

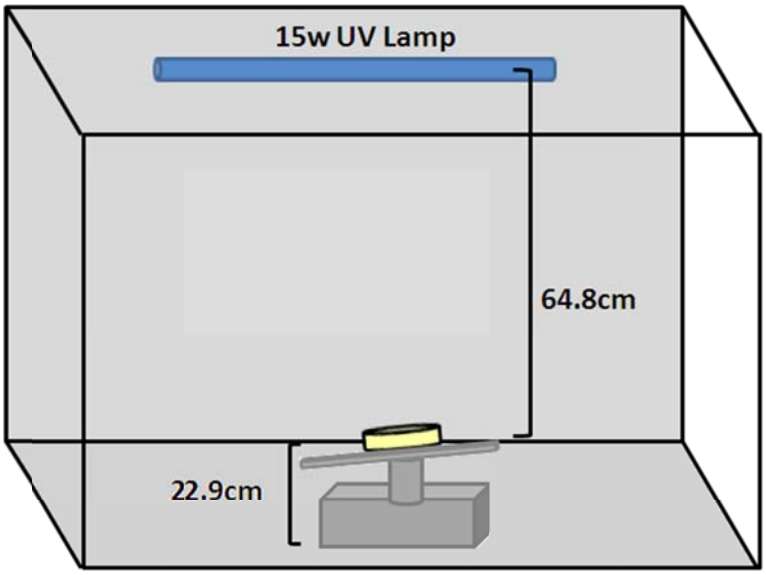

Figure 1. UV Treatment Diagram. Diagram details UV treatment procedure for *E. coli* OP50. *E. coli* was placed on an rocking platform shaker for one hour under a 15w germicidal lamp.

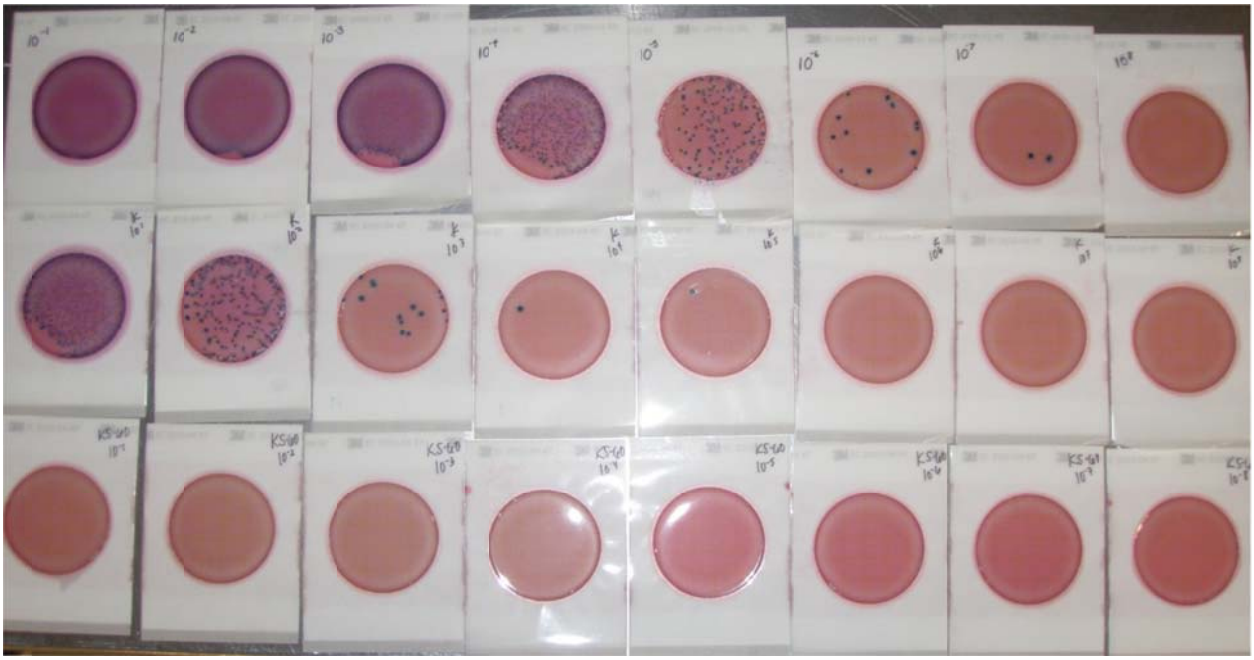

Figure 2 . *E. coli* Serial dilutions. 1st row is *E. coli* OP50 after culturing, 2nd row is *E. coli* OP50 (without agitation) after UV treatment for one hour, and 3rd row is *E. coli* OP50 after being exposed under UV light for one hour with continuous agitation. *E. coli* positive colonies are blue. Dilutions are 10^{-1} on the far left, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7} and 10^{-8} on the far right. The 10^{-6}dilution in the 1st row was used to calculate the *E. coli* concentration.

The *E. coli* control food source was prepared by inoculating the nutritionally rich lysogeny broth (LB broth) with *E. coli* OP50 strain bacteria and incubating (Boekel, Feasterville, PA) at 37ºC for 16 hours to reach a final concentration of approximately 1 x 10^6 cfu/mL. The cultured *E. coli* was then exposed under ultraviolet (UV) light in 8mL aliquots under a 15 watt germicidal lamp at a distance of 68.68cm (ESCO, Hatboro, PA) for one hour while being agitated with a rocking platform shaker at 24 revolutions per minute (RPM) with a tilt angle of 10º (VWR, West Chester, PA) (Figure 1). The UV treated *E. coli* was confirmed by serially diluting 8 times using Petrifilm (3M, St. Paul, MN) and incubated for 16 hours at 37ºC. Serial dilutions revealed negative results for viable *E. coli* colonies at all dilutions after UV treatment with constant agitation (Figure 2).

Material and Solution Preparation

Nematode Growth Media (NGM) Agar was made by combining 3g NaCl, 20g of Bacto-agar (Becton, US), 2.5g of Bacto-peptone (Becton, US), 0.1% cholesterol solution (0.001g/mL 95% ethanol), and 975mL of deionized water (dH_2O) and then this solution was mixed and autoclaved. The following were then added to the autoclaved solution: 1mL of 1M $CaCl_2$, 1mL 1M $MgSO_4$, and 25mL of 1M KPO_4 at pH 6. The agar was pipetted into agar plates (∅ 3mm) while still warm. The plates were cooled and stored at 4°C before use.

Phosphate Buffered Saline (PBS) one liter of PBS was made by adding 8g NaCl, 0.2g KCl, 1.44g Na_2HPO_4, and 0.24g KH_2PO_4 to 800mL dH_2O. The pH was then adjusted to 7.4 with HCl and dH_2O was added to bring the volume to 1 liter. The PBS was then autoclaved.

S-Basal solution was made by combining 5.8g of 1M NaCl, 50mL of 0.05M KPO_4 at pH 6, and 1mL cholesterol, dH_2O was added to this solution to bring the volume to 1L. The solution was then autoclaved.

Nile red solution The stock solution was made by dissolving 0.5mg Nile red per 1mL dH_2O. The working solution was made by adding 0.05mL stock solution to 50mL glycerol/water (75:25). The mixture was stirred well.

Nematode Preparation

Wild Type *C. elegans* Bristol N2 strain, the most common laboratory strain of *C. elegans,* was chosen for this investigation. The *C. elegans* live on NGM agar plates and were prepared using an age synchronized design. Two methods were used to create a synchronized generation of worms. In the first method, mature gravid *C. elegan*s were transferred individually onto agar plates and then treated with a 1:1 (0.4M NaClO: 1M NaOH) solution to kill the mature *C. elegans* but leave the eggs viable. The eggs then hatch after one day and mature *C. elegan*s were present in three days. The *C. elegans* are stored in a 20ºC temperature incubator (Revco Tech., Nashville, NC).

A second age synchronized procedure was also used. Gravid *C. elegans* were washed off of agar plates with 1mL S-Basal, the suspension containing *C. elegans* was centrifuged for 20s at 3,000 rpm and the supernatant was removed. The remaining pellet (*C. elegans*) was re-suspended by vortexing and worms were treated with a 5:3 ratio of 1M NaOH: 0.4M NaClO. The *C. elegans* were treated with intermittent vortexing until the adult gravid nematodes were no longer living and had released their viable eggs, about thirty seconds. This step was confirmed by plating a three micro liter specimen on a glass slide and viewing with a compound microscope (American Optical, Buffalo, NY). The eggs were washed three times with S-Basal solution and then 1mL of S-Basal was added and the solution was placed into 1.5mL lidded vials to hatch while shaking (VWR, West Chester, PA) for 16 hours at 20ºC. The hatched *C. elegans* were centrifuged for 20s at 3,000rmp and approximately 970µL of the supernatant was removed. The worms were re-suspended by vortexing and approximately 3µL of

worms were plated onto each NGM agar plate. The *C. elegans* incubated at 20ºC (Revco Tech., Nashville, NC) until mature *C. elegans* were present in three days.

Feeding and Data Collection Protocol

All groups of *C. elegans* were fed 200µL of *E. coli (ad libitum)*. Three legume treatment concentrations were fed to the experimental groups at 20%, 33.3% and 50% legume solution, corresponding to .2%, .33% and .5% dry legume matter. This diluted the *E. coli* diet 20-50%. The concentrations are shown in Table 3.

Table 3. Diet Treatment and Control Diet Concentrations. Volumes of food that were used to comprise the 20%, 33.3% and 50% legume treatment and control *ad libitum* diets.

	Control	20% Legume Treatment	33.3% Legume Treatment	50% Legume Treatment
UV treated *E. coli*	200µL	200µL	200µL	200µL
Legume solution	-----	50µL	100µL	200µL

The *C. elegans* were fed every four to seven days, depending on the amount of food left in the plates as judged by moisture of the NGM plates. Sterile conditions were maintained throughout the study to ensure no bacterial contamination was present. The nematodes were held at 20ºC in a temperature controlled incubator (Revco Tech., Nashville, NC). All treatment and control groups were created in triplicate with three NGM plates for each treatment level. Ninety NGM plates of *C. elegans* were observed in this study.

Table 4. *C. elegans* diet nutrient composition.

	Legume Diet (mg/mL)	*E.coli* OP50 (mg/mL)	Available Nutrients per Plate in milligrams			
			Control	20% Legume	33.3% Legume	50% Legume
Carbohydrate (mg/mL)	5.6	13	2.6	2.824	2.970	3.160
Fat (mg/mL)	0.13	6.7	1.34	1.345	1.349	1.353
Protein (mg/mL)	2.3	100	20	20.092	20.1532	20.230
Fiber (mg/mL)	0.329	--	--	0.013	0.022	0.033

Fluorescence Data Collection

At the end of the study, the *C. elegans* were washed from their agar plates using S-basal (a liquid medium which supplies key nutrients to the *C. elegans*), and centrifuged for 20s at 3000 rpm. The *C. elegans* were then fixed with 4% paraformaldehyde for 30min and washed with phosphate buffered solution (PBS) three times and held in a volume of 1mL of PBS overnight at 4ºC. The solution was then centrifuged for 20s at 3000 rpm to pellet the *C. elegans.* The supernatant was removed and discarded. Ten micro liters of Nile red solution was added to the *C. elegans* and this solution was vortexed to effectively stain all of the animals. Nile red dye was used because it has lipophilic and fluorescent properties. Ten micro liters of fluoromount-G (Southern Biotechnology Associates, Birmingham, AL) was applied to a glass slide and then 10µL of the Nile red stained *C. elegans* was added to the slide. A cover glass covered the specimen. The slides were allowed to set overnight at

4ºC before being viewed with a fluorescence microscope (Nikon Eclipse *Ti)* which was equipped with a Texas Red filter. Digital images were taken with the Retiga 4000R digital camera (QImaging, Surrey, BC, Canada). The fluorescence images were analyzed for intensity using the line profiling function with Image-Pro 6.2 software (Media Cybernetics, Inc., Bethseda, MD).

