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The Effects of Resistant Starch and Fructooligosaccharides on Mineral Absorption in Rats

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The Effects of Resistant Starch and Fructooligosaccharides on Mineral Absorption in Rats

A Thesis

Submitted to the Honors College of Louisiana State University and Agricultural and Mechanical College in partial fulfillment of the requirements for Upper Division Honors Distinction

In

The School of Human Ecology

By:

Katherine Ann Nolen
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ABBREVIATIONS

°C	degrees Celsius
°F	degrees Fahrenheit
AIN-93M	American Institute of Nutrition modified diet
Al	aluminum
ANOVA	analysis of variance
BHT	butylated hydroxytoluene
C	control diet treatment group
Ca	calcium
Cu	copper
DSS	dextran sodium sulfate
F	fructooligosaccharide diet treatment group
FDA	Food and Drug Administration
Fe	iron
FOS	fructooligosaccharide
g/cm²	grams per centimeter squared
g/kg	grams per kilogram
GERD	gastroesophageal reflux disease
GI	gastrointestinal
GRAS	generally recognized as safe
ICP	inductively coupled plasma spectrophotometry
IUCAC	Institutional Animal Care and Use Committee
K	potassium

LSU A&M	Louisiana State University and Agricultural and Mechanical College
Lb	pound
Lb*in	pound-inch
Mg	magnesium
Na	sodium
Na/K ATPase	sodium potassium adenosine triphosphate synthase
P	phosphorus
pDEXA	peripheral dual energy x-ray absorptiometry
pH	negative log of hydrogen ion concentration
pKa	negative log of K equilibrium constant for acids
ppm	parts per million
PTH	parathyroid hormone
R	resistant starch diet treatment group
RS	resistant starch
RS1	type 1 resistant starch
RS2	type 2 resistant starch
RS3	type 3 resistant starch
RS4	type 4 resistant starch
S	sulfur
SCFA	short chain fatty acids
SD	standard deviation
TPN	total parenteral nutrition
Zn	zinc

DEFINITIONS

Fructooligosaccharide – a short chain carbohydrate composed of one glucose monomer with two to four fructose monomers

Prebiotic – substance that reaches the large intestine where it can be used to support microfloral proliferation

Resistant starch - a class of complex carbohydrates that “resists” digestion in the small intestine; the amount of starch that remains intact, or not hydrolyzed, after two hours of incubation at 37°C with pancreatin, amyloglucosidase, and invertase

RS1 - natural, raw starch that is physically trapped within the whole plant and food matrices

RS2 - raw starch that is poorly gelatinized and slowly hydrolyzed by pancreatic enzymes

RS3 - retrograded starches

RS4 - chemically modified starch

Splanchnic blood flow – blood flow to the intestinal tract

ABSTRACT

Current increases in gastrointestinal problems are more common in cultures consuming Western diet, which is lower in indigestible starches and fiber. It is believed that increasing fiber intake can improve gastrointestinal (GI) health. Two types of indigestible starches of recent interest are high-amylose resistant starch (R2) and fructo-oligosaccharides (FOS). R2 is a type of starch that is poorly digested in the small intestine, and fermentable by colonic bacteria. FOS is composed of short chains of fructose monomers, is indigestible by humans, and completely fermented by colonic bacteria. Fermentation in the gut produces short-chain fatty acids (SCFA) which are the preferable source of energy by colonocytes. Increased SCFA in the colon can lead to healthier epithelial tissue growth and mineral absorption. For twelve weeks, 24 Sprague-Dawley rats were fed one of three diet treatments, 5% RS (R), 5%FOS (F) or control (C). Percent mineral absorption was calculated during metabolism periods at the start and end of the study. Results showed that at week twelve FOS had increased wet fecal mass ($p=0.027$), increased empty cecal mass ($p=0.000$) and lower cecal pH ($p=0.000$). Both RS and FOS had increased cecal content and empty cecal weights. These results suggest that low levels of FOS have a greater effect than low levels of RS on fecal bulking, SCFA production and mineral absorption.

INTRODUCTION

Overview

Western societies are known to be progressively more industrialized and wealthy, with more comfortable lifestyles and better health care. However, epidemiological studies indicate that these nations suffer from a host of serious chronic diseases that are less common in third world nations. From gastrointestinal problems to osteoporosis to metabolic syndrome, a multitude of serious health problems plague more affluent nations, decreasing the quality of life. Countless studies aim to identify the common factor in industrialized societies that leads to these health problems(1,2), but all fall short. Instead of being one precipitating factor, it is the combination of lifestyle and dietary factors such as decreased physical activity, abundance of refined starches and high fat convenience foods, fast foods, large portion sizes, and poor fruit, vegetable and dairy intakes (1,3, 4).

The most likely causes for poor nutrient intakes in wealthy nations ties in with the fast-paced lifestyle and overabundance of unhealthy convenience foods. Individuals living in industrialized societies, like America, are often too busy to take the time to prepare well-balanced meals, and so rely on convenience and fast foods. Such food products are almost all high in fats, particularly trans-fatty acids and saturated fats, highly refined cereal grains, simple sugars, and extremely low in nutrients. Providing empty calories and appetite stimulation, these foods can lead to dramatic increases in energy intake, leading to overweight or obesity over time. Additionally, highly-refined, low-fiber grains are associated with increased risks of gastrointestinal problems such as diverticulitis, hiatal hernias, constipation, and cholelithiasis (1,3,4).

To meet the nutritional needs of a rapidly growing, fast paced society, convenience foods with health benefits must be developed. Individuals are unlikely to change their lifestyle and revert back to a slow-paced daily schedule, incorporating time to prepare well-balanced meals. Also, many low-income individuals cannot afford pricey fruits, vegetables and whole grains, which are more readily available in more affluent residence areas. Therefore, the food industry must create products that have clear nutritional benefits for normal, healthy individuals that can aid in prevention of chronic disease.

Resistant starch (RS) and fructo-oligosaccharides (FOS) are naturally occurring carbohydrates that have clearly reported health benefits. RS is a poorly digested carbohydrate that is fermented in the large intestine, leading to decreased luminal pH and enhanced mineral absorption. RS results in a much smaller increase in postprandial glucose than a white bread standard (5,6,7). It naturally occurs in food sources such as raw bananas and raw potatoes. FOS is a short chain of fructose monomers, indigestible by human enzymes, and is also highly fermented in the large intestine(2). More conclusive evidence, however, is needed to support enhanced absorption of minerals from RS and FOS.

Justification:

The scientific community needs to find functional foods that the food industry can incorporate into convenience food items for multifaceted health benefits without causing further complications.

Objective

The objective of this study is to use rats as a human model to determine the effects of RS and FOS in bone density, mineral retention, and gastrointestinal (GI) health.

Hypothesis

The hypotheses of the study were as follows:

1. RS and FOS will induce no adverse effects on organ weights, body weight or food intake.
2. RS and FOS will decrease cecal pH and increase cecal wall weight
3. RS and ROS will increase fecal mass
4. RS and FOS will increase mineral absorption
5. RS and FOS will increase bone density

Assumptions

1. Rats are a valid model for human physiology.
2. Amounts of RS and FOS in rat diets are relevant to feasible human consumption levels.
3. Sample size was adequate to detect significant differences.
4. Peripheral dual energy x-ray absorptiometry is a valid method for measuring bone density, abdominal fat, and lean body mass.
5. ICP is a valid test for mineral concentration of urine, diet and dried feces.

Limitations

1. Seven-day age difference between Group 1 and Group 2 could result in age-related mineral absorption differences.
2. Young, male Sprague-Dawley rats are not a good model for osteopenia.
3. Food spillage was estimated, which may have contributed to inaccurate food intake measurements.
4. Water was added to urine during metabolism periods to rinse out metabolism cages.
5. A number of times rats spilled all food, resulting in temporary food deprivation.

6. At times, water bottle stoppers had excessive leaking resulting in inadequate water access to rats, and further resulting in minor weight loss over weekends.
7. Slippage of bones during three-point breaking could result in fatigue of bone when force was applied.
8. Variations in the angle of mounted femur for femoral head breaking could result in inaccurate measurements.
9. Biochemical markers to determine changes in bone mineral density were not measured.

REVIEW OF LITERATURE

Rats as a human model

Using animals as human models is widely accepted in scientific experimentation. It is more ethical to perform extensive testing on animals than on humans, and also more practical. Animal experimentation almost always precedes human experimentation. Once products are proved safe and effective on animals, the next step is controlled studies on humans. These are much more difficult because conditions cannot be controlled as closely as in animal experimentation and also are considerably more expensive. Animal models are widely used when the benefits of therapeutic agents need to be investigated (8). When testing juveniles, there are also liability and ethical issues to consider. Furthermore, surgeries such as the ovariectomization and gastrectomy can be performed on rats to simulate situations of nutritional risk in humans, particularly involving mineral loss. One of the problems with rats and dietary measurements is that rats commonly engage in coprophagy, or ingestion of fecal matter. This, however, can easily be controlled by the environment and rat cage used (9).

Resistant Starch

Resistant starch (RS) is a term used to describe a class of complex carbohydrates that “resists” digestion in the small intestine. Pancreatic enzymes poorly digest these compounds, so they reach the large intestine almost intact (2). Some resistant starches are digested easier than others, and many factors contribute to the digestibility of starch products. There are four main categories of RS based on structure and processing. The first, RS1, consists of natural, raw starch that is physically trapped within the whole plant and food matrices (2). This includes partly milled grains and seeds and is characterized by the physical barrier to

hydrolysis. The second category, RS2, is another type of raw starch that is poorly gelatinized and slowly hydrolyzed by pancreatic enzymes due to the structure of the starch granules and molecules (2, 10). It includes raw potato starch, green banana starch and high amylose cornstarch. The type of starch used in this study was high amylose cornstarch. The third category, RS3, includes retrograded starches such as cooked and cooled rice or potato starch (2,10). Retrogradation is a natural process that occurs after cooked starches have gelatinized, and then revert to their original crystalline structures. Retrograded starches are poorly hydrolyzed because of the complex rigid structure. Finally, the last category of RS is a chemically modified starch, RS4, which is chemically esterified or has added ethers, fatty acids or amino acids (2). Modified starches are widely used in processed foods. Other factors may affect the degree of digestibility of RS such as processing, cooking conditions, mastication, acidity, and individual differences in digestive enzymes and peristalsis (11). All of these factors affect digestibility by modifying the starch structure.

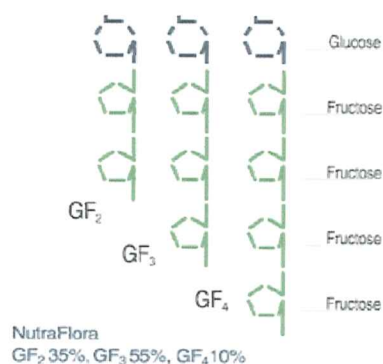
The portion of the starch that reaches the large intestine is fermented by microflora in the colon. Byproducts of microbial fermentation of carbohydrates are various short-chain fatty acids (SCFA) (2,12,13,14,15). RS content of foods is measured chemically by the amount of starch that remains intact, or not hydrolyzed, after two hours of incubation at 37°C with pancreatin, amyloglucosidase, and invertase (2). Unfortunately, the RS content of most foods is not determined except in convenience foods prepared with RS as an ingredient (11). Overall, with naturally occurring RS, it appears the more starch that is eaten, the more that enters the colon (2).

Fructooligosaccharides

Fructooligosaccharides (FOS) are another type of carbohydrate that is poorly digested

by pancreatic enzymes. FOS is considered a type of prebiotic, which is a short-chain length carbohydrate that reaches the cecum of animals by resisting digestion by gastric and pancreatic acids. Naturally, FOS is found in plants such as onions, asparagus, wheat, rye, triticale and Jerusalem artichokes (6,7). Just as RS, FOS is fermented by the colonic bacteria, producing short-chain fatty acids (6,7). Because it is an oligosaccharide, FOS is hygroscopic and sweet to taste. The FOS used in this study is Nutraflora®, which is composed of a glucose monomer connected to two to four fructose monomers by β -1,2 glycosidic bonds (18). Figure 1 is a diagram of the chemical composition of FOS. Nutraflora® is the only brand of FOS that is approved by the Food and Drug Association and Generally Recognized as Safe (GRAS). It is supplemented into beverages, pudding and energy bars (18, 19). The three oligosaccharides in the Nutraflora FOS mixture are kestose (GF₂), nystose (GF₃), and fructosyl-nystose (GF₄) (6).

Figure 1: Nutraflora Fructooligosaccharide (18)



Fermentation of RS and FOS

The numerous benefits of RS and FOS are largely due to the increased fermentation by intestinal microflora. Since they escape digestion in the small intestine, the carbohydrates are fermented in the large intestine by colonic bacteria, producing short-chained fatty acids (SCFA) (2, 20,21). These SCFA are the preferred energy source for colonocytes.