Pharyngeal Pumping Rate Data Collection

The pharyngeal pumping rate of the *C. elegans* was observed and recorded every two to four days throughout the study. Pharyngeal pumping rate was determined by manually monitoring the oscillatory movement at the terminal bulb of each organism's pharynx for 20 seconds under 7.5 x magnification on a stereomicroscope (Nikon SMZ-U). The terminal bulb of the *C. elegans* pharynx can be seen in Figure 3. Pharyngeal pumping rates of 5 *C. elegans* from each NGM agar plate were observed and recorded with the units of pumping rate per minute.

Travel Distance Data Collection

The NGM dishes were viewed at 1.5 x magnification on a stereomicroscope (Nikon SMZ-U). Twenty frame video images were captured using Image Pro 6.2 (Media Cybernetics, Inc., Bethseda, MD) and a Retiga 4000R digital camera (QImaging, Surrey, BC, Canada) at one frame per 490 milliseconds. These videos were analyzed with Image-Pro 6.2 (Media Cybernetics, Inc., Bethseda, MD). Twenty *C. elegans* were manually selected on the first sequence of the 20 frames and then the software was designed to automatically find and track the worms' movement through all 20 frames.

Consumer Study with National School Lunch Program Participants

A study was conducted at McKinley Middle School, Baton Rouge, Louisiana with 34 seventh grade students to determine the attributes that they find important in a hamburger product. Institutional

Review Board (IRB) permission was obtained through an application for exemption from the Louisiana State University Agricultural Center (The approved protocol has exemption number HE09-25). Four ounce (113.4g) meat patties with 40% added light red bean were prepared by hydrating light red beans in excess water at 4ºC overnight. Ground Beef (80% lean) was obtained from a local

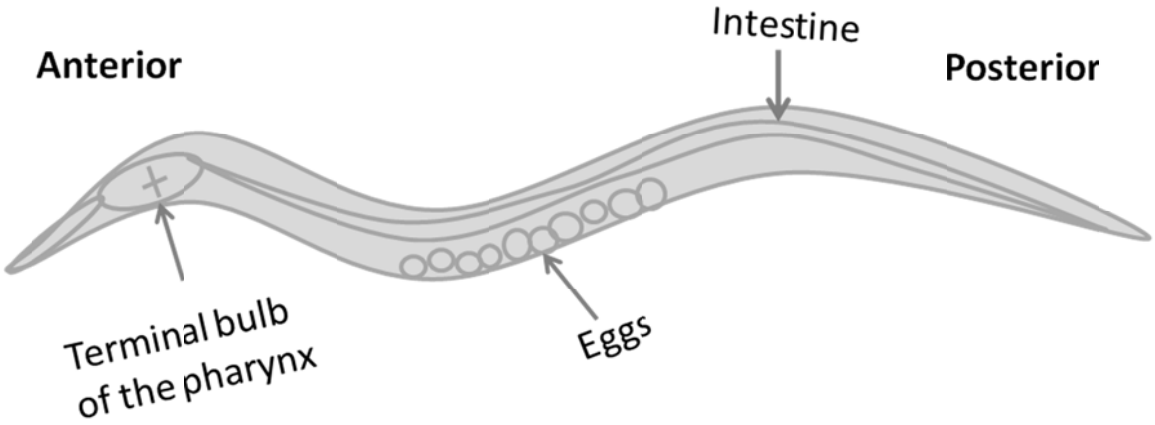

Figure 3. Anatomy of the *C. elegans* showing the terminal bulb of the pharynx which is monitored to determine pharyngeal pumping rate, eggs which are observed to determine if a worm is gravid, and the intestine which transports and absorbs food.

supermarket (Baton Rouge, Louisiana). The hydrated beans were then drained and ground through a 0.48cm plate with a KitchenAid grinder stand mixer attachment (KitchenAid, St. Joseph, MI) and then mixed by hand with the ground beef to make a 42.5:57.5 (bean:beef) patty. The patties were formed into 114g round patties using the Hollymatic Model 200-U Patty Machine (Hollymatic, Countryside, IL). The patties were then frozen at -20ºC in a blast freezer in stacks of three with patty paper interleaved between them. Patties were held at -20 ºC until being baked on commercial half-sheet pans (42cm x 29cm) in a Moffat Turbofan 32 Oven (Moffat, Christchurch, New Zealand) for approximately 15 minutes at 177ºC or until the patties reached an internal temperature of 74 ºC. The patties were cut in half and transported to the middle school in a hot box. White wheat hamburger

buns were generously donated from Flowers Bakery (Flowers Foods, Thomasville, GA). The buns were cut in half before service.

The consumers were given a parental consent form two weeks prior to the study and asked to get parental/guardian consent before participating. The consent form excluded any participation if a consumer was allergic or opposed to eating to wheat, beef or legumes. The consent form also asked that the study participants be participants in the NSLP. On the day of the consumer study, the students were asked to fill out a student research assent form which indicated their willingness to participate in the study. After all forms were collected, the students were given four questions and a question response sheet to help the researchers keep accurate records of consumer responses. The questions included: "what is a hamburger, what is a hamburger made of, what do you like about hamburgers and how often do you eat hamburgers?" Once students had sufficient time to record their responses, we asked the students to verbally share their answer with the rest of the group; this was done one student at a time. After all students had responded to the first question, we moved onto the next question and so on. The response sheets were collected and students were instructed to pick up a hamburger (½ meat patty and ½ bun assembled as ½ a hamburger). The students could then pick from any of 3 condiments to dress their hamburger as they normally would when consuming hamburgers (ketchup, mustard and/or mayonnaise). Students were then asked to taste their hamburger and provide their feedback. The forms were then collected and study participants were rewarded with 12oz bottles of Powerade (Coca-Cola Company, Atlanta, GA). The interview with the children was repeated in the same classroom with a different group of students. The first group had 15 participants and the second group had 19 participants.

Statistical Analysis

Statistical analysis was completed using Statistical Analysis Systems statistical software package version 9.1.3 (SAS Institute, Cary, NC, USA). The *C. elegans* pharyngeal pumping rate and distance analyses were performed by using a two-way ANOVA. To determine the overall trend for pumping rate data, the data was sorted by concentration and the treatments were tested against the pumping rate in a one-way ANOVA; lsmeans output was used to determine an appropriate p-value. The fluorescence analysis was performed by using a one way ANOVA. ANOVA analysis was done using the PROC MIXED. Correlation was determined by correlation analysis using PROC CORR. The level of statistical significance is $p \leq 0.05$.

Results

Legume Nutrient Composition

The protein content of legumes ranged from 20.16±0.15g/100g dry bean in navy beans to 24.64±0.45g/100g in lentils. The fiber content of the legumes ranged from 0.89±0.12g/100g in navy beans to 4.10g±0.31/100g in dark red kidney beans. The fat content in the legumes ranged from 0.76±0.08g/100g in white kidney beans to 1.70±0.015g/100g in navy beans. The carbohydrate content of the legumes ranged from 71.2±0.26g/100g in light red kidney beans to 74.00±0.12 in lentils. (Figure 4)

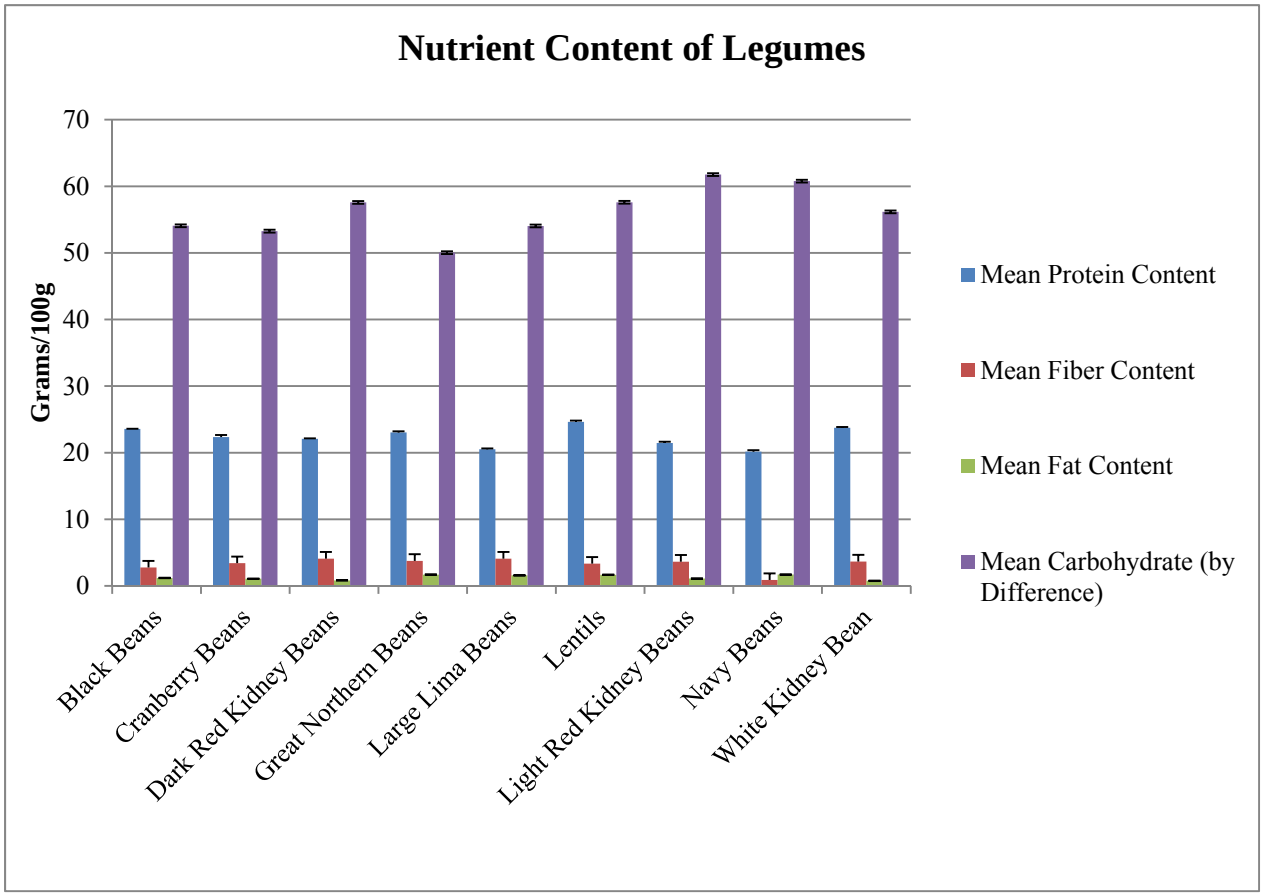

Figure 4. Nutrient Content of Legumes in g/100g for the 9 beans included in this study, analysis completed by dry weight bean flour.

Nile Red Fluorescence

White kidney and cranberry beans at a treatment level of 20% significantly lowered Nile red fluorescence intensity by 6.53% and 4.06%, respectively. Light red beans treatment at concentrations of 20% and 50% significantly decreased the Nile red fluorescence intensity by 24.10% and 29.75% respectively. Black beans at all three treatment levels (20%, 33.3% and 50% legume) significantly diminished the Nile red fluorescence intensity by 15.67%, 13.62%, 10.52%. Navy beans also significantly reduced Nile red fluorescence intensity by 13.59%, 7.95%, 13.66%, at treatment concentrations of 20%, 33.3% and 50%, respectively. (Figure 5) A decrease in Nile red fluorescence correlates to decreased fat deposition because Nile red dye is a lipophilic and fluorescent dye. The fluorescent intensity of the *C. elegans* images is shown in Figure 6; the control group exhibits a larger fluorescent intensity than the navy and white kidney beans. The legumes that significantly lowered Nile red fluorescence and the overall trend in pharyngeal pumping rate had a correlation coefficient of 0.712. Nile red fluorescence was not correlated with the legume treatment levels (20%, 33% and 50%) with a correlation coefficient of 0.058.

Pharyngeal Pumping Rate

C. elegans fed a diet of great northern beans at 20%, 33.3% and 50% treatment levels exhibited a 17.20%, 20.54% and 33.50% increase in pharyngeal pumping rate compared to the control throughout the study, respectively (Table 5). During the study, the cranberry bean treatment group of 33.3% displayed an increase in pumping rate of 24.44% and at treatment level of 50% had a significant increase in pumping rate of 29.39%, as compared to the control groups. Lentil treatment group of 50% exhibited a significant increase of 34.14% compared to the control group. Dark red kidney beans showed a significant increase over the control in pumping rate at the 50% treatment group at 16.83%.