(2,21,22,23,) There are over 400 species of bacteria identified in human feces, and the greatest bacterial proliferation is in the proximal large intestine (2). Of the colonic anaerobes, the most common are bacteroides, bifidobacteria, eubacteria, streptococci and lactobacilli. May et al and Topping et al (2,24) reported that diet is the primary determinant of the effect of fiber on large intestine microflora and SCFA concentration. *Bifidobacteria* is one genus that is known to be beneficial in the GI tract, and is stimulated by dietary FOS (23,19,21). Also, the low pH due to SCFA helps create an unfavorable environment for proliferation of pathogenic bacteria, especially in high-risk patients, who have decreased GI immunity (23). Greater amounts of fermentable carbohydrate entering the large intestine aids in bacterial proliferation (2, 25). Thus, the more abundant colonic microflora produces greater amounts of SCFA's in the large intestine. As the undigested starch passes through the lumen of the proximal colon, the bacteria ferment the substrate producing large amount of acetate, propionate and butyrate. Short chain fatty acids are relatively weak acids, with pKa values around 4.8 (2,24). Greater degrees of fermentation resulting in higher concentrations of SCFA's in turn lower the pH of intestinal digesta.(2) Other dietary components that reach the large intestine, such as calcium, are known to have a buffering capacity in the gut. However, carbohydrate fermentation is found to have an overriding effect (2).

Less than 5% of SCFA's are found in fecal samples due to colonic uptake. (2) SCFA's are taken up by the intestinal mucosa by two mechanisms; passive diffusion and ionized cotransport. The SCFA's can passively diffuse across the epithelial membrane in the proximal colon based on a concentration gradient of protonated fatty acids. Since the protonated SCFA's passively diffuse into colonocytes in a concentration-dependent manner,

a progressive decline in concentration is seen along the large intestine (26,27). The other mechanism for absorption is by Na^+ and K^+ cotransport with ionized SCFA. Rectal infusion with SCFA's has displayed greater mineral uptake and increased splanchnic blood flow (2,22,25). The increased SCFA supply due to fermentation stimulates growth of mucosal and colorectal cells. Animal studies have shown that supplementation of SCFA in TPN can slow mucosal atrophy commonly seen after massive bowel resections (21). Progressive fermentation promotes colonocyte health, making the body more resistant to disease, and also helping those in compromised gastrointestinal states to return to their previous colon health.

Since over 95% of SCFA's are absorbed, in vivo studies in humans are difficult. Information from rat studies is generally limited to measurements of fecal matter and digesta after a period of RS or FOS feeding. Many studies report an increased weight of large bowel digesta and decreases in pH of cecal or large intestinal contents (2,12,13,15). Controlled human studies are generally limited to measurements in feces or by the hydrogen breath test. Hydrogen gas is an intermediate fermentation product of many different types of intestinal bacteria (2). The amount of H_2 produced is proportional to the bacterial proliferation. Hydrogen gas is easily dissolved in the bloodstream and then expelled through the lungs. A lack of effect of RS and FOS is reported at low doses, possibly because they are fermented rapidly and then the fermentation byproducts are absorbed in the proximal colon.

Functionality of RS and FOS

Currently, due to insufficient research in humans, there are no FDA recommendations for either RS or FOS. The RS and FOS content of foods remains largely unknown.(2, 11) Nevertheless, much research is being done on the functionality of these compounds in food applications.

Novelose®, a high amylose cornstarch (RS2) was found to have improved textural benefits in baked goods (11). The high-amylose starch has a low water-holding capacity, which is good for crispness and can give a favorable tenderness to the crumb. Such items are appropriate for convenience foods such as breakfast foods or snacks. Yue and Waring report extensive studies on the effects of RS2 in food applications. They compared RS to control (wheat flour) and oat bran, wheat bran and cellulose in bread, snack cakes, butter cookies, French toast, waffles and crackers. The 40%RS bread loaf had the highest sensory rating overall in terms of flavor, moisture perception, grittiness and tenderness 24 hours after baking. The 40% RS muffins remained softer than the controls after a two-week storage period, indicating a more desirable effect on texture. Also, when compared to the other fiber sources, RS had the least impact on pancake batter viscosity. Overall the RS waffles and French toast scored better than the control and other fibers. RS has even been used in thickening opaque beverages with a favorable outcome (11).

As mentioned previously, Nutraflora™ is the only brand of FOS that is GRAS and approved for human consumption. FOS is sweet to taste, ranging from 30% to 60% as sweet as sugar, and is also very hygroscopic, giving it positive attributes in food processing (18). Furthermore, FOS has only 1.5 kcal/g, which comes from the SCFA fermentation byproducts, compared to 4kcal/g for sucrose or fructose, coming solely from carbohydrate (18). It acts as a sweetener but dilutes the energy density. Additional properties in food processing include its humectancy and minimal participation in the Maillard reaction. Humectancy is a property that reduces undesirable water separation, or syneresis, and results in improved texture after storage.

One of the most notable uses of FOS is in Ross® formularies. Enteral feedings are

supplemented with FOS, and the amount of FOS is listed on the food label as fiber (19). It is useful as a functional food additive because of its fiber-like properties and high fermentability. FOS is found to be safe for individuals in severe immunocompromised states.

Glycemic Response

Both FOS and RS are carbohydrates, but unlike other oligosaccharides and complex carbohydrates, they do not raise blood glucose. RS and FOS are not digested in the stomach or small intestine. The majority of FOS and RS reaches the large intestine and is then fermented by intestinal microflora to SCFA. Only the SCFA's are absorbed into the body, which cause no increase in blood glucose. Maintenance of normal blood glucose is of utmost importance for diabetics and pre-diabetics.

Diabetes mellitus is a disorder in which the pancreas fails to produce adequate insulin in response to increased blood glucose (29). Type 1 diabetes is caused by autoimmune factors in which the beta cells of the pancreas are destroyed resulting in no insulin production. In most type 2 diabetics, the pancreas is still able to produce insulin, but is not as sensitive to increases in blood glucose. Eventually, however, type 2 diabetics may require insulin injections. The three key elements of diabetic treatment are medicine, diet therapy and exercise (30). Diet therapy is based on controlled carbohydrate intake throughout the day. Carbohydrate intake by the gram must be monitored for type 1 diabetics. Insulin and insulin sensitizers are the main medical treatments for diabetes, and dosage is based on carbohydrate intake (30).

Diabetics need carbohydrate-containing snack foods that do not cause increases in the blood glucose. The euglycemic properties make RS and FOS useful as food additives for the

diabetic. Supplementation of RS or FOS in snack items such as cookies or snack cakes aid diabetics in compliance with their strict diets. Such snack foods would provide diabetics more choices for carbohydrate foods, without causing the usual increase in blood glucose (11,5,6).

Short-chain fatty acids and colon health

RS and FOS are believed to have protective effects in prevention of colorectal cancer and remission of colitis (2). In epidemiological studies, RS consumption correlates negatively with colorectal cancer (2). Two theories currently stand about the mechanism behind the observed benefits of cancer reduction. First, because of the indigestibility of the starch, harmful carcinogenic toxins may bind to the undigested starch molecules and are excreted in the feces. Secondly, the greater SCFA supply to colonocytes promotes healthy cell growth and increased splanchnic blood flow (2,31,32).

Colonocyte proliferation is observed by measuring cecal wall weight in animal models. In rats, the cecum is the main site of fermentation and thus the greatest amount of SCFA's are produced in the cecum. Undigested starches are fermented and remain in the cecum until passing into the colon. Fermentable carbohydrate ingestion led to cecal wall weights at least two times greater than the control rats (2, 12,13,31,32). Adult male Wistar rats were fed 15% RS (12). Another study by Ohta et al used five-week-old male Sprague-Dawley rats fed 5% and 10% FOS. They reported increased proximal small intestine and cecum weights of rats fed FOS, regardless of the level.

Of the three main SCFA's produced in microbial carbohydrate fermentation, butyrate displays the greatest degree of colonic health benefits (2,25, 26). Butyrate has the ability to induce proliferation in normal cells at the crypt base, enhancing healthy tissue turnover and

mucosal maintenance.(2) Furthermore, butyrate stimulates healthy regeneration of inflamed mucosa, as occurs in ulcerative colitis and Crohn's disease. It is the major intestinal fuel (2) and butyrate enemas directly induced remission of ulcerative colitis (26). Unfortunately, the positive effect of butyrate disappears when administered by the mouth. Butyrate enemas are not a desirable treatment in humans because they are too invasive (26). However, administration of RS that is fermented to produce butyrate seems a very promising treatment. Moreau et al used dextran sodium sulfate (DSS) to induce colitis in male Sprague Dawley rats. After three weeks of DSS treatment, they achieved stable chronic colitis in the rats. Rats were then treated with FOS, RS3 or a control diet. They found remission of colitis in rats treated with RS3 based on lower cecal mucosa , less blood in feces, less colonic mucosa thickening, and less colonic ulceration (26). Patients with ulcerative colitis are also at increased risk of developing colon cancer (32).

Moarita et al compared the effect of different protein sources in diets with RS or FOS against controls. They found that rice protein increased the concentration of butyrate produced from FOS fermentation when compared to casein and soy (22). Studies show that fermented RS consistently favors butyrate production (2, 21). Also, butyrate is found to have increased antineoplastic effects, possibly linking RS to decreased risk of colorectal cancer. Thus, the protective mechanism of RS against colorectal cancer is presumed to be through the SCFA products of fermentation.

Pathophysiology of Gastrointestinal Problems

Gastrointestinal disorders such as diverticulitis, gastroesophageal reflux disease (GERD), gallstones and colorectal cancer are much more prevalent in affluent nations, such as North America (1,33). Between 60 and 70 million Americans are affected by all digestive

diseases (34). Costs for treating these diseases in 1992 were \$107 billion, with \$87 billion in direct costs (34), and since then have continued to rise. Cancer is the second leading cause of death in America, with colorectal cancer being ranked third in prevalence in men and women (30). The exact pathophysiology of colorectal cancer is largely unknown, but as mentioned in the previous section, SCFA's are linked with reduced risk of colorectal cancer incidence. It is likely that many different factors contribute to colorectal cancer, as well as other GI problems.

In 1985 it was estimated that between 3 and 7% of Americans suffered from GERD (34); however, currently the lifetime prevalence of GERD is between 25 and 35% of all Americans (30). The leading cause of GERD, commonly known as acid reflux, is the hiatal hernia. The hiatal hernia is caused by a portion of the stomach being pushed up through the diaphragm, and in most cases, this results in GERD. A likely cause for both hiatal hernias and diverticulitis is increased constipation due to inadequate fiber intake.

The reduction of dietary fiber intake in Western lifestyles is paired with the stark increase in gastrointestinal disorders and disease. Of these, constipation is the most common digestive complaint in the United States (1). Constipation is defined as absence of a stool for three days. Painter's hypothesis suggests that constipation can cause excessive pressures on the lumen of the colon during defecation (3). Daily straining over time could very well be responsible for the pressure necessary to cause herniation or diverticuli. In fact, straining to pass small firm stools can raise intraabdominal pressures to around 200cm H₂O (1).

Gastrointestinal Benefits of Fermentation

A number of gastrointestinal benefits have been linked to RS and prebiotics. The most noted are decreased intestinal transit time, increased fecal bulking, remission of

bacterial induced diarrhea and cholera, protection against harmful bacteria overgrowth, and prevention of antibacterial induced digestion problems (2,23,19).

Insoluble fibers are long known to decrease intestinal transit time and promote fecal bulking. Although classified as soluble dietary fibers, FOS and RS are associated with more regular stools and softer stools without diarrhea (35). Animals fed soluble fermentable fibers have increased colon weight, increased fecal bulk, and normalized motility in the colon. All of these factors indicate general GI health and decreased occurrence of constipation.

Both RS and FOS are highly fermentable, so fecal bulking is variable. Normal fecal bulking is largely caused by indigestibility and promoting laxation (2). The stimulation of microbial growth causes an increase in microbial biomass, resulting in the increased bowel content stimulating peristalsis (21). Additionally, SCFA's promote greater splanchnic blood flow, which can reduce impairments in peristalsis from poor circulation. FOS, particularly, can have an osmotic effect on intestinal contents leading to greater wet fecal mass. When the intake of water soluble carbohydrates, such as FOS, exceeds the fermentative capacity of intestinal microflora, osmotic diarrhea occurs (2).

Another theory reported by Wolf is that increased colonic bacterial proliferation leads to fecal bulking due to microbial biomass loss in the feces. This phenomenon is explained by the increased fecal nitrogen excretion observed (36). Regardless of the mechanism, decreased intestinal transit time results in more frequent defecation and softer stools. The 1982 Nurses' Health Study, a cohort study among over 120,000 registered nurses in the US, indicated that higher dietary fiber was associated with decreased prevalence of constipation ($p < 0.0001$) (37). Animals fed FOS and RS have increased colon weights and fecal bulk as mentioned previously, and normalized motility in the colon.

Johns Hopkins University School of Medicine conducted a study on the effects of FOS supplementation in infant cereal (38). A sample of 52 infants completed the study for 28 days, lending a total of 2,723 stooling episodes. More frequent and softer stools, without diarrhea, occurred in the FOS group. There were only twenty-one “watery” stooling episodes, of which 95% occurred in the control group. Thus, supplementation of dietary fiber as FOS or RS could promote healthy GI motility and decreased constipation, resulting in a decreased risk of developing associated disorders.

Remission of bacterial induced diarrhea and cholera is another observed GI benefit of RS and FOS. It is believed that the low pH induced by fermentation byproducts inhibits the overgrowth of harmful bacteria (2). For example, propionate has been shown to kill *E. coli* or *Salmonella* at an acidity of approximately a pH of 5 (2,23,39).

Additionally, SCFA can prevent antibacterial induced digestion problems such as diarrhea. Antibiotics can destroy healthy intestinal microflora. Absence of intestinal microflora leaves individuals more susceptible to infectious diseases and osmotic diarrhea. Elevation of SCFA in the active phase of cholera stimulates remission by up to 50% and reduces fluid loss substantially. Nondigestible food ingredients beneficially affect the host by selectively stimulating the growth of one or a limited number of bacterial species (2).

Short-chain Fatty Acids and Mineral Retention

The production of SCFA from RS and FOS fermentation lowers luminal pH, and thus increases mineral solubility. The decreased pH from fermentation leads to increased mineral retention, particularly in nutritionally compromised states such as gastric surgery and menopause (40,41,42). Increased absorption is more apparent in the analysis of macrominerals such as calcium and magnesium. Coudray et al showed an increase in

calcium and magnesium absorption using 7.5% RS from crude potato starch, a type of RS2 (43). However, Behall et al reported no differences in mineral retention between RS and control wheat starch diets in hyperinsulemic men (44). They used a 70% RS diet based on percentage of starch component.

The effects of FOS in enhancing mineral retention are more conclusive. Wolf et al showed that varying degrees of FOS supplementation increased Mg absorption linearly using 1%, 3%, and 5% FOS (36). Studies consistently report enhanced Mg absorption due to FOS supplementation (12,16,31). Other studies on rats have shown enhanced absorption of Ca as well in short term feeding studies (42,45,46). One mechanism proposed is that the SCFA's stimulate mineral absorption by a combination of increased blood flow, increased mineral solubility and increased colonocyte health.

Each mineral has a different mode of absorption, and the body has a unique mechanism for regulating mineral uptake, largely based on bioavailability (47). The two minerals studied most commonly in controlled feeding experiments are calcium and magnesium. Both calcium and magnesium are critical in bone health. In order for these minerals to be absorbed and enter circulation, they must be in solution in the GI tract (47,48).

Calcium passes across the intestinal epithelium in two ways during digestion, transcellularly and paracellularly. Transcellular movement happens through the cells and is regulated by vitamin D, occurring largely in the duodenum (48,49). Increased vitamin D circulating in the bloodstream enhances transcellular absorption. Paracellular absorption occurs throughout the intestine and is not selective to any particular segment (48).

Paracellular absorption is a concentration gradient based diffusion. Dietary factors, such as dairy foods and phytate, can affect mineral absorption. Dairy calcium has greater

bioavailability than other supplemental calcium (47,48), but phytate has a strong inhibitory effect on mineral absorption (50,51).

Another dietary aspect that influences mineral bioavailability is the digestion of food and solubility of the mineral (48). In fact Lopez et al reports the ability of fermented RS to increase dietary mineral retention, even when phytate is present (52,53). The more completely that food is digested, the more calcium available for absorption. Poor digestion occurs in post-gastrectomy patients because of the lack of stomach acid. Post-gastrectomy patients also have poor mineral absorption which frequently results in osteopenia (41,42.). As pH of digesta increases, calcium may precipitate. At the neutral or alkaline pH of the large intestine, only about 4mol of CaCO_2 will dissolve per liter (48). FOS and RS fermentation byproducts can have a beneficial effect in increasing solubility of minerals, and thus increasing mineral bioavailability. (41,52)

Transcellular entry into mucosal cells occurs in three steps; entry across the brush border, intracellular movement, and extrusion at the basolateral membrane.(47,48) Overall absorption rate of calcium is constant throughout the small intestine. Lactose increases the absorption of calcium, possibly due to hypermolar distention. In hypermolar distention, fluids are retained in the colon because digesta is hyperosmotic compared to the splanchnic blood flow. Ions, such as calcium contribute to the osmolality of fluid, thus creating a concentration gradient favoring absorption of minerals. FOS may increase mineral absorption through hypermolar distention, because it is an oligosaccharide and does not occur in granules as RS does. Granular starch does not increase the osmolality of digesta as simple sugars do (30).

Magnesium absorption is not dependent on hormones, such as calcitriol, as is calcium

(48,49). Animal studies indicate that magnesium is better absorbed in the ileum and colon. It is secreted across the duodenum to neutralize digesta pH, but reabsorbed in the ileum. Two types of magnesium absorption occur, passive and nonpassive cellular transport (48). In humans, the rate of absorption is influenced by dietary concentration and dietary components. Dietary components such as phytate, may bind minerals, thus reducing their bioavailability (48,51). In healthy adults, 35% to 68% of magnesium is excreted (48).

Bone Composition and Mineralization

Bone is a metabolically active tissue, constantly being broken down and reformed, even in old age. There are three main cellular components of bone; osteoblasts, osteocytes and osteoclasts (29,49). Osteoblasts are located on the side surfaces of bone and are responsible for building bone mass. Osteoid is an organic compound secreted from osteoblasts, which is deposited on bone surface. Bone mineral is then deposited over the osteoid matrix, forming mineralized bone (29,49). As bone mineralization occurs, osteoblasts become embedded in the bone matrix. During mineralization, calcium from the bone fluid is precipitated and converted to the crystalline compound hydroxyapatite, $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$ (49). Osteoclasts are responsible for bone resorption. They are large cells located on the side of bones and secrete acids and proteolytic enzymes when stimulated (29). The resulting acidic environment increases the solubility of bone mineral, and the proteolytic enzymes destroy the organic protein matrices, thus causing loss of bone mass. Osteoclasts hold a very important role in calcium homeostasis and regulate the buffering capacity of bone through resorption. Bone mineralization and resorption are part of normal, healthy bone metabolism. However, if osteoclasts are continually stimulated greater than osteoblasts, abnormal loss of bone density results, leading to osteoporotic bones.

Bone density is highly regulated by a number of hormones. Parathyroid hormone (PTH), calcitriol [1,25(OH)₂D₃], calcitonin and vitamin D are all regulators in bone mineralization and resorption (29,49). PTH is stimulated by low plasma calcium and phosphorus concentrations. PTH increases plasma calcium concentrations by stimulating osteoclasts in bone and enhancing calcium reabsorption in the kidney tubules. Also, PTH stimulates production of calcitriol. Stimulation of calcitriol production in the kidneys also results in synthesis of calbindin, which acts on the gastrointestinal tract to increase calcium absorption. Calcitriol interacts with the nuclear receptors in enterocytes and increases cellular transcription of genes that code for calbindin (49). On the other hand, calcitonin acts to lower serum calcium by inhibiting osteoclast action and stimulate osteoblast mineralization. These alterations result in lower plasma calcium. Impairments of normal calcitonin action are associated with hypertension (29).

Gastrectomization

Osteopenia commonly occurs in humans after a gastric resection (27,41,42). In addition, there has been reported a high prevalence of bone disorders following total gastrectomy (27). Poor mineral absorption in postgastrectomy patients is commonly believed to be due to the lack of stomach acid, thus poor digestion and dissolving of minerals (41). However, water-insoluble calcium compounds can be absorbed without gastric acid secretion (41). Thus, the exact mechanism of the stomach's role in calcium homeostasis is unknown.

Animal models of gastrectomization display decreased bone mass, anemia and decreased intestinal absorption. In a number of studies, gastrectomized Sprague-Dawley rats were used to simulate surgery in humans, sham-operated rats serving as the controls.

Increased bone mass in FOS supplemented treatment groups is widely reported (27,41,42,54). Ohta et al found that absorption of calcium and phosphorus was higher in FOS-fed rats (41,42). The mineral absorption in the gastrectomized control rats was lower than the other three groups, indicating that the animal models effectively simulate the problems found in humans. It is possible that factors other than stomach acid, such as hormone secretion, are responsible for the decreased mineral absorption and bone mineralization. Furthermore, Morohashi et al found that in gastrectomized rats, dietary FOS prevented reduction of cortical and trabecular bone (27).

Another complication of gastric surgery is postgastrectomy anemia. A study by Ohta et al (41) reported that rats fed 7.5% FOS had greater recovery from gastrectomy-induced anemia by increasing iron absorption. Serum iron in gastrectomized rats fed FOS was significantly higher than the sham-operated control group. Total iron binding capacity was significantly lower in both gastrectomized groups. In conclusion, gastrectomized rats are a useful model for the postgastrectomy malnutrition observed in humans. FOS displayed a clear ability to enhance mineral absorption and prevent anemia and osteopenia in this critical state (41,42).

Ovariectomization

Menopause is another health state that is strongly linked to poor mineral absorption and osteopenia. Estrogen has a protective effect on bone health, and postmenopausal women experience rapid bone resorption. Ovariectomized retired female breeder rats are a common model for human postmenopausal osteopenia (55,56,57). Following ovariectomy, rat models experience cancellous bone mass decreases comparable to post-menopausal bone loss (58,59).

Osteoporosis

Osteoporosis, also known as “brittle bone disease” is caused by rapid bone resorption and is characterized by osteopenia, or loss of bone mass, frequent fractures and skeletal pain (58,59). Osteopenia weakens bones, predisposing them to fractures, which can occur spontaneously in humans. Figures 3 and 4 depict healthy bone structure compared to osteoporotic bone. Osteoporosis is a chronic disease often associated with the elderly, affecting 55% of adults over 50 years of age (60). Postmenopausal women, especially small-framed women, have an increased risk of developing osteoporosis (30,60). In fact, eighty percent of those affected by osteoporosis are women (60). As explained previously, lack of estrogen is associated with active bone resorption. Research in prevention of osteoporosis is increasingly important as society continues to age. The population of the elderly is rapidly increasing, and associated chronic disease is increasing as well. Osteoporosis is responsible for over 1.5 million fractures annually (60). It is necessary to enhance mineral absorption in individuals at risk for osteoporosis, thus reducing bone resorption.

Figure 2: Normal Bone (61)

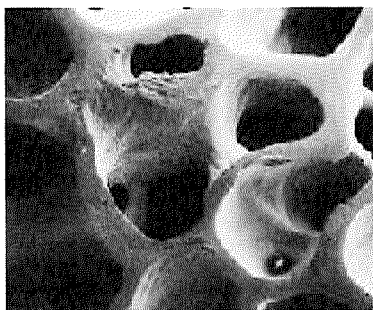


Figure 3: Osteoporotic Bone (61)



Measurement of Bone Density

Bone densiometry is the measurement of bone density, and is a useful indicator for sites that are more porous. Low density predisposes bones to fracture, and although it is not

the only factor in determining bone strength, it is the most important. Equipment such as the dual energy x-ray absorptiometer and computed tomography scans are noninvasive methods of predicting sites that are at high risk of fracture (62). Bone density can be expressed either superficially, as grams per centimeter squared (g/cm^2), or volumetrically, as grams per centimeter cubed (g/cm^3). Regardless of the technique chosen to measure bone density, it is crucial that measurements are taken using the same instrument and method in comparing samples (63).

Peripheral Dual Energy X-ray Absorptiometer

The dual energy x-ray absorptiometer (DEXA) uses an x-ray tube to emit two gamma rays. Radiation waves are absorbed and reflected back to the source differently based on density of the sample. This method can measure fat mass as well as total bone mineral (64). A peripheral DEXA (pDEXA) machine is appropriate for measuring individual bones of small animals, such as rats, as well as small animal body scans. Currently, the DEXA is thought to be the best method for assessing bone mineral density (64).

Biomechanical Bone Measurements

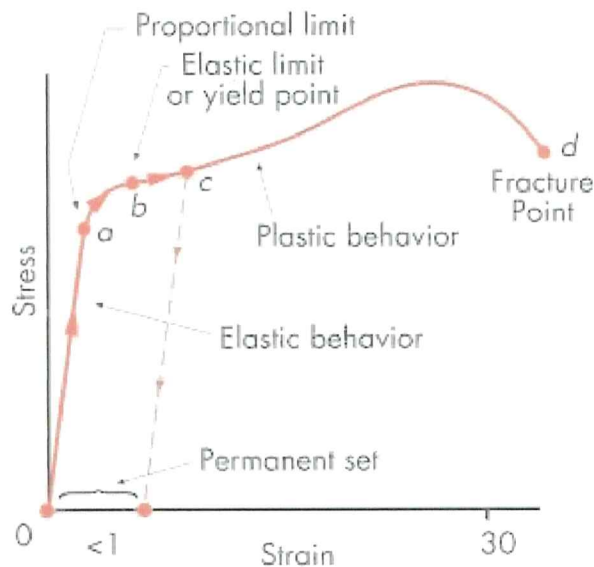
Stress testing on animal models is a common way to evaluate bone strength. Although bone densitometry is commonly used to evaluate bone density or content, direct biomechanical testing provides information about the mechanical integrity and strength (65). Stress and strain are fundamental concepts in bone biomechanics. Stress is defined as the force per unit area and is reported in units of the Pascal. Three types of stress are compressive, tensile and shear stresses, and always occur in combination. Strain is defined as the percentage change in length during deformation. Bone has elastic properties, thus

when force is applied, it is either compressed or stretched. Bones can withhold a certain amount of strain before breaking.