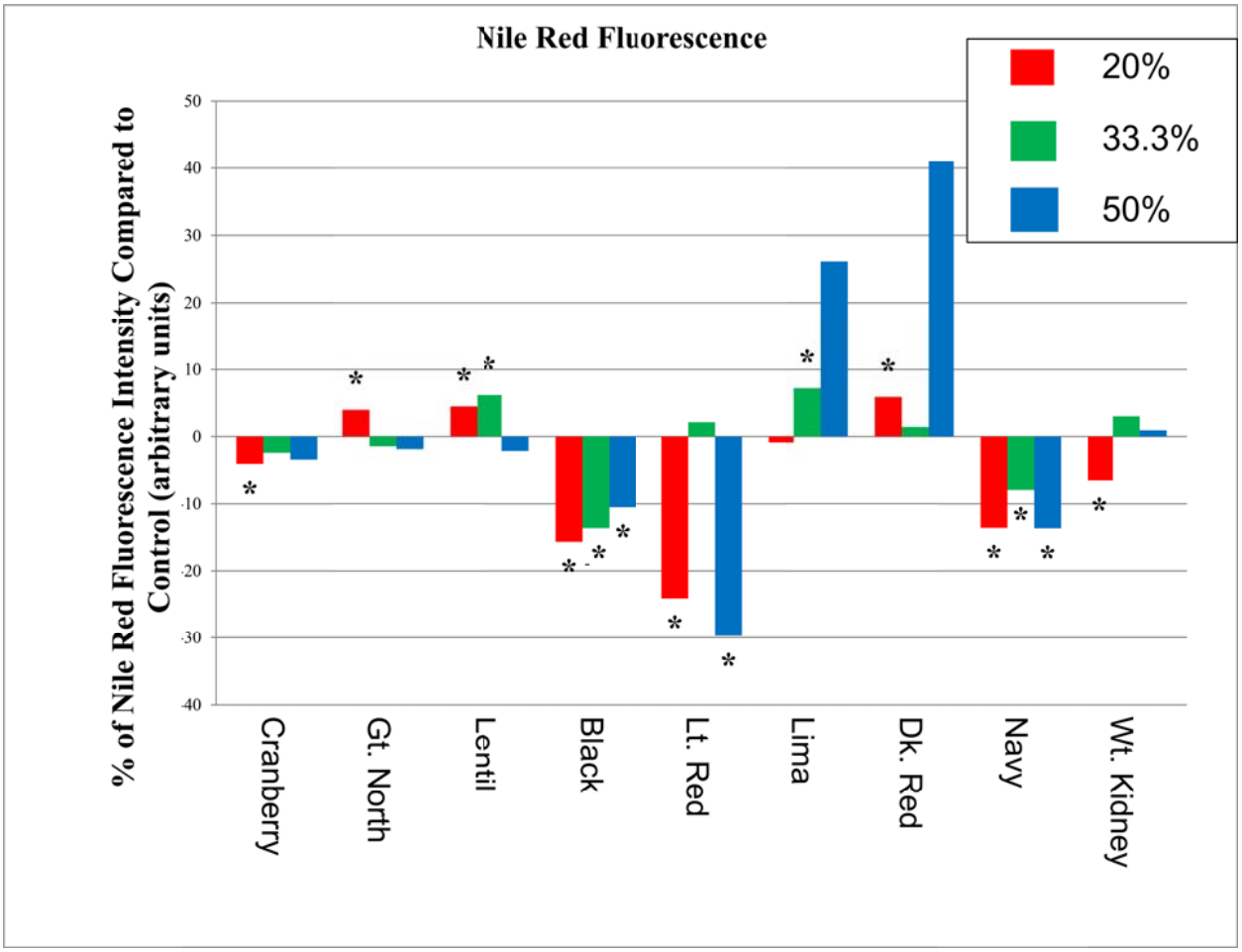

Figure 5. Nile red fluorescence percent change of legume treatment groups normalized to the control group. * indicates a significant increase or decrease in Nile red fluorescence. $p \leq 0.05$. Arbitrary units are due to the process of converting the Nile red images into computerized grayscale images to determine fluorescence intensity.

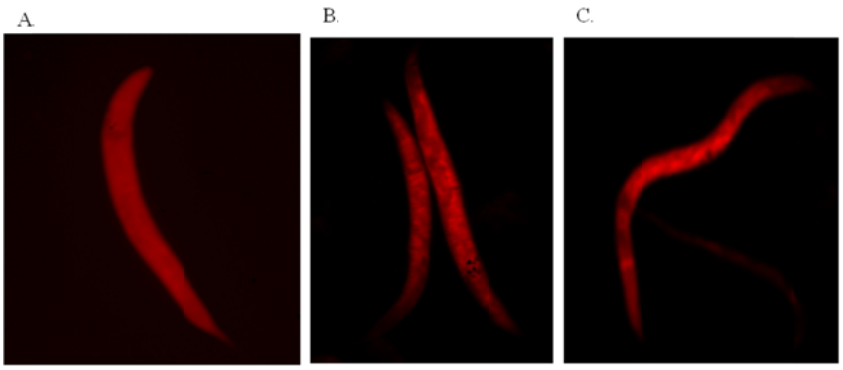

Figure 6. Images of *C. elegans* stained with Nile red dye. Image A is a *C. elegans* in the control group. Image B is of 2 *C. elegans* fed Navy beans at 50% treatment level. Image C is of 2 *C. elegans* fed White Kidney beans at 20% treatment level.

The overall trends in pumping rate over the control reflect that there was, in general, a more significant increase in pumping rate when the *C. elegans* were fed a diet of 50% legume. The lima bean regression graph (Figure 12) shows a trend of the control having a lower pharyngeal pumping rate than the 20% lima, 33.3% lima and 50% lima and a trend of the 50% group having the highest pharyngeal pumping rate. In the light red kidney bean pharyngeal pumping rate regression graph (Figure 11), the regression lines are close together as seen in Table 5 the 33.3% and 50% light red bean show an overall trend higher than the control, although not significantly. Figure 10 shows the 50% black bean pharyngeal pumping rate per minute trended high throughout the study. Figure 9 depicts *C. elegans* pharyngeal pumping rate when treated with lentils; the 50% legume treatment group produced higher pharyngeal pumping rates than the control group. The cranberry bean pharyngeal pumping rate regression graph also shows a higher rate in the 50% treatment group over the control (Figure 8). The great northern bean pharyngeal pumping rate regression graph (Figure 7) shows a higher pharyngeal pumping rate than the control group than in the 50% great northern treatment group. The white kidney and navy bean pharyngeal pumping rate regression graphs (Figures 14 and 15, respectively) appear similar without much difference between the groups. The dark red kidney bean pharyngeal pumping rate regression graph shows that the 50% dark red kidney treatment had the greatest effect on pharyngeal pumping rate (Figure 13).

Travel Distance

Travel distance is the mean distance traveled by *C. elegans* in micro meters across the NGM agar plates. A significant difference in all three concentrations (20%, 33.3% and 50%) of black bean and all three concentrations of light red kidney beans compared to the control group was seen on the 5th day. However, as the worms had not yet received treatment, this is seen as a natural deviation in the *C. elegans;* this was not seen at the latter travel distance observation day. (Table 6) There were no other

Table 5. Percent Change of Pumping Rate in Bean Treatment Groups Compared to the Control Group.
* Indicates a statistically significant increase in pumping rate ($p \leq 0.05$)

Percent Concen -tration	Legume	Day 4-9	Day 10-14	Day 15-19	Day 20-22	Overall Trend
20	Great Northern	6.91	11.95	32.12*	17.83	17.20*
33.3		5.11	13.25*	33.13*	30.66*	20.54*
50		1.35*	61.32*	26.27*	45.05*	33.50*
20	Cranberry	3.99	1.07	-39.39*	11.76	-5.64
33.3		6.31	22.01*	0.61	68.81*	24.44*
50		4.08	20.12*	32.73*	60.61*	29.39*
20	Lentil	7.51*	13.25*	-11.11	-1.78	1.97
33.3		-3.14	20.83*	42.83*	15.15	18.92
50		5.19	21.30*	43.23*	66.84*	34.14*
20	Light Red	-3.65	0.75	4.19	-6.47	-1.30
33.3		3.52*	1.51	13.84	6.63	6.38
50		7.38*	23.52	21.21	-21.08	7.76
20	Black	3.65	-17.5	-27.56	-48.59*	-22.50*
33.3		23.52*	-42.08*	-37.18	-12.77	-17.13*
50		24.38*	24.43	-5.77	16.46	14.88
20	Lima	-3.19	19.68	-5.51	30.80*	10.45
33.3		-4.9	5.85	-9.44	49.90*	10.35
50		-5.08	20.89*	-5.08	41.15*	12.97
20	Dark Red Kidney	5.29	0.56	-5.29	9.89	2.61
33.3		2.6	1.92	-5.57	16.86*	3.95
50		-3.02	9.74	23.14*	37.44*	16.83*
20	White Kidney	4.78	0.68	-10.86	-14.42	-4.96
33.3		4.36	2.14	3.86	7.29	4.41
50		4.36	13.09*	-0.71	17.34*	8.52
20	Navy	4.45	2.6	6.29	-6.48	1.72
33.3		-1.59	10.72*	0.86	-1.62	2.09
50		3.52	7.56	3.43	9.08	5.90

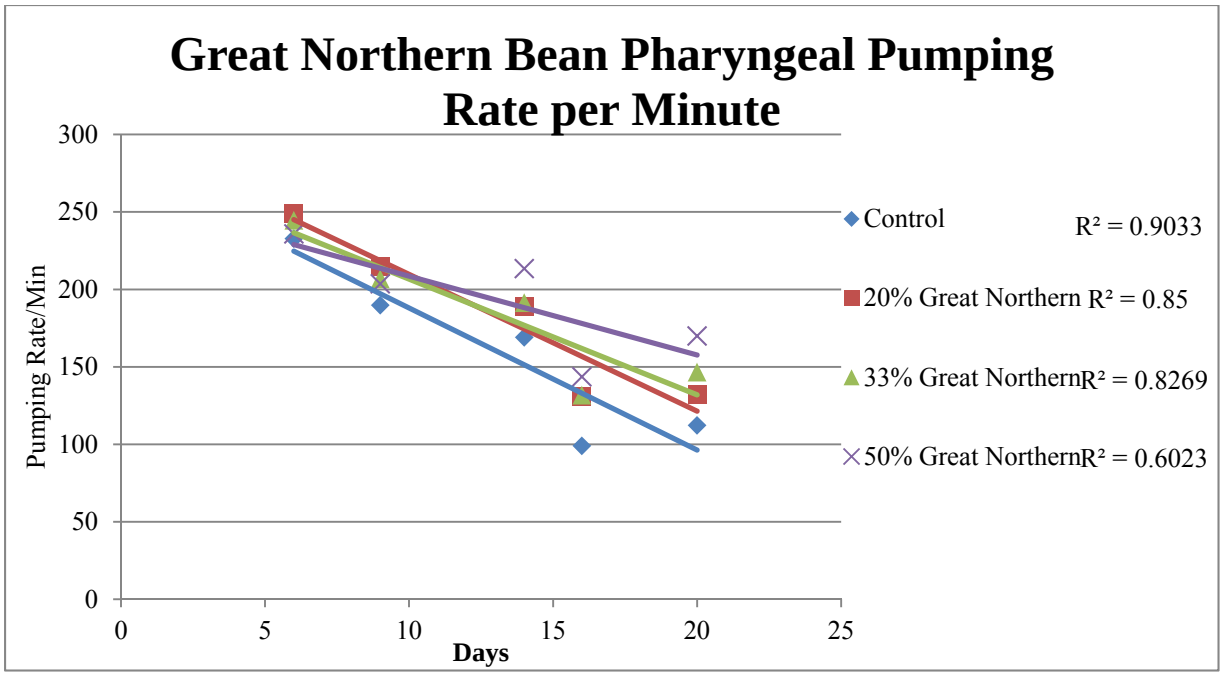

Figure 7. Regression Analysis of Pharyngeal pumping rate of *C. elegans* when fed great northern beans. Line colors correspond to marker color.