As force is applied, deformation in response to the load occurs. The load-deformation curve describes the relationship between the load applied to a specimen and the responsive deformation. Two regions comprise the load-deformation curve; elastic deformation and plastic deformation (65). In the elastic deformation region of the curve, the bone reflects its spring-like properties during strain. A linear deformation increase is observed with an increased load. The two regions are divided by a yield point, or a boundary beyond which permanent damage occurs. This permanent damage is called plastic deformation. The yield point indicates the maximum stress that bone can withstand before fracture. At this point, the curve becomes non-linear.

When load is converted to stress and deformation is converted to strain, the load-deformation curve becomes the stress-strain curve. The relationship between stress and strain in bone deformation is accurately described by the stress-strain curve. Within the elastic region, or linear region, the slope is called Young's modulus, which is a measure of the stiffness of material. The area under the stress-strain curve is a measure of the amount of energy required to cause bone fracture. In bone biomechanics, this energy is referred to as toughness. A tough bone will have a greater energy absorption, and will be more resistant to fracture (65).

Figure 4: Stress-Strain Curve (66)



Biomechanical Testing Methods

Mechanical testing methods can accurately create a stress-strain curve of bone deformation using a controlled applied force. Bending, or flexure tests are useful for measuring mechanical properties of bone in rodent specimens (65,66). However, the cross-sectional moment of inertia is necessary to calculate the bending stress. For cross-sectional moment of inertia, the cross-sectional area must be known, which is difficult in irregularly shaped bones. Stress is the applied force divided by cross-sectional area of the bone at the focal point (65). For irregularly shaped bones, the best measurement of cross-sectional area is achieved using a digitizing system. Cross-sectional moment of inertia can also be determined using dual energy x-ray absorptiometry (DEXA). Conversely, Crenshaw et al reported that the moment of inertia for pig femur and humerus bones could be closely approximated by the equation for an ellipse. Figure 2 displays three-point loading, a common flexure test for rodent bones. It is recommended that length (L) be about 16 times the thickness of the bone specimen.

Three point flexure tests are useful for trabecular bone, but another method was

described for femoral neck strength. Testing of the femoral neck is important because hip fractures are common among osteoporotic individuals. Fracture most likely takes place at the femoral neck because it is the weakest point of the hip joint. The method described by Reeves uses one focal point of constant force applied to the femoral head until fracture (68). Energy absorption and total force load are used to compare toughness and bone strength.

The stress-strain curve is greatly influenced by procedural factors such as sample preservation and temperature of testing environment. Surprisingly, freezing in saline soaked gauze at -20°C caused no change in bone bending properties (56,65). Bone specimens must be stored in iso-osmotic saline to avoid residual water loss. For the most accurate measurements, bone should be tested at 37°C . A temperature difference of 14°C can cause a 2-4% difference in Young's modulus (65). Even if optimal temperature is not achieved, variability in biomechanical measurements can be limited by temperature control. Unfortunately, the lack of standardized testing procedures for bone biomechanics has resulted in considerable variation in reported values.

Inductively Coupled Plasma Spectrophotometry

Mineral concentrations of various samples, solid and liquid, can accurately be determined using inductively coupled plasma spectrophotometry (ICP). ICP mass spectrophotometry has effectively been applied to determine the concentration of over 60 different minerals. Method 6020 has demonstrated acceptability for determining concentrations of aluminum, cadmium, calcium, chromium, cobalt, copper, iron, magnesium, manganese, nickel, potassium, selenium, sodium and zinc (69). ICP is widely used in urinalysis and mineral analysis of feces in rat feeding studies (31,41,42).

METHODS

The Louisiana State University Institutional Animal Care and Use Committee (IACUA) approved the animal protocol (Appendix A) for this study on February 13, 2003.(Appendix B)

Animals and Timeline

Twenty-four four-week old Sprague-Dawley rats were acquired from Harlan Co. Immediately upon receipt, the rats were housed in stainless steel cages, 24 X 28 centimeters in groups of four. Once the study began the rats were housed individually in the stainless steel cages, except for two one-week periods in the metabolism cages. The Animals were kept in room 658 of Life Science's animal care facility of Louisiana State University and Agricultural and Mechanical College (LSU A&M) at 22° C with a humidity level of 60% and 12-hour light/dark schedule.

The rats were stratified by weight and randomly assigned to treatment groups. There was no significant difference among the mean arrival weights of the three treatment groups. In the time period before treatment began, all rats were fed control diets. After seven days, an initial pDEXA was taken. Twelve of the rats, numbers 1 to 4 of each group, immediately began the diet treatment and were placed in metabolism cages for collection period 1. Feces and urine were collected for seven days, which was week one. On the eighth day (beginning of week two) the remaining twelve rats, those numbered 5 to 8 in each group, began the diet treatments and were placed in the metabolism cages for seven days. For the consecutive nine weeks, all twenty-four rats were monitored in the stainless steel cages. At week six, a second pDEXA was taken of each rat. At week twelve, rats numbered 1 to 4 were placed back into the metabolism cages for collection period 2. During week thirteen the rats numbered 1 to 4

were sacrificed and rats numbered 5 to 8 were placed in the metabolism cages for seven days.

At the conclusion of week thirteen, rats numbered 5 to 8 were sacrificed.

Diets

The three treatments were a control diet (C), a diet containing by weight 5% Nutraflora® fructo-oligosaccharide (F), and a third diet containing by weight 8.4% Hi-Maize® high amylose cornstarch (R). The starch used was estimated to be 60% resistant starch, thus the diet mixture was approximately 5% RS. All of the diets were AIN-93G (70) approved powdered diets. Trained student workers made batches of diet in the diet-mixing room of Human Ecology. Diet was made fresh and stored in the freezer in gallon-sized Zip-lock® bags clearly labeled. Each batch yielded 6 kg of diet. A sample of each diet batch was stored at 0C in a small Zip-lock® bag for ICP analysis. Table 1 shows the ingredients of each diet for the three treatment groups.

Table 1: Diet Content in g/kg

	Control (C)	FOS (F)	Resistant Starch (R)
RS	0	0	84
FOS (NutraFlora®)	0	50	0
Cornstarch	530.7	480.7	446.7
Casein	200	200	200
Sucrose	100	100	100
Soybean Oil with 0.015% BHT	70	70	70
Cellulose	50	50	50
AIN97 Mineral Mix	35	35	35
AIN97 Vitamin Mix	10	10	10
Choline Chloride	1.3	1.3	1.3
L-Cystine	3	3	3

Macronutrients were added to a 20-quart stainless steel mixing bowl and mixed at low speed for 10 minutes using a Hobart mixer (model no. A-200-FD; Hobart Mfg. Co.; Troy, OH) with a number 14 paddle attachment. After mixing for 10 minutes, the bowl was

scraped and the mixture was mixed for an additional 5 minutes. Micronutrients were added to a small mixing bowl and simultaneously mixed for 10 minutes. Next, the micronutrient mixture was sieved into the macronutrient mixture, and mixed for 10 minutes. The bowl was scraped and mixed for another 10 minutes. Soybean oil containing 0.015% butylated hydroxytoluene (BHT) was added to the dry ingredient mixture, and then mixed for 5 minutes. The bowl was scraped and mixed for another 10 minutes. Immediately the diet was stored in the Zip-lock ® bags and placed in the freezer.

For ten weeks total, the rats were kept in the stainless-steel cages, and for the two metabolism periods in the plastic metabolism cages. Throughout the study, rats were fed on Monday, Wednesday and Friday *ad libitum*. Also, on days when fed, rats were given fresh distilled water dispensed from plastic bottles with rubber stoppers and stainless steel sippers. During the two weeks when in the metabolism cages, the distilled water level was checked daily.

Measurements During Diet Treatment

Peripheral Dual X-ray Absorptiometer

Throughout the study, a total three pDEXAs (Norland Medical Inc.) were taken of each rat; one immediately before diet treatment, one at week six, and one immediately prior to sacrifice. The pDEXAs were used to measure bone density of the spinal cord. Rats were anesthetized by Isofluorene gas via a nose cone for the duration of the pDEXA scan. Isofluorene at a concentration of 5 -6% was administered with 20% oxygen at a rate of 1cc/sec. Each rat was monitored carefully to ensure that there were no complications or abnormalities during anesthesia. Figure 5 displays the x-ray procedure.

Figure 5: pDEXA Scans of Rats



Food Intake and Body Weight

On Monday, Wednesday and Friday at 9AM rats were weighed and fed. Body weight was measured by a balance scale and rounded to the nearest gram. Empty and full food cups were weighed to the nearest gram as well. Clean food cups were exchanged weekly. While in the metal cages, paper weigh-backs were placed underneath the cages to monitor food spillage. In the metabolism cages, food spillage accumulated in the collection tubes, and was cleaned from feces samples.

Metabolism Cages

For one week at the start of the study and for the final week, rats were placed in plastic metabolism cages (Figure 6). For seven consecutive days, food intake was monitored and urine and feces were collected daily. Food spillage was filtered from the urine with a paper filter, and compiled for each rat for each metabolism period. Filter papers were weighed before and after use to determine food spillage weight. Filtered urine was weighed and volume was measured, then stored in the freezer in airtight plastic containers until further

analysis. Feces collection tubes were weighed and then food spillage was cleaned off with distilled water. After air-drying for an hour, fecal matter was weighed and then stored in the freezer in airtight plastic containers. Each day feces samples were added to the previous days accumulation.

Figure 6: Metabolism Cages



Measurements after Diet Treatment

At Sacrifice

After taking the final pDEXA, rats were administered Isofluorene gas and then bled by cardiac puncture, removing up to 10mL of blood. Blood was collected in heparinized collection tubes and stored at -80°C . The heart was removed first, then the gastrointestinal tract. The GI tract was weighed full, then separated into stomach, small intestines, cecum, and large intestines. Organ were weighed full, then cleaned with distilled water and patted dry with a Kimwipe®. The pH was taken of cecal contents, and then the cecum was cleaned and weighed. The kidneys, liver, spleen, abdominal fat, perirenal fat and brown adipose

tissue were also removed and weighed.

Mineral Analysis

Diet samples from each batch of each type of diet were combined to be analyzed for mineral content. Samples were sent to the Louisiana State University Soil Testing Department to analyze via Inductively Coupled Plasma Spectrophotometry (ICP) (CIROS, Spectra Inc., Germany). Each type of diet was digested by the ICP, results displaying boron, zinc, iron, aluminum, copper, calcium, phosphorus, potassium and sodium.

Urine for each rat over the seven-day metabolism period was combined and thoroughly mixed via a vortexer. The total amount of urine was weighed and the volume was measured. Small aliquots were sent to the Soil Testing Laboratory for mineral content in parts per million. The results were multiplied by total urine volume output to determine urinary mineral output.

Feces were weighed, and then dried overnight in the freeze-dryer. Samples were re-weighed and then finely ground via acid-washed mortar and pestle. Small aliquots of feces from each rat during metabolism periods 1 and 2 were sent to the LSU Soil Testing Department to be analyzed via ICP. Results for minerals were reported as parts per million, and then multiplied by the dry weight of fecal output to determine fecal mineral output. This was compared to mineral intake from the diet to determine percent absorption in the following equations.

Equation 1: Mineral intake = (mineral content of diet)(grams of food intake)

Equation 2: Mineral output = (mineral content of feces)(grams of dry fecal output)

Equation 3: % Absorption =
$$\frac{[(\text{mineral intake}) - (\text{mineral output})]}{\text{mineral intake}}$$

At sacrifice, the left legs were removed and stored in the freezer. Legs were thawed and then humerus, tibia and femur bones were thoroughly cleaned, with all cartilage removed. The same ICP mineral analysis procedure was used to determine mineral composition of bones as for urine, diet and feces. Equation 4 was used to determine urinary mineral output, and equation 5 was used to determine mineral intake.

Equation 4:

Urinary Mineral Output = (ppm of urinary mineral conc.)(mL of urine output)

Equation 5: Mineral Intake = (mineral ppm of diet)(grams of intake)

Bone Stress and Strain

The right tibia, femur and humerus were removed at sacrifice and stored in a 0.9 % isotonic saline solution at 0°C. The day of analysis for bone strength, bones were thawed and cleaned with surgical scissors and a scalpel to remove all muscle and tendons. A pDEXA scan was taken and then humerus and tibia bones were broken via three-point break using the Q-tester. The breaking procedure is outlined in Appendix I. In the few hours between pDEXA scans and breaking, bones were stored at room temperature in the isotonic saline solution. The strength of the hip joint was tested by breaking off the femoral head. The femur bone was stationed upright in 1" copper tubing and then fixed by hot glue. Pressure from the Q-tester (Q/T5 Q Test, MTS Systems Corp., Triangle Park, NC) was placed on the femoral head until breakage. Measurements recorded were peak load, break load, energy to peak, energy to break and the flexure modulus.

Statistical Analysis

All results were analyzed via SPSS® computerized software. An ANOVA was used to determine significant differences among the three groups, and the LSD post-hoc test was used to determine differences between groups. A 95% confidence interval was constructed

to determine significance, with $p < 0.05$.

RESULTS

Food intake and diet composition

There was a significant difference in grams of food intake among the three treatment groups ($p=0.002$), but only for cumulative intake over the entire twelve weeks. R had significantly greater intake than both F and C ($p=0.001$, $p=0.003$ respectively). There were no significant differences in food intake during metabolism period 1 or 2. Table 2 displays the mean cumulative food intake and food intake during metabolism periods. During period 1 and period 2 there were no significant differences in food intake among the three groups. Also, there were no significant differences in mineral content of the three diets. There were not any significant differences in the intakes of Al, Fe, Cu, Zn, Ca, P, K, Na, or Mg during period 1 or period 2. Table 3 shows the mineral content per gram of diet and mineral intakes during periods one and two.

Table 2: Grams of Food intake ($g \pm SD$)

	C	F	R
Cumulative food intake	1539 \pm 88.75 _a	1559 \pm 93.83 _a	1713 \pm 95.42 _b
Intake Period 1	105.263 \pm 4.239	102.425 \pm 12.180	111.875 \pm 16.983
Intake Period 2	111.587 \pm 9.027	114.737 \pm 12.957	97.237 \pm 15.039

*means with different subscripts are significantly different

Table 3: Mineral Content of Diet Treatments ($n \pm SD$)

	C	F	R
Aluminum (ppm)	8.165 \pm 1.977	10.195 \pm 1.968	9.046 \pm 1.862
Calcium (per cent)	0.6337 \pm 0.0255	0.683 \pm 0.0620	0.623 \pm 0.0376
Copper (ppm)	7.442 \pm 0.418	6.200 \pm 1.452	6.624 \pm 0.824
Iron (ppm)	47.777 \pm 1.484	47.045 \pm 3.344	45.798 \pm 6.399
Magnesium (per cent)	0.0563 \pm 0.0154	0.0480 \pm 0.00755	0.0343 \pm 0.00306
Phosphorus (per cent)	0.424 \pm 0.0295	0.455 \pm 0.0191	0.475 \pm 0.0357
Potassium (per cent)	0.345 \pm 0.0570	0.378 \pm 0.0585	0.365 \pm 0.0459
Sodium (ppm)	1564.4 \pm 31.205	1530.9 \pm 291.356	1409.4 \pm 200.370
Sulfur (per cent)	0.342 \pm 0.0291	0.336 \pm 0.0329	0.328 \pm 0.0258
Zinc (ppm)	42.904 \pm 0.164	42.502 \pm 3.508	42.787 \pm 5.134

*means with different subscripts are significantly different

Table 4: Mineral Intake During Period 1 (grams $\times 10^{-2} \pm$ SD)

	C	F	R
Aluminum	$8.595 \times 10^{-2} \pm 3.455 \times 10^{-3}$	$0.104 \pm 1.244 \times 10^{-2}$	$0.101 \pm 1.537 \times 10^{-2}$
Calcium	66.740 ± 2.683	69.958 ± 8.335	69.703 ± 10.585
Copper	$7.834 \times 10^{-2} \pm 3.149 \times 10^{-3}$	$6.349 \times 10^{-2} \pm 7.565 \times 10^{-3}$	$7.141 \times 10^{-2} \pm 1.125 \times 10^{-3}$
Iron	$0.503 \pm 2.022 \times 10^{-2}$	$0.482 \pm 5.741 \times 10^{-2}$	$0.512 \pm 7.780 \times 10^{-2}$
Magnesium	5.932 ± 0.238	4.9085 ± 0.585	3.842 ± 0.583
Phosphorus	44.607 ± 1.793	46.579 ± 5.550	53.050 ± 8.056
Potassium	36.320 ± 1.460	38.712 ± 4.613	40.782 ± 6.193
Sodium	16.469 ± 0.662	15.678 ± 1.868	15.767 ± 2.394
Sulfur	35.990 ± 1.447	34.373 ± 4.095	36.659 ± 5.567
Zinc	$4.516 \pm 1.816 \times 10^{-2}$	$4.352 \pm 5.186 \times 10^{-2}$	$4.786 \pm 7.269 \times 10^{-2}$

*means with different subscripts are significantly different

Table 5: Mineral Intake During Period 2 (grams $\times 10^{-2} \pm$ SD)

	C	F	R
Aluminum	$9.111 \times 10^{-2} \pm 7.372 \times 10^{-3}$	$11.697 \times 10^{-2} \pm 1.830 \times 10^{-2}$	$8.796 \times 10^{-2} \pm 1.360 \times 10^{-2}$
Calcium	70.750 ± 5.724	78.374 ± 12.261	60.587 ± 9.370
Copper	$8.305 \times 10^{-2} \pm 6.720 \times 10^{-3}$	$7.113 \times 10^{-2} \pm 1.113 \times 10^{-2}$	$6.441 \times 10^{-2} \pm 9.962 \times 10^{-3}$
Iron	$0.533 \pm 4.314 \times 10^{-2}$	$0.540 \pm 8.444 \times 10^{-2}$	$0.445 \pm 6.888 \times 10^{-2}$
Magnesium	6.288 ± 0.509	5.499 ± 0.860	3.340 ± 0.517
Phosphorus	47.287 ± 3.826	52.182 ± 8.163	46.112 ± 7.132
Potassium	38.503 ± 3.115	43.370 ± 6.785	35.448 ± 5.483
Sodium	17.458 ± 1.413	17.564 ± 2.748	13.705 ± 2.120
Sulfur	38.153 ± 3.087	38.509 ± 6.024	31.8652 ± 4.928
Zinc	$0.479 \pm 3.874 \times 10^{-2}$	$0.487 \pm 7.628 \times 10^{-2}$	$0.416 \pm 6.435 \times 10^{-2}$

*means with different subscripts are significantly different

Whole body, fat and organ weights

There were no significant differences in the final body weight ($p=0.927$) or organ weights at sacrifice. Also, there were no significant differences in the body weights at any point in the growth of the rats. The growth curves for the rats of each treatment group are displayed in Graph 1. No significant differences were found in the following organ measurements; spleen ($p=0.520$), kidneys ($p=0.118$), heart ($p=0.491$), or liver ($p=0.848$).

There were no significant differences in the abdominal fat ($p=0.997$), perirenal fat ($p=0.774$), epididymal fat ($p=0.661$), total white adipose ($p=0.859$), or brown adipose tissues ($p=0.329$).

Table 6 shows mean organ weights for each treatment group.

Figure 7: Growth of rats over 12-week study

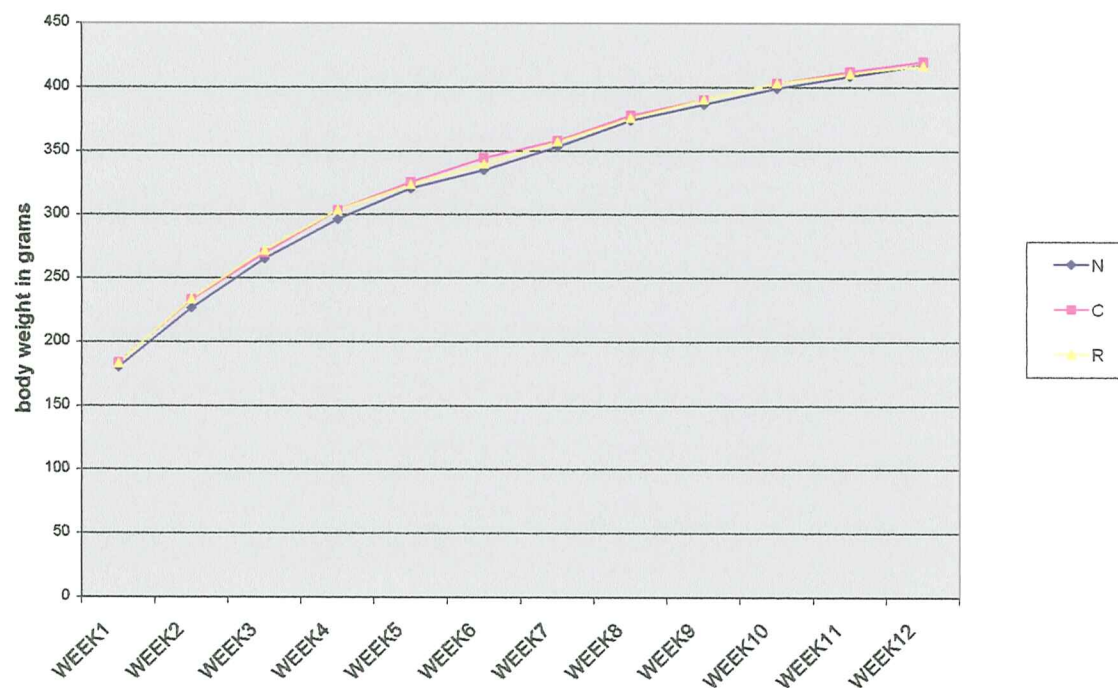


Table 6: Organ weights at Sacrifice ($g \pm SD$)

	C	F	R
Heart	1.2437 \pm 0.06359	1.1846 \pm 0.1567	1.2546 \pm 0.1331
Kidney	2.4794 \pm 0.09568	2.2826 \pm 0.2284	2.4774 \pm 0.2609
Spleen	0.7702 \pm 0.05486	0.7682 \pm 0.04398	0.8008 \pm 0.08331
Liver	12.8234 \pm 1.017	12.7679 \pm 1.9517	12.4235 \pm 1.3897

*means with different subscripts are significantly different

Gastrointestinal measurements

There were significant differences in full GI weight, full cecal weight, empty cecal weight, cecal pH, total GI contents and cecal contents. The weight of the full GI tract was significantly greater in F than in C ($p=0.000$) and R ($p=0.000$), however there was no

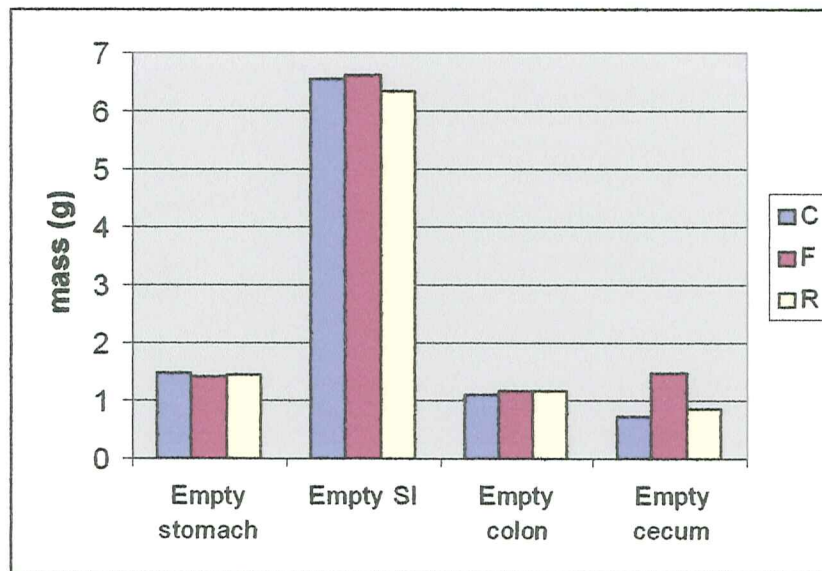
significant difference between C and R ($p=0.126$). The weight of the full cecum, empty cecum, and cecal contents were significantly greater in F than in C and R ($p=0.000$ for all). There were no differences between C and R in these measurements. There were no significant differences in full or empty stomach small intestine, or large intestine weights. Cecal pH was significantly lower in F than in C ($p=0.000$) and R ($p=0.000$). Table 7 displays GI measurements.