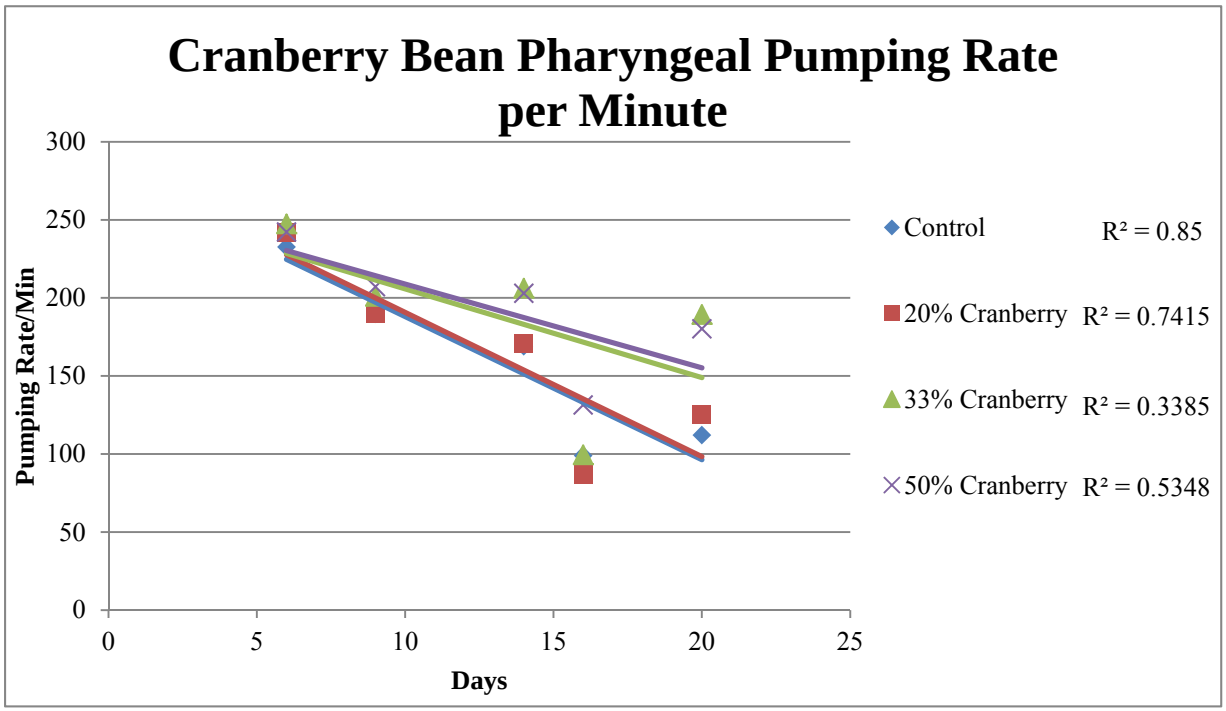

Figure 8. Regression Analysis of Pharyngeal pumping rate of *C. elegans* when fed cranberry beans. Line colors correspond to marker color.

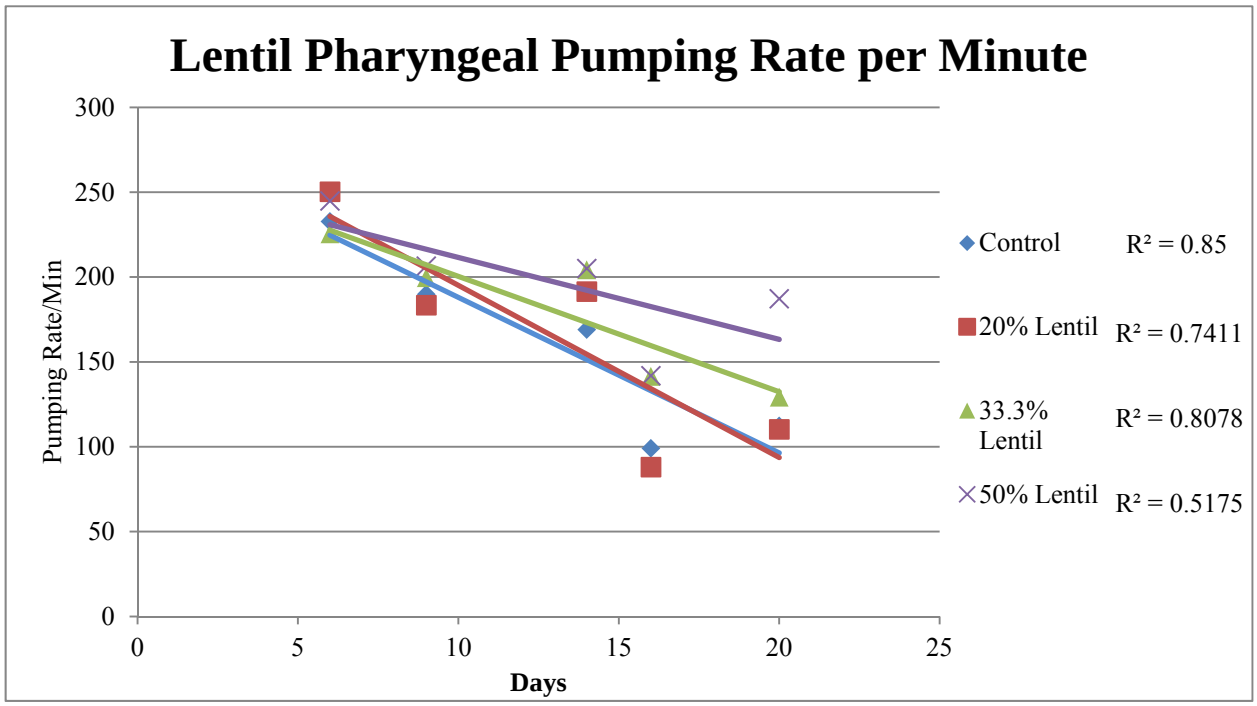

Figure 9. Regression Analysis of Pharyngeal pumping rate of *C. elegans* when fed lentils. Line colors correspond to marker color.

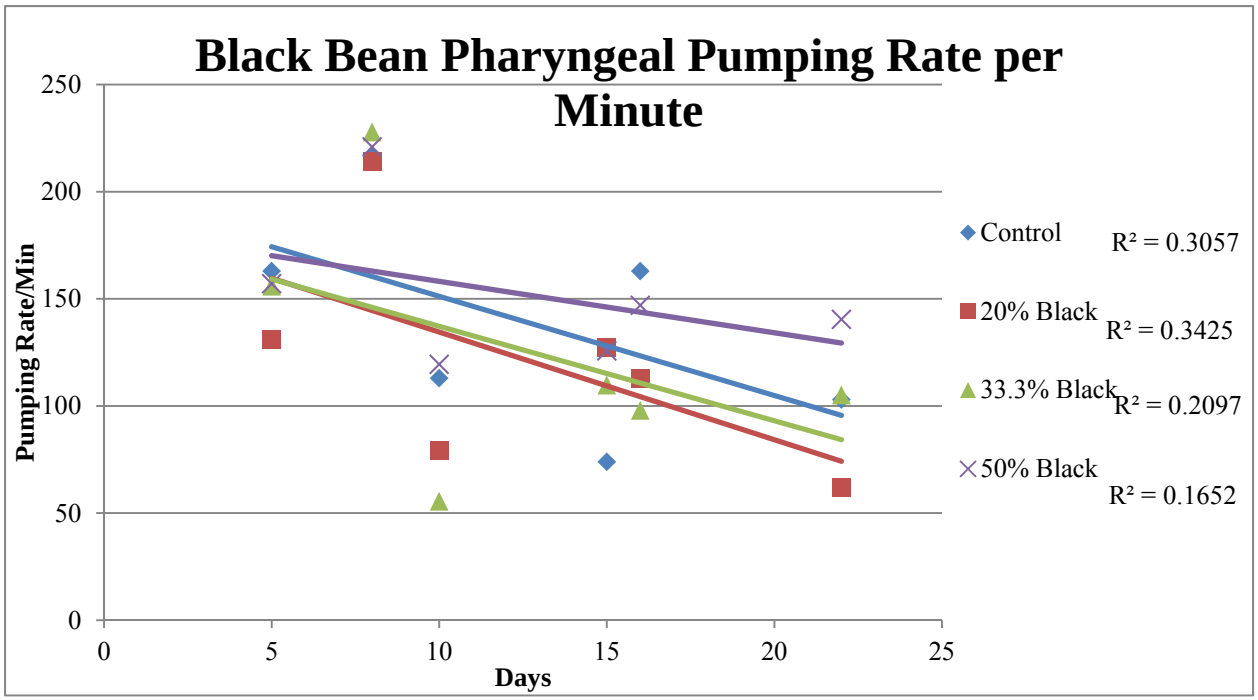

Figure 10. Regression Analysis of Pharyngeal pumping rate of *C. elegans* when fed black beans. Line colors correspond to marker color.

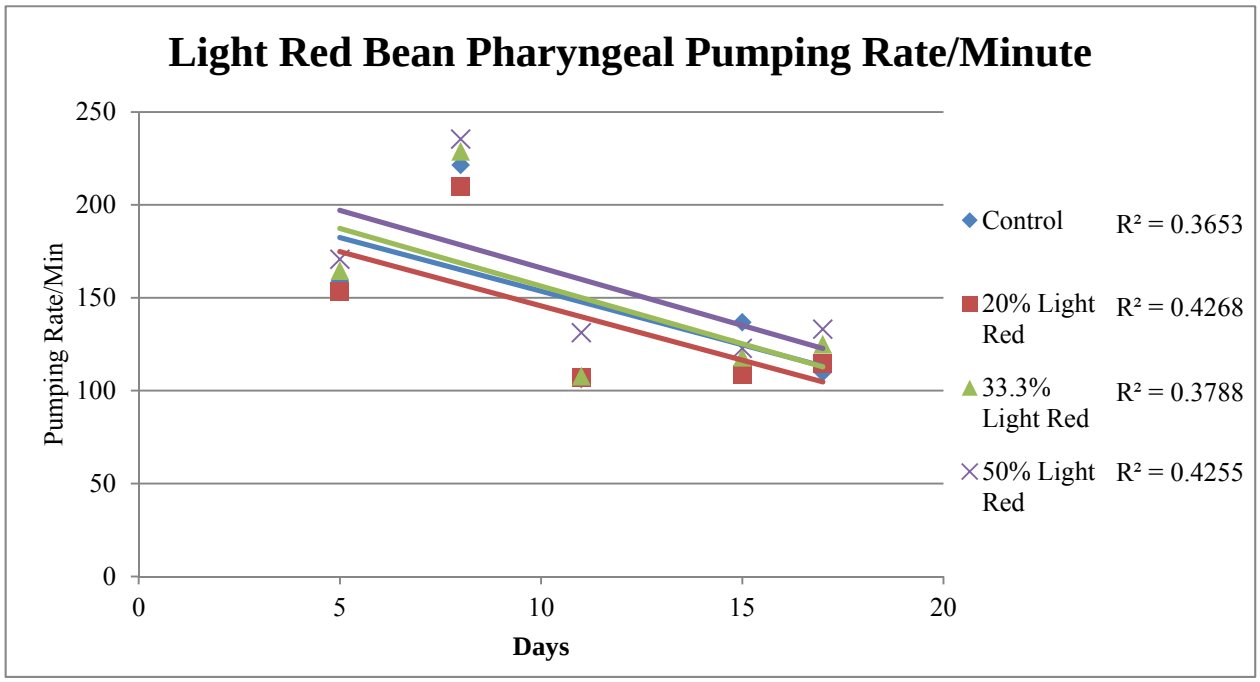

Figure 11. Regression Analysis of Pharyngeal pumping rate of *C. elegans* when fed light red kidney beans. Line colors correspond to marker color.