Table 7: Gastrointestinal weights at sacrifice ($g \pm SD$)

	C	F	R
Full GI weight	$14.977 \pm 2.051_a$	21.828 ± 3.184	$16.837 \pm 1.431_a$
Full stomach	2.642 ± 0.978	3.198 ± 1.469	2.868 ± 0.552
Full small intestine	6.804 ± 0.870	7.304 ± 0.871	7.220 ± 1.016
Full large intestine	2.282 ± 0.215	2.713 ± 1.147	2.812 ± 0.507
Full cecum	$3.114 \pm 0.758_a$	8.521 ± 1.099	$3.767 \pm 0.740_a$
Empty stomach	1.474 ± 0.113	1.404 ± 0.173	1.459 ± 0.142
Empty SI	6.535 ± 0.614	6.637 ± 0.880	6.343 ± 0.496
Empty colon	1.102 ± 0.105	1.173 ± 0.1833	1.175 ± 0.180
Empty cecum	$0.709 \pm 0.1969_a$	1.475 ± 0.309	$0.865 \pm 0.182_a$
Cecal pH	$8.213 \pm 2.41_a$	6.600 ± 6.45	$7.825 \pm 0.515_a$
GI length	15.700 ± 0.728	16.075 ± 0.736	16.475 ± 0.665

*means with different subscripts are significantly different

Figure 8: Empty GI organ weights (g)



Fecal bulking

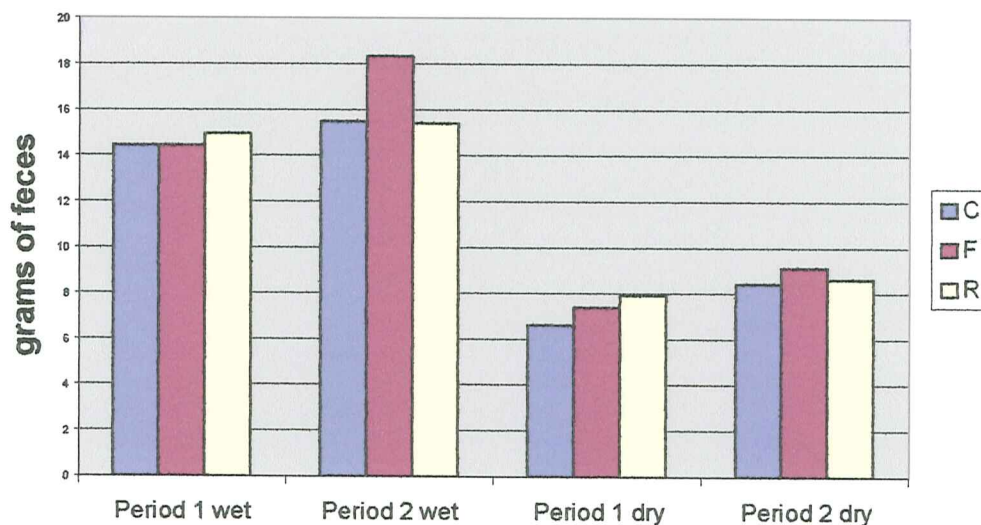
During period 1, there were no significant differences in wet or dry fecal masses among the groups. In period 2, however, the F group had a significantly greater wet fecal mass than the C ($p=0.028$) and R (0.026) groups at the 95% confidence interval, but not at the 99% confidence interval. There was no significant difference between C and R wet fecal weights ($p=0.978$) during period 2. After dehydration, however, there were no significant differences in dry fecal mass among the groups ($p=0.445$) Table 8 and Figure 9 display the differences in fecal masses.

Table 8: Fecal Masses (g \pm SD)

	C	F	R
Period 1			
Wet fecal mass	14.453 \pm 2.385	14.405 \pm 1.893	14.997 \pm 3.292
Dry fecal mass	6.580 \pm 0.990	7.389 \pm 0.869	7.881 \pm 1.682
Period 2			
Wet fecal mass	15.458 \pm 2.465 _a	18.329 \pm 2.732	15.423 \pm 2.030 _a
Dry fecal mass	8.411 \pm 1.314	9.132 \pm 1.144	8.577 \pm 1.011

*means with different subscripts are significantly different

Figure 9: Fecal Masses



Mineral absorption

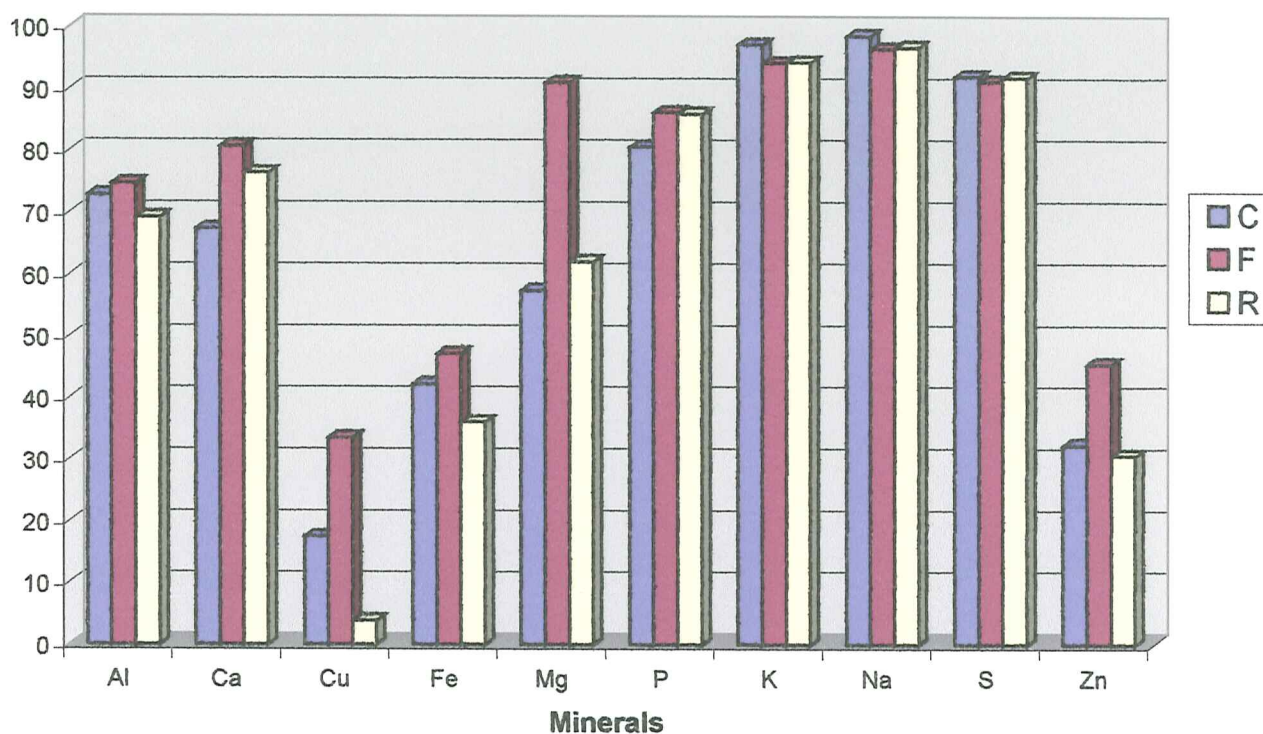
During period 1 there were significant differences in percent absorption of calcium, copper, iron, magnesium, phosphorus, potassium, sodium, and zinc. There were no significant differences in aluminum or sulfur. Table 9 and figure 10 displays the mean mineral absorption for the minerals during period 1. Calcium absorption was significantly increased by the F ($p=0.000$) and R ($p=0.002$) groups compared to C. There was no significant difference in calcium absorption between F and R ($p=0.109$). For copper, C had greater absorption than R ($p=0.002$), and F had greater absorption than both C and R ($p=0.004$ and $p=0.000$, respectively). Iron absorption was significantly lower in R than F ($p=0.039$). For magnesium, F had a significantly greater absorption than both C and R ($p=0.000$, $p=0.000$ respectively). Both F and R had significantly lower absorptions of sodium ($p=0.000$, $p=0.001$) and potassium ($p=0.000$, $p=0.000$). Zinc absorption was significantly increased by F as compared to R ($p=0.007$), and slightly increased from C ($p=0.014$)

Table 9: Period 1 Percent Mineral Absorption (% \pm SD)

	C	F	R
Aluminum	72.756 \pm 2.855	74.642 \pm 7.477	69.189 \pm 4.744
Calcium	67.358 \pm 4.839 _a	80.675 \pm 4.939 _b	76.346 \pm 5.705 _b
Copper	17.428 \pm 8.728 _a	33.46 \pm 11.736 _b	3.804 \pm 9.457 _c
Iron	42.156 \pm 8.926	47.091 \pm 11.003 _a	35.959 \pm 10.280 _b
Magnesium	57.316 \pm 6.785 _a	90.959 \pm 2.585 _b	62.02 \pm 9.886 _a
Phosphorus	80.599 \pm 4.565 _a	86.205 \pm 4.157 _b	85.915 \pm 3.455 _b
Potassium	97.163 \pm 0.906 _a	94.164 \pm 1.328 _b	94.268 \pm 1.558 _b
Sodium	98.541 \pm 0.649 _a	96.436 \pm 0.863 _b	96.737 \pm 1.223 _b
Sulfur	91.98 \pm 1.176	91.156 \pm 1.832	91.79 \pm 1.081
Zinc	32.271 \pm 8.978 _a	45.416 \pm 11.175 _b	30.581 \pm 9.282 _a

*means with different subscripts are significantly different

Figure 10: Period 1 Percent Mineral Absorption



Period 2 mineral absorption is displayed in table 10. There were significant differences in mineral absorption of copper, iron, magnesium, potassium, sodium and zinc. Copper absorption was significantly lower in the R group ($p < 0.000$). Additionally, zinc absorption was significantly lower in R opposed to F ($p = 0.007$) and C ($p = 0.013$). Iron was slightly significantly lower in R than in the F group ($p = 0.015$) and highly significantly lower than C ($p = 0.006$). Magnesium absorption was significantly greater in the F group compared to C ($p = 0.000$) and R ($p = 0.000$). Sodium and potassium absorption were both lower in N and R compared to C. Potassium was significantly greater in C compared to F ($p = 0.005$) and

R (p=0.017). Sodium absorption was greater in C compared to F (0.001) and R (0.029).

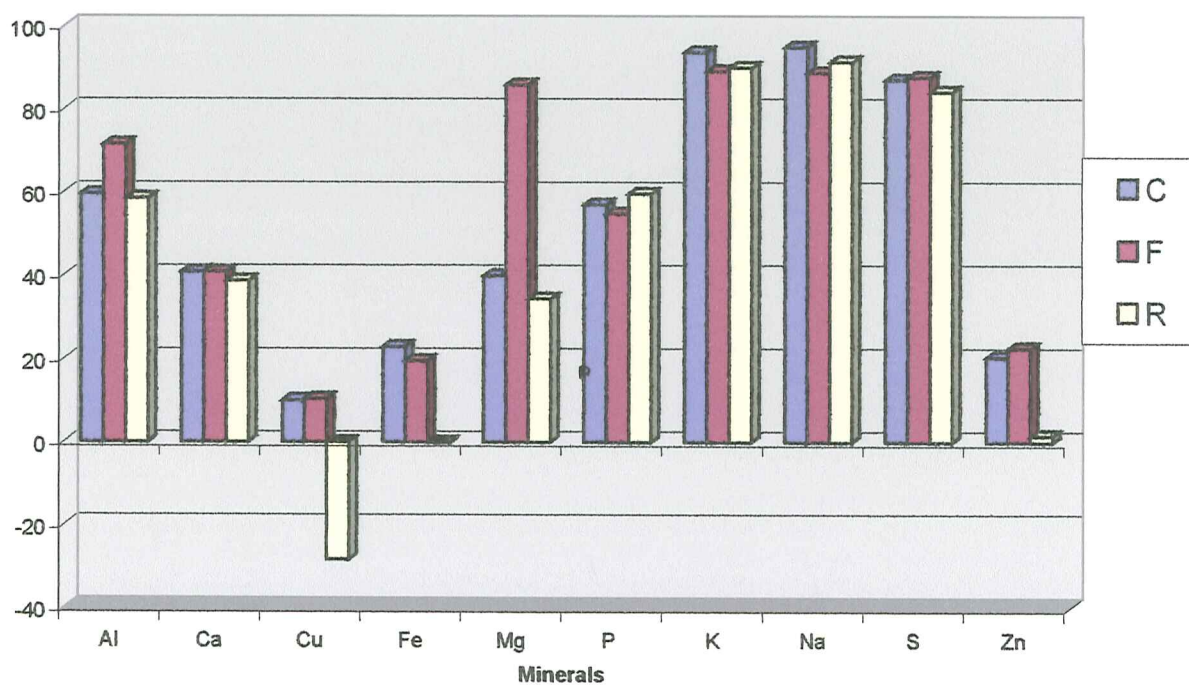
Figure 11 displays the mean mineral absorption of each mineral by diet treatment during period 2.

Table 10: Period 2 Percent Mineral Absorption (% \pm SD)

	C	F	R
Aluminum	59.543 \pm 6.600 _a	71.454 \pm 3.225 _b	58.431 \pm 15.118 _a
Calcium	40.8 \pm 7.833	40.856 \pm 8.032	38.643 \pm 11.897
Copper	9.993 \pm 12.445 _a	10.301 \pm 11.794 _a	-28.327 \pm 23.453 _b
Iron	22.825 \pm 11.569 _a	19.488 \pm 11.474 _a	-0.244 \pm 20.139 _b
Magnesium	39.837 \pm 18.323 _a	85.738 \pm 3.204 _b	34.335 \pm 15.467 _a
Phosphorus	56.876 \pm 4.615	54.821 \pm 6.564	59.566 \pm 8.525
Potassium	93.651 \pm 2.823 _a	89.103 \pm 3.289 _b	89.908 \pm 2.503 _b
Sodium	94.915 \pm 2.497 _a	88.786 \pm 3.382 _b	91.278 \pm 3.337 _b
Sulfur	87.013 \pm 1.955 _a	87.67 \pm 1.689 _a	84.15 \pm 3.446 _b
Zinc	20.324 \pm 10.991 _a	22.505 \pm 10.481 _a	1.415 \pm 18.939 _b

*means with different subscripts are significantly different

Figure 11: Period 2 Percent Mineral Absorption



Urine mineral excretion

There were a number of significant differences in urinary mineral output in both period 1 and period 2. R had greater Al excretion than F and C ($p=0.000$ and $p=0.000$ respectively). Ca excretion was less in F than in R ($p=0.046$). Potassium excretion was greater in F than C ($p=0.017$), but Mg absorption was less in F than C ($p=0.004$). Urinary excretion of Na, P and S were all greater in F than in C and R.

Table 11: Period 1 Urinary Mineral Output (g \pm SD)

	C	F	R
Al	$2.01 \times 10^{-3} \pm 2.238 \times 10^{-4}_a$	$1.03 \times 10^{-3} \pm 1.812 \times 10^{-4}_a$	$61.52 \times 10^{-5} \pm 8.584 \times 10^{-6}_b$
Ca	$2.71 \times 10^{-3} \pm 1.533 \times 10^{-3}_a$	$5.13 \times 10^{-3} \pm 3.853 \times 10^{-3}$	$9.19 \times 10^{-3} \pm 9.751 \times 10^{-3}_b$
Cu	$2.05 \times 10^{-5} \pm 3.583 \times 10^{-6}$	$2.00 \times 10^{-5} \pm 3.545 \times 10^{-6}$	$1.69 \times 10^{-5} \pm 4.752 \times 10^{-6}$
Fe	$1.42 \times 10^{-5} \pm 1.07 \times 10^{-5}$	$2.54 \times 10^{-5} \pm 2.467 \times 10^{-5}$	$1.10 \times 10^{-5} \pm 1.151 \times 10^{-5}$
Mg	$2.45 \times 10^{-3} \pm 3.058 \times 10^{-3}_a$	$1.31 \times 10^{-2} \pm 7.884 \times 10^{-3}_b$	$7.88 \times 10^{-3} \pm 7.650 \times 10^{-3}$
P	$6.07 \times 10^{-3} \pm 4.991 \times 10^{-3}_a$	$1.42 \times 10^{-3} \pm 1.244 \times 10^{-3}_b$	$2.20 \times 10^{-3} \pm 1.225 \times 10^{-3}_b$
K	$0.219 \pm 2.527 \times 10^{-2}_a$	$0.186 \pm 4.460 \times 10^{-2}$	$0.170 \pm 4.119 \times 10^{-2}_b$
Na	$8.15 \times 10^{-2} \pm 9.538 \times 10^{-3}_a$	$6.36 \times 10^{-2} \pm 1.417 \times 10^{-2}_b$	$6.30 \times 10^{-2} \pm 1.608 \times 10^{-2}_b$
S	$1.04 \times 10^{-1} \pm 1.41 \times 10^{-2}_a$	$8.43 \times 10^{-2} \pm 2.367 \times 10^{-2}_b$	$7.81 \times 10^{-2} \pm 1.725 \times 10^{-2}_b$
Zn	$4.98 \times 10^{-5} \pm 1.268 \times 10^{-5}$	$5.69 \times 10^{-5} \pm 2.302 \times 10^{-5}$	$4.46 \times 10^{-5} \pm 2.479 \times 10^{-5}$

*means with different subscripts are significantly different

Table 12: Period 2 Urinary Mineral Output (g \pm SD)

	C	F	R
Al	$5.58 \times 10^{-5} \pm 3.581 \times 10^{-5}$ _a	$1.26 \times 10^{-4} \pm 1.067 \times 10^{-4}$ _b	$8.93 \times 10^{-6} \pm 6.751 \times 10^{-6}$ _a
Ca	$5.83 \times 10^{-4} \pm 2.141 \times 10^{-4}$ _a	$3.78 \times 10^{-3} \pm 3.46 \times 10^{-3}$ _b	$2.84 \times 10^{-3} \pm 2.402 \times 10^{-3}$
Cu	$1.89 \times 10^{-5} \pm 3.596 \times 10^{-6}$ _a	$1.56 \times 10^{-5} \pm 5.116 \times 10^{-6}$	$1.17 \times 10^{-5} \pm 2.254 \times 10^{-6}$ _b
Fe	$1.36 \times 10^{-5} \pm 6.076 \times 10^{-6}$	$1.56 \times 10^{-5} \pm 1.076 \times 10^{-5}$	$8.39 \times 10^{-6} \pm 4.018 \times 10^{-6}$
Mg	$5.86 \times 10^{-5} \pm 3.766 \times 10^{-5}$	$1.87 \times 10^{-3} \pm 3.612 \times 10^{-3}$	$1.13 \times 10^{-3} \pm 1.527 \times 10^{-3}$
P	$6.80 \times 10^{-2} \pm 1.445 \times 10^{-2}$	$3.57 \times 10^{-2} \pm 1.457 \times 10^{-2}$	$3.83 \times 10^{-2} \pm 8.442 \times 10^{-3}$
K	$2.03 \times 10^{-1} \pm 3.658 \times 10^{-2}$ _a	$1.47 \times 10^{-1} \pm 5.209 \times 10^{-2}$ _b	$1.19 \times 10^{-1} \pm 2.882 \times 10^{-2}$ _b
Na	$7.18 \times 10^{-2} \pm 1.423 \times 10^{-2}$ _a	$4.75 \times 10^{-2} \pm 1.492 \times 10^{-2}$ _b	$4.08 \times 10^{-2} \pm 9.549 \times 10^{-3}$ _b
S	$1.14 \times 10^{-1} \pm 2.528 \times 10^{-2}$ _a	$8.55 \times 10^{-2} \pm 3.139 \times 10^{-2}$ _b	$6.61 \times 10^{-2} \pm 1.344 \times 10^{-2}$ _b
Zn	$1.77 \times 10^{-5} \pm 1.080 \times 10^{-5}$	$2.28 \times 10^{-5} \pm 1.304 \times 10^{-5}$	$1.33 \times 10^{-5} \pm 9.659 \times 10^{-6}$

*means with different subscripts are significantly different

Bone density measured by pDEXA

There were no significant differences in the whole body pDEXA scans at the start of the study.(Table 11) No significant differences were determined in vertebral bone mineral composition, abdominal density, vertebral lean mass, vertebral fat mass, abdominal lean mass or abdominal fat mass. At the midpoint of the study, vertebral bone mineral density was greater in R than in F ($p=0.005$), as well as bone mineral concentration ($p=0.006$). Vertebral fat mass and abdominal fat mass were lower in F than in R ($p=0.005$ and $p=0.004$ respectively). However, no significant differences were determined in whole body pDEXA scans at sacrifice.

There was a slightly significant difference in the tibia bone mineral density, with the R having greater density than F ($p=0.014$). There were no significant differences in tibia midshaft, humerus, humerus midshaft, humerus shoulder, femur, or femur midshaft.

Table12 displays the mean pDEXA measurements with standard deviations.

Table 13: Initial pDEXA Whole Body Measurements (g/cm² ± SD)

	C	F	R
Vertebral BMD	0.0826 ± 0.00505	0.0797 ± 0.00725	0.0800 ± 0.00223
Abdominal BMD	0.0765 ± 0.00549	0.0745 ± 0.00498	0.0749 ± 0.00621
Vertebral lean mass	10.413 ± 0.898	10.153 ± 0.999	9.891 ± 0.774
Vertebral fat mass	0.804 ± 0.436	1.031 ± 0.403	0.937 ± 0.369
Abdominal lean mass	48.494 ± 5.306	46.555 ± 4.289	46.669 ± 3.840
Abdominal fat mass	3.839 ± 2.077	4.788 ± 2.124	4.401 ± 1.673

*means with different subscripts are significantly different

Table 14: Midpoint pDEXA Whole Body Measurements (g/cm² ± SD)

	C	F	R
Vertebral BMD	0.151 ± 0.00567	0.146 ± 0.00335 _a	0.154 ± 0.00595 _b
Abdominal BMD	0.134 ± 0.00307	0.132 ± 0.00615	0.137 ± 0.00419
Vertebral lean mass	20.748 ± 1.831	21.086 ± 0.950	19.740 ± 1.539
Vertebral fat mass	2.483 ± 0.485	1.862 ± 0.558 _a	2.852 ± 0.769 _b
Abdominal lean mass	135.317 ± 9.161	136.387 ± 11.385	133.875 ± 8.207
Abdominal fat mass	16.305 ± 3.747	12.080 ± 3.833	19.453 ± 5.667

*means with different subscripts are significantly different

Table 15: Final pDEXA Whole Body Measurements (g/cm² ± SD)

	C	F	R
Vertebral BMD	0.174 ± 0.00984	0.176 ± 0.00892	0.179 ± 0.0106
Abdominal BMD	0.164 ± 0.00423	0.163 ± 0.00878	0.166 ± 0.00697
Vertebral lean mass	23.604 ± 2.436	23.770 ± 2.005	20.370 ± 8.051
Vertebral fat mass	4.798 ± 1.383	4.522 ± 1.407	4.914 ± 1.454
Abdominal lean mass	159.300 ± 13.470	160.375 ± 17.128	158.575 ± 11.648
Abdominal fat mass	32.465 ± 9.718	30.725 ± 10.556	34.572 ± 11.279

*means with different subscripts are significantly different

Table 16: pDEXA of Bones (g/cm² ± SD)

	C	F	R
Humerus	0.160 ± 0.00539	0.16 ± 0.00669	0.159 ± 0.00736
Humerus Midshaft	0.181 ± 0.00821	0.181 ± 0.00832	0.181 ± 0.00955
Shoulder	0.178 ± 0.0125	0.184 ± 0.0079	0.174 ± 0.00723
Tibia	0.163 ± 0.00518	0.159 ± 0.00669 _a	0.169 ± 0.00988 _b
Tibia Midshaft	0.164 ± 0.00640	0.158 ± 0.00875	0.164 ± 0.00522
Femur	0.203 ± 0.0118	0.201 ± 0.0109	0.199 ± 0.0118
Femur Midshaft	0.208 ± 0.119	0.204 ± 0.00917	0.205 ± 0.0129

*means with different subscripts are significantly different

Bone strength

There were no significant differences in the bone breaking measurements. No significant difference in maximum peak load, energy to peak, energy to break, or flexure modulus were determined in the humerus, tibia or femoral head. For break load, R was significantly greater compared to C for the tibia bone (p=0.028)

Table 17: Humerus Bone Strength

	C	F	R
Peak Load (lbs)	14.896 ± 1.055	14.492 ± 1.579	14.786 ± 1.965
Break load (lbs)	14.115 ± 1.349	13.866 ± 1.896	13.951 ± 2.205
Energy to peak (lb*in)	0.131 ± 0.0211	0.223 ± 0.156	0.172 ± 0.101
Energy to break (lb*in)	0.132 ± 0.0204	0.229 ± 0.166	0.174 ± 0.101
Flexure Modulus	6.97 E4 ± 9.65E3	5.29E4 ± 1.27E4	5.85E4 ± 1.49E4

*means with different subscripts are significantly different

Table 18: Tibia Bone Strength

	C	F	R
Peak Load (lbs)	16.546 ± 1.095	15.725 ± 2.684	18.093 ± 2.209
Break load (lbs)	13.421 ± 0.997 _a	14.066 ± 2.651	16.391 ± 3.322 _b
Energy to peak (lb*in)	0.4085 ± 0.116	0.388 ± 0.0842	0.456 ± 0.116
Energy to break (lb*in)	0.458 ± 0.112	0.445 ± 0.0962	0.486 ± 0.113
Flexure Modulus	9.89E5 ± 3.04E5	1.02E6 ± 3.15E5	7.67E5 ± 3.12E5

*means with different subscripts are significantly different

Table 19: Femoral Head Strength

	C	F	R
Peak Load (lbs)	22.803 ± 5.458	22.422 ± 5.832	22.219 ± 5.322
Break load (lbs)	20.995 ± 6.005	20.164 ± 6.607	19.164 ± 4.257
Energy to peak (lb*in)	0.711 ± 0.215	0.703 ± 0.385	0.634 ± 0.390
Energy to break (lb*in)	0.762 ± 0.225	0.772 ± 0.409	0.726 ± 0.498

*means with different subscripts are significantly different

DISCUSSION

The aim of this study was to determine whether or not low levels of FOS and RS incorporated in the diet have a significant effect on bone mineral density and mineral absorption. It was designed to determine whether 5% FOS or 5% RS stimulated mineral absorption through SCFA production, as measured by cecal hypertrophy and digesta pH. Overall, the purpose for studies such as this one, is to determine the functionality of food ingredients for use in convenience foods. Mineral retention was measured during two metabolism periods in order to observe the differences both short term and long term, after body adjustment to dietary FOS and RS. Results indicated no significant differences in food intake during metabolism periods, vital organ weights, body weight, or GI tract weights, except for the cecum. There were significant differences in mineral absorption and urinary mineral output, both short term and long term. However, there were no significant differences in bone mineral density or bone strength.