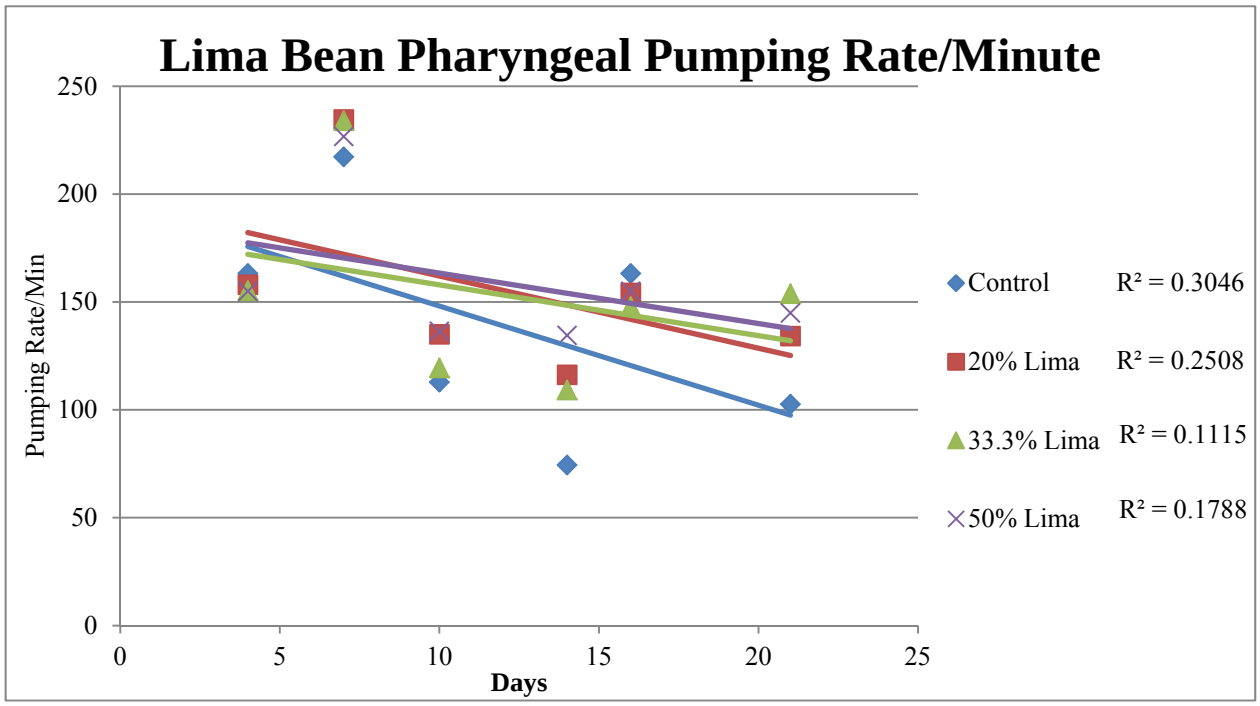

Figure 12. Regression Analysis of Pharyngeal pumping rate of *C. elegans* when fed lima beans. Line colors correspond to marker color.

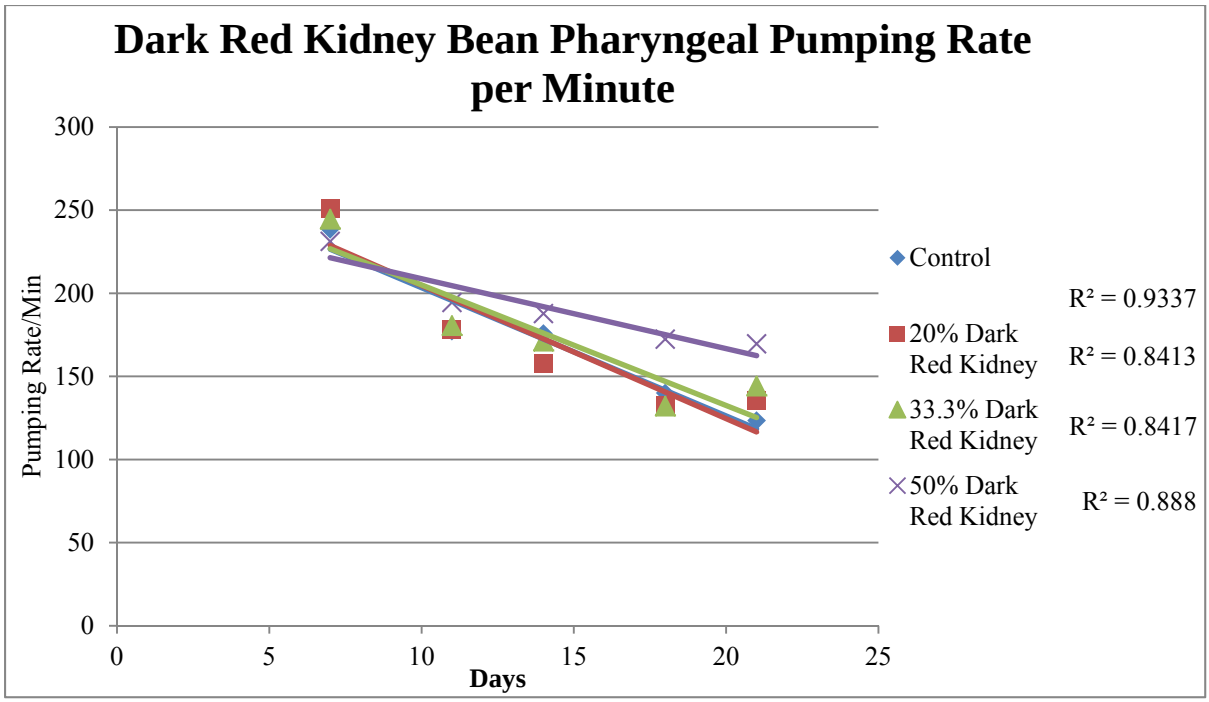

Figure 13. Regression Analysis of Pharyngeal pumping rate of *C. elegans* when fed dark red kidney beans. Line colors correspond to marker color.

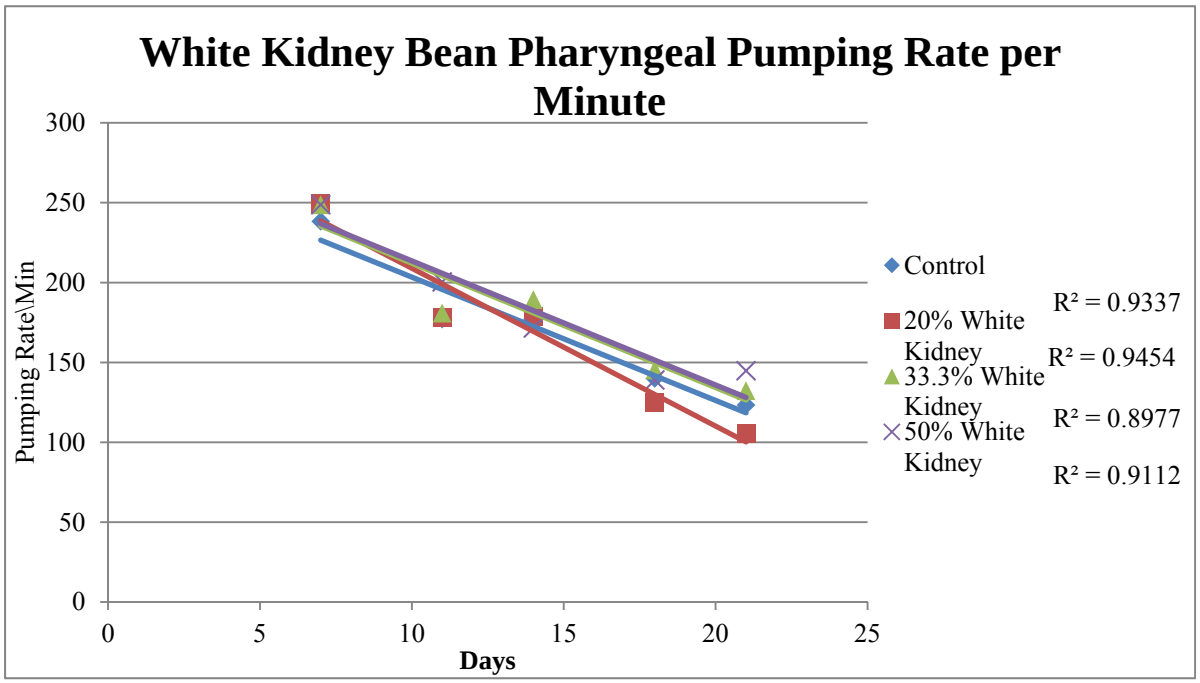

Figure 14. Regression Analysis of Pharyngeal pumping rate of *C. elegans* when fed white kidney beans. Line colors correspond to marker color.

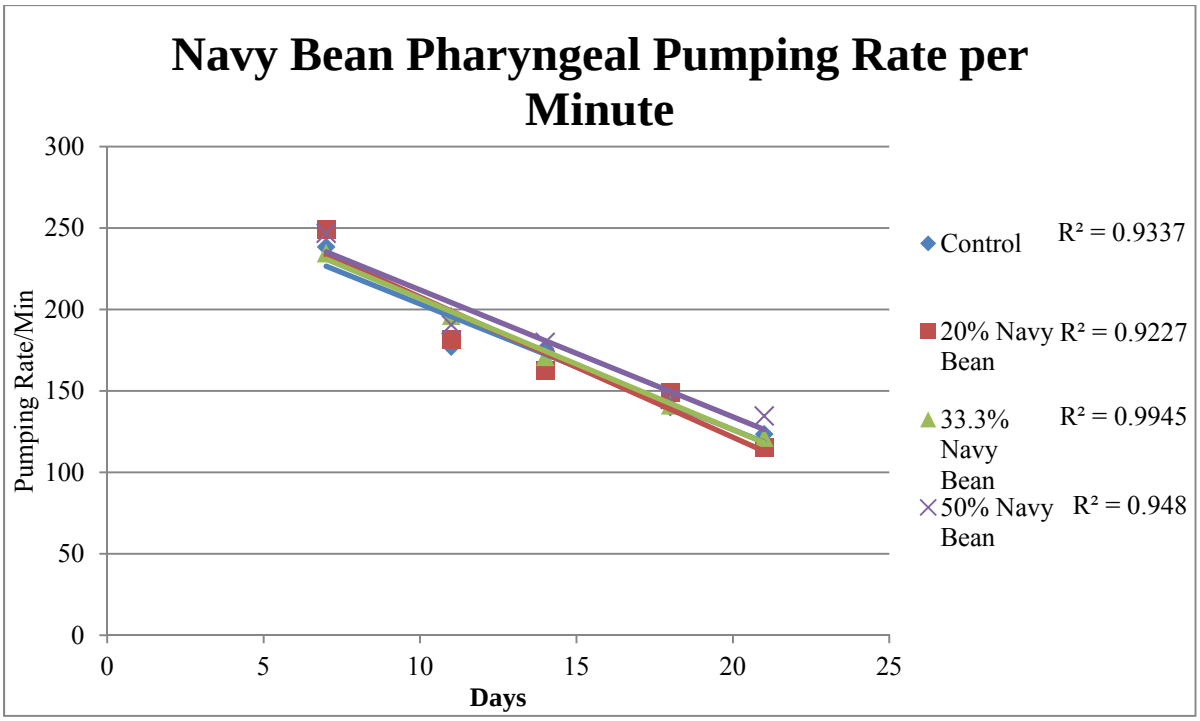

Figure 15. Regression Analysis of Pharyngeal pumping rate of *C. elegans* when fed navy beans. Line colors correspond to marker color.

statistically significant differences between legume and control groups. Lentil, cranberry, great northern, lima, lentil, cranberry and great northern beans show a similar travel distance among all groups on all days of observation.

Consumer Study with National School Lunch Program Participants

In the first question "What is a hamburger?" most students indicated that hamburgers are meat (beef or turkey) with bread and condiments. In the second question "What is a hamburger made of?" a majority of students indicated beef, turkey, bread, seasonings and condiments. In the third question "What do you like about hamburgers?" participants responded: cheese, seasonings, condiments, and taste/flavor. In the fourth question "How often do you eat hamburgers?" the results were more varied ranging from once a day to once a month. The major attributes of hamburgers identified from this study with 7th graders include: spices, taste/flavor, condiments, cheese, meat, and bread.

Table 6. The distance traveled in micro meters by *C. elegans* across the NGM agar plates. *Indicates data that is not available due to computer error. This data is expected to be similar to other data points on these days because *C .elegans* had not yet received treatment. †Compare to Control 1. ▪Compare to Control 2. §Compare to Control 3. **Indicates statistically significant ($p<0.05$)

Treatment		Day 4-6		Day 16-18	
		Mean	SEM	Mean	SEM
20%	†Lentil	30.29	3.41	10.80	1.40
33.3%		28.33	3.47	14.92	1.66
50%		26.01	3.20	22.71	1.72
20%	†Cranberry	32.70	3.86	11.40	2.27
33.3%		28.14	2.47	15.03	2.08
50%		24.19	2.33	20.42	1.96
20%	†Great Northern	29.60	2.91	12.74	1.85
33.3%		22.12	2.08	15.06	1.76
50%		24.14	2.54	17.38	1.94
Control 1		28.91	3.76	10.50	2.24
20%	▪Black	44.87**	5.49	17.32	1.69
33.3%		34.55**	2.13	17.45	1.51
50%		42.27**	3.32	17.77	1.87
20%	▪Light Red Kidney	36.17**	4.02	16.65	1.47
33.3%		43.47**	4.26	20.17	1.63
50%		82.96**	8.90	17.01	1.86
20%	▪Lima	28.04	4.08	19.92	1.50
33.3%		44.74	3.11	20.21	1.74
50%		48.51	4.30	16.80	1.68
Control 2		19.81	1.83	17.23	1.61
20%	§Lentil	*	*	18.12	1.58
33.3%		*	*	15.18	1.54
50%		*	*	21.94	2.15
20%	§Cranberry	*	*	17.17	1.73
33.3%		*	*	18.54	1.95
50%		*	*	19.90	1.62
20%	§Great Northern	*	*	19.75	1.77
33.3%		*	*	19.86	2.07
50%		*	*	17.38	2.88
Control 3		*	*	16.83	1.40

Discussion

Nile Red Fluorescence

Nile red dye is an excellent medium to quantify fat deposition because it has fluorescent and lipophilic properties.[82] The method of Nile red staining has been evaluated for accuracy by measuring the actual fat content of the *C. elegans* by extraction of the fat from the animals, separation of the lipids with thin layer chromatography and quantification with gas chromatography, resulting in equivalent data to Nile red fluorescence intensity.[83]

Nile red fluorescence intensity was significantly decreased in cranberry beans, black beans, light red beans, navy beans and white kidney beans. (Figure 5) The correlation factor between the legumes that significantly reduced fat deposition and their respective pharyngeal pumping rates is 0.712. This correlation factor indicates that there are likely additional factors other than the pharyngeal pumping rate (feeding rate) influencing fat deposition. Preceding theories in this field are that as the pharyngeal pumping rate increases, fat deposition increases; however fat deposition is not solely dependent on feeding rate but it is a complexly regulated pathway with hundreds conserved regulatory genes.[83] Srinivasan, *et al.* exogenously administered serotonin targeted at 5-HT receptors, which regulate feeding rate, fat deposition, and other metabolism and food related behaviors, which caused an increase in pharyngeal pumping rate but a decrease in fat deposition.[84] This study confirms that feeding rate (pharyngeal pumping rate) is not solely responsible for fat deposition and that hormones such as serotonin may have had an effect on fat deposition.

The decreased fat deposition in *C. elegans* in this study could be due to a combination of the phenolic content, the micro and macronutrient content of the legumes and the oligosaccharide content. The

mean macronutrient content of the legume solution is as follows: 0.000126g/mL fat, 0.00228g/ml protein, 0.0056g/ml carbohydrate and 0.00033g/mL fiber (calculated from unpublished data from the Foods for Health Laboratory). The mean macronutrient content of the *E. coli* OP50 at $1x10^6$cfu/mL is as follows: 0.0067g/mL fat, 0.1g/mL protein and 0.012g/mL carbohydrate.[85] The macronutrient content of the legumes are all very similar while the micronutrient and phenolic content of the legumes are different[49, 86-87]; this indicates that the micronutrient and phenolic content of the different legumes may play a role in decreasing fat deposition. However, this theory is inconclusive in literature.[49, 86-87] The literature that analyzed the phenolic content of black, navy and cranberry beans (the three beans that significantly lowered Nile red fluorescence) indicates that black beans have an anthocyanin content of 44.5mg/100g while navy and cranberry beans have been reported to contain little to no flavonoids.

The resistant starch content of the following legumes that have been cooked and lyophilized is as follows: common bean 2.33±1.23g/100g and lentils 2.46±0.16g/100g; the resistant starch content of common beans and lentils is not statistically different.[88]

The decreased fat deposition in *C. elegans* in this study is thought to be due to the oligosaccharide content of legumes and the ensuing gut fermentation. When the oligosaccharides present in legumes are fermented in the gut, short-chain fatty acids are produced. Zheng, *et al.* reported a lower intestinal fat deposition in *C. elegans* after feeding short-chain fatty acids.[89] Gut fermentation of dietary resistant starch in rodents has been evidenced to cause the production of Glucagon-like peptide-1(GLP-1) and peptide YY (PYY) which function as anti-diabetes/anti-obesity hormones.[90] Humans fed a highly viscous, fermentable fiber for 14 weeks along with a restricted calorie intake had increases in GLP-1 and PYY.[91] The gut fermentation of beans by *C. elegans* may have an effect of

lowering intestinal fat deposition. Therefore, it is hypothesized that the micronutrient, phenolic or oligosaccharide content of black and navy beans could be the cause of decreased fat deposition.

Pharyngeal Pumping Rate

Pumping rate in the terminal bulb of the pharynx is a direct correlate of the feeding rate of the organism. However this feeding mechanism is also highly correlated to aging and it is proven be higher at a younger age and lower at a more advanced age.[29, 92-93] Pharyngeal pumping rates in young *C. elegans* can be as high as 250-300 per minute. It is hypothesized that the decline in pumping rate may be due to sarcopenia.[94-95] By comparing the mean pumping rate of each treatment group to the mean pumping rate of the control group, the effect of legumes on pharyngeal pumping rate was observed. Under the conditions of the present study, an ideal pumping rate for *C. elegans* in the first five days of life may be as high as 250-300 pumps per minute. In the adult phase (days 6-15) an ideal pumping rate for the *C. elegans* would be 150-240 pumps per minute. As the *C. elegans* reach the end of their lives an ideal pumping rate would be 100-150 pumps per minute. A pharyngeal pumping rate in these ranges may correlate to increased longevity. The trend in cranberry beans, lentils, and dark red kidney beans of higher pumping rate compared to the control indicates that the legumes had an influence on decreasing the signs of aging in *C. elegans*. This suggests legumes may improve longevity in *C. elegans*.

There are several genes responsible for longevity in *C. elegans*. Insulin signaling, serotonin receptors, and Ca MKII pathway for osmotic stress resistance are all implicated as mechanisms for increased longevity.[96] The genes *age-1* and *daf-2* are also hypothesized to be involved in regulating longevity through the mechanism of insulin signaling.[97-98] Lipid metabolism is implicated in having a role in longevity through the regulation of CTBP-1.[99]

It is hypothesized that the change in diet composition from a diet from 100% *E. coli* OP50 to a diet diluted with 20%, 33.3% or 50% legumes appears to have a role in increasing longevity. The *E. coli* solution is higher in protein than the legume solution; the change in macronutrient content could have had an impact on longevity. The composition of *E. Coli* is approximately 43% protein 3% fat and 7% carbohydrates by dry weight.[85] Unpublished data from the Foods for Health Laboratory at Louisiana State University quantifies legumes to be 20-25% protein (lentils at 24.64±0.45% and navy beans at 20.16±0.15% with the other legumes in the study ranging between these two values) and carbohydrates (by difference) were found to be 50-62%.[100] From the results of this study it is known that a diet of 50% legume solution increased pharyngeal pumping rate which is a surrogate marker of lifespan. Previous studies have shown that the bacterial lawn influences longevity in *C. elegans* because of food preference and choice of the *C. elegans* for bacteria.[101] In other studies that altered the food source of *C. elegans* to include other experimental foods, blueberry proanthocyanidin significantly increased lifespan by 28%.[102] In another study, acrylamide was fed at the low dose 0.5 μg/L, and was found to significantly decrease lifespan.[27] These blueberry and acrylamide studies show that longevity in *C. elegans* can be positively or negatively affected by the diet.

Travel Distance

There was no significant difference in travel distances among treatment and control groups and in the majority of legume treatment groups. These results indicate that there wasn't a significant disturbance in the diet quality when fed legume material. *C. elegans* have the ability to locate their food and then determine if it is a high quality food source based on its nutritional value.[101] Because the travel distance wasn't significantly different among the *C. elegans* on days other than the first day of

observation when worms had not yet received any diet treatment, this suggests that the food source was adequate and did not lead to a diet restricted state.

A large travel distance has been observed by others as "roaming". [103] *C. elegans* tend to "dwell" when there is a high concentration of food and tend to "roam" when food is scarce, and according to the data published, the amount of bacteria (*E. Coli* OP50) used in this experiment in both treatment and control groups should have kept the worms in a "dwelling" state. However, it is also reported that an internal metabolic perception of the foods nutritional value has to be assessed by *C. elegans* to induce "dwelling" behavior.[104] This "roaming" behavior was not seen in this study.

Consumer Study

The consumer study in 7th graders revealed the attributes that middle school students in Baton Rouge, Louisiana find important in a hamburger product. The students felt strongly about the inclusion of strong flavors from seasonings, cheese and condiments in a hamburger product. After presenting the students with a meat and bean patty, students in both groups commented that the patty needed more seasoning and suggested "Tony Chachere's." However, this may be a regional preference for salty and spicy food. More research is needed in other parts of the country to determine the preferences in different regions. Many of the students indicated an alternative meat such as turkey when asked "What a hamburger is made of?" which indicates their potential willingness to accept a hamburger product that is not 100% beef. The results from this student interview give guidance to future research.

Implications into Human Diet and Health

In accordance with the theory of Darwinian Fitness, *C. elegans* and other low entropy species such as mice have a greater potential for their lifespans to be affected by changes such as altering

macronutrients, micronutrients and food quantity such as in a diet restriction state.[105] Therefore, in the *C. elegans* model we may observe a magnified response in lifespan change with an alteration in diet. This magnified response may reveal mechanisms of life extension and health that are undetectable in humans. The *C. elegans* model has confirmed the role of beans in a healthy diet at levels of 20%, 33.3% and 50% legume. These results are difficult to translate into the human diet because an equivalent amount of legume fed at 50% of the diet as a 1% solution for a normal human would be a small amount of legume, which was be predicted to be insignificant. This highlights the sensitivity of the model system *C. elegans.* In previous studies 69-150 grams/day of legumes were fed to human subjects with the results of reducing cardiovascular risk and obesity.[106] However, because the results of this study indicate that the pharyngeal pumping rate of *C. elegans* was increased by the consumption of legumes, particularly at 50% of the diet, and it is known from previous studies that legumes promote good health[10, 13, 30-31, 34-35] it is extrapolated that the mechanism for increased longevity seen in *C. elegans* is seen in humans as general health benefits (lower weight, lower cholesterol, and improved blood glucose control). The low entropy species, *C. elegans,* allows us to screen the effects of legumes on increased longevity and decreased obesity where as these effects haven't been as largely visible in humans.

Black beans, light red beans and cranberry beans are selected as the top 3 beans in this screening study for their role in reducing fat deposition and increasing pharyngeal pumping rate. Also shown in this screening study is that a diet of 50% legumes contributed to increased health benefits in *C. elegans* model.

Conclusions

This data is evidence that legumes may contribute to a healthy diet, based on reduced fat deposition and sustained lifespan (increased pumping rate) of *C. elegans* possibly through the regulation of the insulin signaling pathway, serotonin receptors, and Ca MKII pathway for osmotic stress resistance, and lipid metabolism. It is hypothesized that macronutrient change in the diet, phenolic content of the legume diet, or the oligosaccharide content of legume diet were responsible for the health benefits seen in *C. elegans*. More research is needed to determine the exact mechanism of action for life extension and reduced fat deposition in *C. elegans*.

Furthermore, the *C. elegans* model has been proven useful for screening whole foods for potential health benefits. This model system could be employed to screen a variety of whole foods that are reported to have health benefits such as olive oil, chocolate, grapes, acaí berries, tomatoes, beets, etc..

In the present study legumes have been proven to sustain lifespan and decrease intestinal fat deposition in *C. elegans*. Previous studies demonstrate that legumes are beneficial in the diet of humans. It is suggested that legumes be consumed frequently as part of a varied diet to improve health. This applied project can help consumers and potentially children participating in the NSLP to consume more legumes and to obtain better health.

Future Work

The consumer study conducted with 7th grade middle school students in Baton Rouge, LA served the purpose of obtaining attributes that are important in hamburgers to this population. These attributes will enable us to conduct additional sensory testing including difference and preference testing. The list of important attributes generated in this study will help determine the acceptability of the meat and bean hamburger with this population. Further sensory testing, including difference and preference testing is expected to be completed at Louisiana State University on the meat patty project. Because of the many health benefits of legumes, they lend themselves well to application in many food products. Currently the Foods for Health Research Team has developed the following foods with included legumes: sausage patties, meat patties, hot dogs, bagels, bread, pasta, brownies, cookies, nutritional bars and pizza crust. These products are aimed at the National School Lunch Program population because of their nutritional benefits (legumes are high in fiber, low in fat, and a good source of protein) as well as their reduced cost over conventional products. The meat patty was chosen to fully optimize and to conduct sensory research with because it is one of the most difficult foods to optimize. An entire school breakfast and lunch menu is being developed with the goal of providing at least 1 hidden serving of legumes per day totaling to 5 servings a week. These menus will highlight the foods developed in the Foods for Health Lab. A clinical trial to demonstrate the health benefits of consuming a diet high in legumes (>177g/d delivered by analogs to conventional foods developed by the Foods for Health Research team) is also planned.

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Appendix: Supplementary Codes, Charts and Forms

SAS Code

```
dm'output;clear;log;clear';
TITLE1 'FLUORESCENCE Analysis of Variance One-way ANOVA';
options ps=256 ls=99 nocenter nodate nonumber nolabel;

ods rtf file= 'E:\C. elegans\one way anova.rtf';

proc print data=WORK.FLUORESCENCE;
run;

proc mixed data=WORK.FLUORESCENCE;
class TREATMENT;
Title2 'FLUORESCENCE 1 WAY ANOVA with PROC MIXED';
model INTENSITY=TREATMENT  /HType=3 ddfm=Satterth outp=resids;
LSMEANS TREATMENT / PDIFF ADJUST=TUKEY;
ods output diffs=ppp lsmeans=mmm;
run;

proc univariate data=resids normal plot; var resid;
TITLE4 'Univariate analysis of Residuals';
run;

ods rtf close;


dm'output;clear;log;clear';
TITLE1 'Distance Analysis of Variance Two-way ANOVA';
options ps=256 ls=99 nocenter nodate nonumber nolabel;

ods rtf file= 'E:\C. elegans\two way anova.rtf';

proc print data=WORK.DISTANCE;
run;

proc mixed data=WORK.DISTANCE;
class DAY TREATMENT;
Title2 'DISTANCE 2 WAY ANOVA with PROC MIXED';
model DISTANCE=TREATMENT DAY TREATMENT*DAY /HType=3 ddfm=Satterth outp=resids;
LSMEANS TREATMENT DAY TREATMENT*DAY / PDIFF ADJUST=TUKEY;
ods output diffs=ppp lsmeans=mmm;
run;

proc univariate data=resids normal plot; var resid;
TITLE4 'Univariate analysis of Residuals';
run;

ods rtf close;


dm 'output;clear;log;clear';
Title1 'Simple Correlation, Fluorescence and Pumping Rate;
```

```
options ps=256 ls=99 nocenter nodate nonumber nolabel;

ods rtf file= 'E:\C. elegans\SAS\correlation fluorescence and PR.rtf';

data FLUORESCENCE_PUMPINGRATE;
INPUT Fluorescence PumpingRate;
cards;
4.06 -3.96
6.53 -5.78
24.1 14.52
29.75 34.88
15.67 -5.08
13.62 -4.40
10.52 21.45
13.59 -0.75
7.95 1.42
13.66 7.85
;

proc print data=FLUORESCENCE_PUMPINGRATE;
run;

proc corr data=FLUORESCENCE_PUMPINGRATE;
Title2 'Correlation of Significantly Lower Nile Red Fluorescence to Pumping Rate
Overall Trend';
run;

ods rtf close;


dm 'output;clear;log;clear';
Title1 'Simple Correlation, Fluorescence and Treatment';
options ps=256 ls=99 nocenter nodate nonumber nolabel;

ods rtf file= 'E:\C. elegans\SAS\correlation fluorescence and treatment.rtf';

proc print data=FLUORESCENCE_TREATMENT;
run;

proc corr data=FLUORESCENCE_TREATMENT;
Title2 'Correlation of Nile Red Fluorescence to Treatment';
run;

ods rtf close;


dm'output;clear;log;clear';
TITLE1 'CRAN, LENTIL, GN Analysis of Variance Two-way ANOVA';
options ps=256 ls=99 nocenter nodate nonumber nolabel;

ods rtf file= 'E:\C. elegans\two way anova.rtf';

proc print data=WORK.CRGNLN;
run;

proc mixed data=WORK.CRGNLN;
```

```
class DAY TREATMENT;
Title2 Cran, lentil, Great Northern 2 WAY ANOVA with PROC MIXED';
model RATE=TREATMENT DAY TREATMENT*DAY /HType=3 ddfm=Satterth outp=resids;
LSMEANS TREATMENT DAY TREATMENT*DAY / PDIFF ADJUST=TUKEY;
ods output diffs=ppp lsmeans=mmm;
run;

proc univariate data=resids normal plot; var resid;
TITLE4 'Univariate analysis of Residuals';
run;

ods rtf close;



dm'output;clear;log;clear';
TITLE1 'Black Bean, Lentil, Light Red Analysis of Variance Two-way ANOVA';
options ps=256 ls=99 nocenter nodate nonumber nolabel;

ods rtf file= 'E:\C. elegans\two way anova.rtf';

proc print data=WORK.BKLRL;
run;

proc mixed data=WORK.BKLRL;
class DAY TREATMENT;
Title2 'Black, Lt. Red, Lentil 2 WAY ANOVA with PROC MIXED';
model RATE=TREATMENT DAY TREATMENT*DAY /HType=3 ddfm=Satterth outp=resids;
LSMEANS TREATMENT DAY TREATMENT*DAY / PDIFF ADJUST=TUKEY;
ods output diffs=ppp lsmeans=mmm;
run;

proc univariate data=resids normal plot; var resid;
TITLE4 'Univariate analysis of Residuals';
run;

ods rtf close;


dm'output;clear;log;clear';
TITLE1 'White Kidney, Navy, Dark Red Analysis of Variance Two-way ANOVA';
options ps=256 ls=99 nocenter nodate nonumber nolabel;

ods rtf file= 'E:\C. elegans\two way anova.rtf';

proc print data=WORK.WKNDR;
run;

proc mixed data=WORK.WKNDR;
class DAY TREATMENT;
Title2 'White Kidney, Navy, Dark Red 2 WAY ANOVA with PROC MIXED';
model RATE=TREATMENT DAY TREATMENT*DAY /HType=3 ddfm=Satterth outp=resids;
LSMEANS TREATMENT DAY TREATMENT*DAY / PDIFF ADJUST=TUKEY;
ods output diffs=ppp lsmeans=mmm;
run;

proc univariate data=resids normal plot; var resid;
```

```
TITLE4 'Univariate analysis of Residuals';
run;

ods rtf close;



dm'output;clear;log;clear';
TITLE1 'Sorted by 20%,33.3 and 50% Analysis of Variance One-way ANOVA';
options ps=256 ls=99 nocenter nodate nonumber nolabel;

ods rtf file= 'E:\C. elegans\sorted one way anova.rtf';

proc print data=WORK.ALLBEANS;
run;

proc sort data=WORK.ALLBEANS; by CONCENTRATION; run;

proc print data=WORK.ALLBEANS;
run;

proc mixed data=WORK.ALLBEANS;
class TREATMENT;
Title2 'FLUORESCENCE 1 WAY ANOVA with PROC MIXED';
model RATE=TREATMENT  /HType=3 ddfm=Satterth outp=resids;
LSMEANS TREATMENT / PDIFF ADJUST=TUKEY;
ods output diffs=ppp lsmeans=mmm;
run;

proc univariate data=resids normal plot; var resid;
TITLE4 'Univariate analysis of Residuals';
run;

ods rtf close;
```

Parental Research Consent Form

I, _____, agree to allow my child _____to
Please Print Please Print
participate in the research entitled "Foods for Health, Meat Patty with Added Legumes" which is being conducted by Dr. John Finley of the Department of Food Science at Louisiana State University, phone number (225)578-5207. For this particular research about 45 minutes of time will be required during an elective period at school. I understand that my child's participation is entirely voluntary and whether or not they participate will not affect my child's status with their school or LSU. It will be a short focus group where my child will sample a meat patty with added beans and give his/her opinion.
The following will **EXCLUDE** my child:

1. A beef or dry bean (pinto, etc.) **ALLERGY**.
2. A religious or social preference against eating beef.
3. Child **DOESN'T** like **HAMBURGERS** or doesn't participate in the **SCHOOL LUNCH PROGRAM**.

The only **RISKS** foreseen in this study are complications due to beef or dry bean allergy.
The following **PRECAUTIONS WILL BE TAKEN** to protect your child:

- The meat patty will be cooked to an internal temperature of 160◦F, measured with a thermometer, just as in the school cafeteria.
- Excluding children from the study who are allergic to wheat, beef or dry beans.

Privacy:
The results of this study will not be released in any identifiable form without my prior consent unless required by law.

Questions:
The child's teacher has explained the project and the investigator will answer any further questions about the research, either now or during the course of the project. Carla Sandlin (225)578-5207. Email: csandl1@lsu.edu

The study has been discussed with me, and all of my questions have been answered. I understand that additional questions regarding the study should be directed to the investigators Carla Sandlin or Dr. John Finley. In addition, I understand the research at Louisiana State University AgCenter that involves human participation is carried out under the oversight of the Institutional Review Board. Questions or problems regarding these activities should be addressed to Dr. David Morrison, Assistant Vice Chancellor of LSU AgCenter at (225)578-4182.
I agree with the terms above.

_____ _____
Signature of Parent Date

Student Research Assent Form

I _____ am willing to participate in the focus group
Please Print

with prior permission from my parent/guardian. I have given this permission form to the investigator. The study has been discussed with me and all of my questions have been answered.

_____ _____
Student Signature Date

_____ _____
Investigator Signature Date

Student Response Sheet for Consumer Study

1. What is a Hamburger?

_____

_____

2. What is a hamburger made of?

_____

_____

3. What do you like about hamburgers?

_____

_____

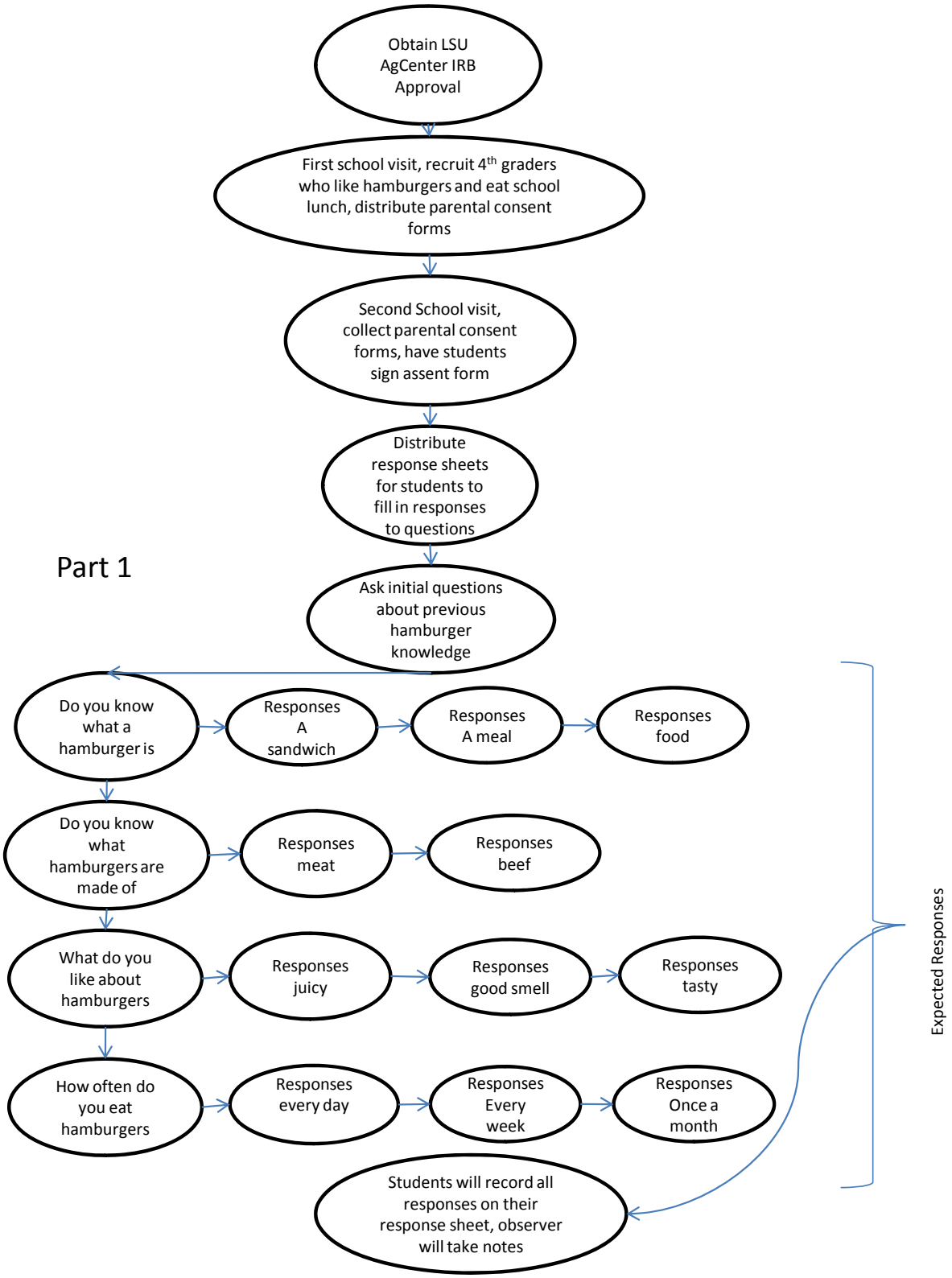
4. How often do you eat hamburgers?

_____

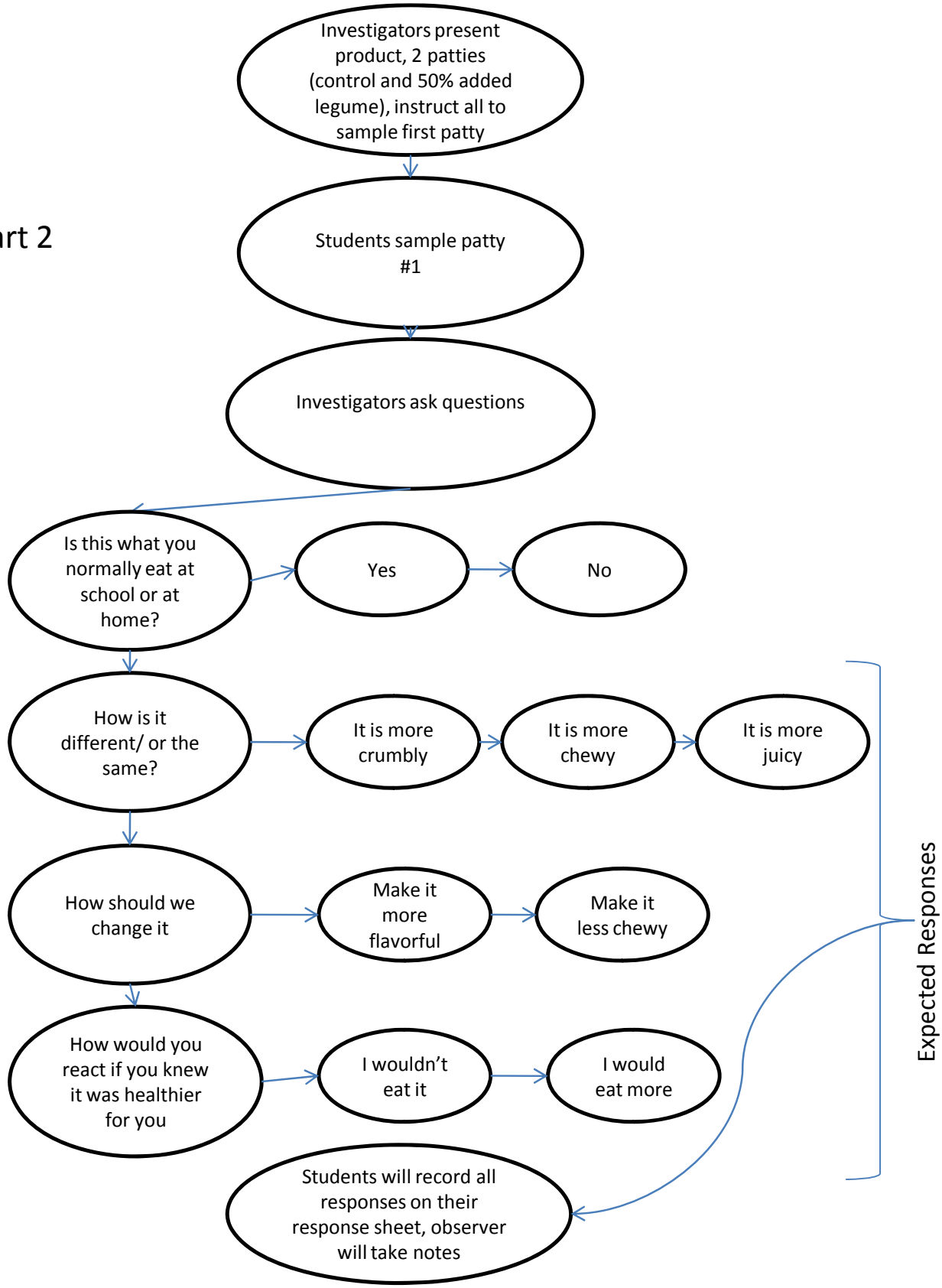
_____

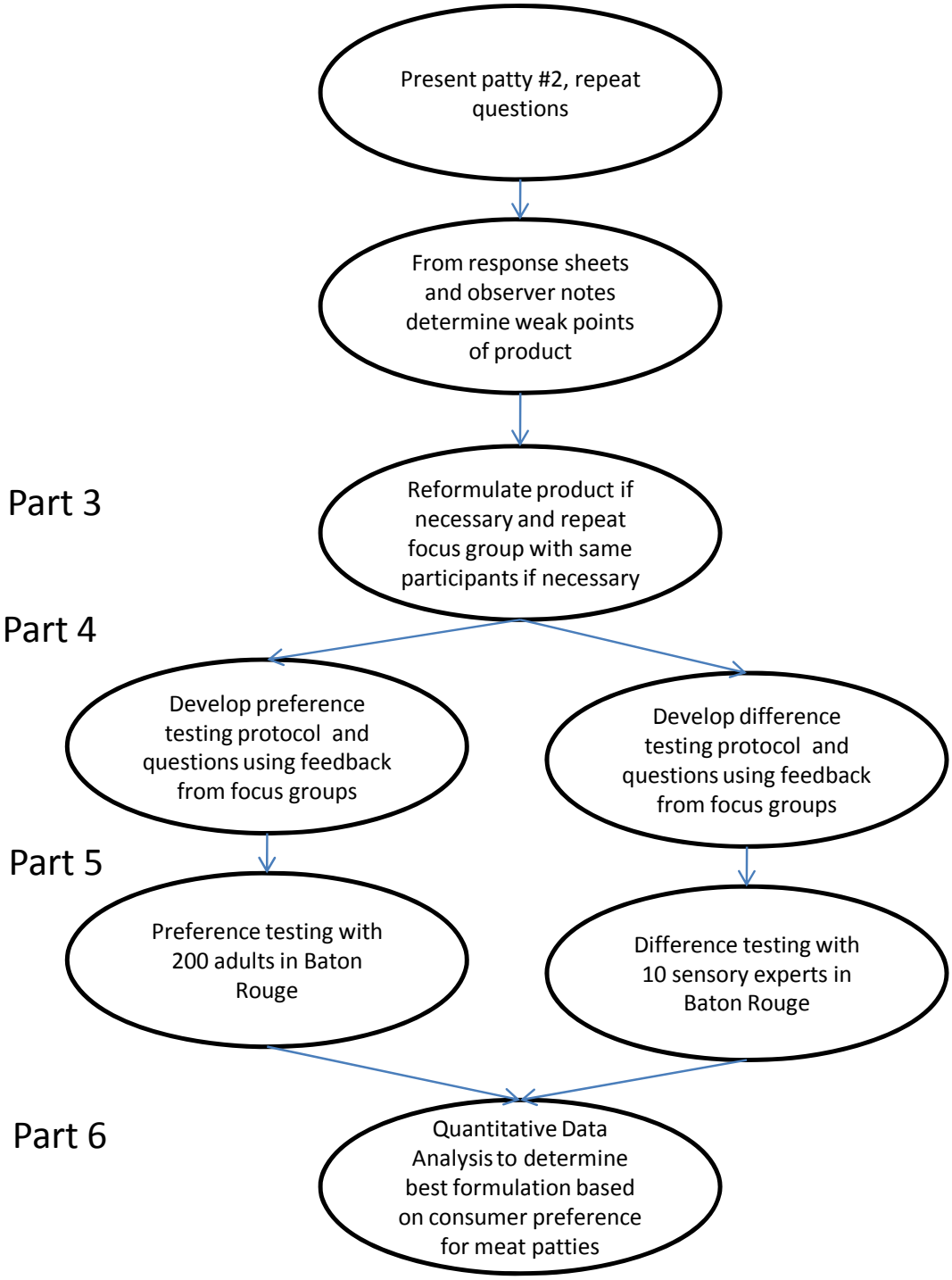
Sensory Design

1) Protocol:



Part 2


Investigators present product, 2 patties (control and 50% added legume), instruct all to sample first patty
Students sample patty #1
Investigators ask questions
Is this what you normally eat at school or at home?
Yes
No
How is it different/ or the same?
It is more crumbly
It is more chewy
It is more juicy
How should we change it
Make it more flavorful
Make it less chewy
How would you react if you knew it was healthier for you
I wouldn't eat it
I would eat more
Students will record all responses on their response sheet, observer will take notes
Expected Responses


Present patty #2, repeat questions
From response sheets and observer notes determine weak points of product
Part 3
Reformulate product if necessary and repeat focus group with same participants if necessary
Part 4
Develop preference testing protocol and questions using feedback from focus groups
Develop difference testing protocol and questions using feedback from focus groups
Part 5
Preference testing with 200 adults in Baton Rouge
Difference testing with 10 sensory experts in Baton Rouge
Part 6
Quantitative Data Analysis to determine best formulation based on consumer preference for meat patties

Vita

Carla Michele Sandlin was born in November of 1986, in Jackson, Mississippi. Her parents are Lisa and Gary Sandlin. She moved to Port Lavaca, Texas, at the age of three where she later graduated high school.

She then attended the University of Texas at Austin where she graduated with a bachelor's degree in nutrition. Here, she completed her dietetic internships, passed her examination and received her dietetic registration.

She is a candidate for a Master of Science from the Department of Food Science at Louisiana State University and Agricultural and Mechanical College, which will be awarded in December 2010.