Food Intake and Diet Composition

There was a significant difference in the cumulative food intake at the end of the study but there were not any significant differences in food intake during the metabolism periods. This indicates that although 5%FOS did not have a significant effect on satiety, 5% RS reduced satiety. Almost all studies indicated similar food intakes among treatment groups, regardless of whether short-term or long-term (6,7,20). Neither FOS nor RS will interfere with normal food intake, which is crucial in patients at risk for malnutrition. Both can be supplemented in the diet without patients experiencing adverse effects in nutrient intake or premature satiety (5). In patients at nutritional risk, decreased food intake can lead to complications such as anemia and osteopenia (30,41). Two groups at high risk for

malnutrition are postgastrectomy patients and the elderly. Postgastrectomy patients almost always experience severe loss of appetite, and the elderly often experience reduced appetites as side effects of medications or secondary to health complications.

Weight Gain and Organs

Weight gain was constant throughout the study among the groups. Although RS contributed to significantly greater cumulative food intake, it did not contribute to increased body weights. Thus, the RS diet must be less energy dense in comparison to the control or FOS, or stimulate digestion enough to increase the metabolism. It is more likely that the RS is less energy dense, and thus acts as a dietary bulking agent. As demonstrated in Graph 1, growth curves of the three groups are almost superimposed. In addition, there were no significant differences in the heart, kidney, liver or spleen masses. These findings support the hypothesis that low levels of FOS and RS would have no effect on normal weight gain and organ development.

Neither FOS nor RS negatively affect normal organogenesis. The rats were fed diet treatments during the active period of growth and experienced no malformations or organ hypertrophy. No known study has demonstrated abnormal organogenesis in FOS or RS-treated animals. Due to the long duration of the feeding treatment, twelve weeks, the animals had almost completely ended normal growth cycle. Comparable human models to the young male Sprague-Dawleys are children and adolescents, since they are in active periods of growth. This indicates that both FOS and RS are safe for child and adolescent consumption. Controlled feeding studies have been completed in male adolescents and infants, with no reported negative effects from FOS supplementation (38,71,72,73).

GI Measurements

As hypothesized, diet treatments did not result in increased stomach or small intestine masses. The low levels of FOS and RS did not affect this part of the GI tract differently than the control starch. A steady fuel source for mucosal cells is needed to observe hypertrophy. Due to natural sloughing off, intestinal epithelial cells completely regenerate about every two days.(30) Maintenance of normal stomach and intestinal weights and increased cecal weight support the theory that FOS and RS not digested in the small intestine, but are fermented in the cecum.

Undigested FOS and RS reach the cecum, where they are broken down by microflora into SCFA. The SCFA products are readily absorbed by colonocytes for energy fuel (2). As anticipated, F had greater empty cecal weights than the control group. However, the cecal weights of R were not significantly greater than those in C, although the mean value of R was greater than C. Increased empty cecal weight is indicative of cecal hypertrophy and increased colonocyte growth, indicating a greater energy supply for epithelial cells. Increased growth rates of colonocytes contributes to overall colon health by lending the ability for damaged cells to quickly regenerate and restore tissue.

Also, FOS exhibited the ability to lower cecal pH in the F group. Lower pH is directly indicative of increased fermentation byproducts, or SCFA (2,24). Thus, FOS is readily available to intestinal microflora to be fermented into SCFA, which are quickly absorbed into colonocytes, enhancing growth. There was no significant difference in mass of the large intestine, thus SCFA's are absorbed at the primary site of fermentation, which is the cecum. It is likely that less than 5% of SCFA produced by fermentation will reach the distal colon (2). Since a reduction in cecal pH and increase in empty cecal mass was observed in F

but not R, FOS is determined to be more readily fermented by intestinal microflora than RS. At low levels, FOS has a greater GI benefits than RS.

Fecal Bulking

The 5% FOS promoted fecal bulking, as was demonstrated in the increased wet fecal mass during period two. Since there were no significant differences in the dry fecal mass, the increased mass was likely due to water retention. A small amount of fluid retention in the feces aids in softening stool and reducing constipation. Excessive fluid retention in the feces results in diarrhea. It should be noted that no watery stools were collected during the metabolism periods. This indicates that low levels of FOS promote healthy defecation and reduced constipation by softening the stool, but do not result in diarrhea or uncomfortable watery stools. Reduced constipation is associated with reduced risk of developing hiatal hernias, diverticulitis, and even colon cancer (1,3).

There was no significant difference in fecal masses during period one, and the most probable cause is that the GI tract needed to adjust to the FOS. After a short period of time, healthy intestinal microflora was established, and the increased SCFA supply resulted in enhanced colonocyte proliferation. Healthy colon growth can increase splanchnic blood flow, which helps support regular peristalsis (2,30). Also, the increased bacterial mass contributes to greater mass of digesta, which may stimulate short reflex nerves on the wall of the digestive tract, resulting in contraction of visceral smooth muscle (30).

Although the dry fecal masses were not significantly different, the FOS group had the highest mean fecal mass in both period one and period two. A likely explanation is that increased bacterial biomass resulted in slightly increased dry fecal masse, but the

experimental parameters were not powerful enough to detect a significant difference. A larger sample size may have the power to indicate significantly different results.

Resistant starch, on the other hand, did not significantly promote laxation or increase fecal mass. Although the mean mass of wet and dry feces were greater than the control means in metabolism period 1 and 2, these differences were not significant. One likely explanation mentioned previously is that the RS is not fermented as extensively as FOS, which is supported by the lower cecal pH in the R groups. Poor fermentative ability would result in less colonocyte proliferation compared with the highly-fermented FOS. The concentration of SCFA's would not be as high and would not increase splanchnic blood flow. Also, bacterial proliferation would not be as great, and a large portion of the fecal mass is likely from bacterial biomass.

Mineral Absorption

During period 1, the R group experienced lower percent absorption of copper. Both RS and F appeared to increase the calcium absorption, and F increased the absorption of magnesium. This further indicates that the FOS was fermented to a greater degree than the RS, thus having a greater impact on the solubility of minerals. By the end of the study, at metabolism period 2, there failed to be a significant difference in calcium absorptions. The higher percent absorptions during period 1 could be result of greater calcium needs as opposed to period 2. During that time frame, the young male rats were experiencing more rapid growth rates. When the body is experiencing rapid growth, it becomes more sensitive to calcium, in order to enhance absorption to meet the increased needs. Calcitriol production is stimulated when calcium needs are elevated, and one of calcitriol's main effects is to enhance calbindin production in enterocytes (29,30,49). During times of increased needs,

calbindin increases, thus increasing the body's ability to absorb calcium. Thus, during times of increased needs, there is a more profound, or significant, effect of the FOS and RS on calcium absorption.

In their research on gastrectomized rats, Ohta et al found that dietary FOS increased calcium absorption and mucosal calbindin-D9K (74,75). Calbindin-D9K is a major intestinal mucosal protein that enhances calcium absorption in the rat intestine. In their study, they used 4-week-old male Sprague Dawley rats that were either gastrectomized or sham operated. Their results indicated calbindin-D9K was increased in the cecum and intestine, but decreased in the proximal and distal small intestine of gastrectomized rats. Calcium absorption in FOS supplemented gastrectomized rats was increased to the level of the control, sham rats. Thus, their results indicate that FOS increases calcium absorption in a high-risk group through a mechanism involving increased calbindin-D9K in the cecum and colon (74,75). FOS supplementation has the potential to increase calcium absorption, and thus decrease gastrectomy-induced osteopenia.

The RS group displayed significantly decreased absorption of copper in both period one and two, and an additional decreased absorption in period 2 of iron and zinc. One explanation for reduced absorption is mineral binding to the starch, resulting in reduced bioavailability. These three minerals are all row one transition metals, which are electronegative and highly reactive. Metals will covalently bind to ligands, forming large organometallic compounds. Undigested carbohydrate in the colon, such as phytate, readily binds minerals (48,50,51). However, values for copper and iron were negative, indicating greater fecal output than intake. The only way this is possible is through excessive wastes such as bilirubin, a byproduct of hemoglobin degradation (31), or microbial biomass losses.

Another possible cause of negative percent absorptions for these minerals is contamination of fecal samples or R diet that was analyzed via ICP. Copper, iron and zinc are all microminerals, which are present in very small amounts in biological compounds (49). Minute contaminations could cause large differences in percent absorption.

Urinary Mineral Output

Surprisingly, there were a number of significant differences in the urinary mineral output. However, these almost all correspond with mineral intakes. The greater the percent absorption, the greater the mineral output. This demonstrates powerfully the body's ability to maintain homeostasis. Largely, the reason why significant differences were not observed in bone mineralization and strength is because the study was not designed to most effectively determine the power of FOS and RS. Although both FOS and RS may demonstrate the ability to increase mineral bioavailability and percent absorption, if the body is not in a nutritionally compromised state, the excess mineral that is absorbed is excreted in the urine. Percent absorption of magnesium was significantly greater in F during period 1, as was the magnesium urinary output. Also, if percent absorption is slightly depressed, as observed in the R group during period 2 for copper, iron and zinc, urinary excretion may be significantly reduced, as was the urinary copper excretion for R. Three mineral output values that remained the same from period 1 to period 2 were sodium, phosphorus and sulfur, which were all greater in C than F and R. Sodium intake was greatest in C in both periods, but there is no relation to phosphorus or sulfur. However, the kidneys do not actively monitor serum P or S as they control Na and K concentrations (30). Thus, it is likely that although the F and R groups decreased sodium percent absorption, they also had decreased urinary excretion in order to maintain homeostasis.

Also, the accuracy of the urinary mineral measurements is questionable due to possible contamination by the diet. When urine samples were collected, some diet spillage had to be filtered out. However, some of the minerals from the diet could dissolve in the urine, thus causing differences in mineral content.

Bone Mineralization

The only significant difference observed in bone mineral analyzed by ICP was in the potassium, which does not contribute to mineralization of bone. Potassium is an electrolyte found in the blood, and is highly regulated by renal function. A likely explanation is that these mineral compositions reflex serum concentrations. With this theory, however, sodium concentrations in the N group would be expected to be lower as well, but this is not the case.

Perhaps the serum sodium was more highly regulated by the kidneys and therefore less affected by GI absorption. Of the two main electrolytes, potassium is the major intracellular osmotically active solute, and sodium is the major extracellular electrolyte. However, these concentrations are highly regulated by cellular Na/K ATPase and other membrane channel proteins. Although it is possible for differences in serum potassium and intracellular potassium to affect the concentrations in the bone mineral, the most likely explanations are slight differences in the dehydration of bones or chemical contamination of samples.

Bone Density

Neither FOS nor RS had an effect on bone density; thus, although they do not increase bone density in healthy rat models, they do not have a negative effect either. Therefore, both FOS and RS are safe for human consumption in children and adolescents, without causing any adverse effects on normal, healthy bone growth and development.

Bone Strength

Since there were no significant differences in bone stress test measurements, neither low levels of FOS nor RS have the ability to increase bone strength in normal rats. It can be extrapolated to assume that neither carbohydrate would lead to stronger bones in normal, healthy young adults. As studies by Ohta et al suggest, (41,42) a better model for the potentiating effects of FOS and RS on bone strength would be gastrectomized rats or ovariectomized rats (55). In order to see significant benefits of FOS and RS on bone health, it appears that there needs to be a critical state of osteopenia. In situations of nutritional risk or malnutrition, FOS and RS have demonstrated the ability to increase mineral retention, bone strength and bone density.

CONCLUSION

FOS and RS are practical supplements to increase the functionality of convenience foods. Both are proven safe and effective in increasing GI health. Although FOS appears more readily fermented by intestinal bacteria, RS still demonstrates active fermentation, as measured by quantitative cecal pH. Normal neutral or alkaline pH of the colon leads to the poor mineral absorption ordinarily experienced. Supplementation of FOS or RS in the diet can increase the absorption of minerals such as calcium via paracellular absorption in the large intestine.

First, the fermentation of the FOS results in decreased luminal pH, which increases the solubility of minerals. Increased solubility gives rise to increased absorption through passive transport mechanism. This effect is more profound in subjects in rapid periods of growth, such as children or adolescents, or in a state of nutritional deficiency, such as gastrectomized or ovariectomized subjects. Higher concentrations of soluble mineral in the colon will give rise to greater absorption rates, because of the concentration gradient-dependent passive transport.

Without any measurement of biological markers, such as calbindin-D9K, or SCFA analysis of digesta, the mechanism behind the increases and decreases in mineral absorption must be limited to assumptions. Future studies should target quantification of intestinal microflora increases, cecal SCFA levels and biochemical markers of changes in physiology.

Thorough research has consistently indicated that both Nutraflora® fructooligosaccharide and Hi-Maize® resistant starch are fit for human consumption. The next step is to further evaluate the functionality of both compounds in food applications.

Extensive actions need to be taken to standardize measurements of naturally occurring RS and FOS in foods. Also, prebiotic content of foods should be listed separately on food labels, since they have such different physiological effects than ordinary dietary fiber or nondigestible dietary starch (NDS). A person whose intestinal microflora is not accustomed to abundant carbohydrate supply in the colon may experience flatulence, cramping, or loose stools due to osmotic diarrhea. Although consumers need to be aware of these potential side effects, they also need to be educated on the benefits of dietary FOS and RS. Low levels, 5%, of both RS and FOS have proven safe for human consumption, and neither have resulted in the gastrointestinal problems observed at higher supplementation levels. Thus,

As functional foods in food applications, both RS and FOS can increase the health benefits of a variety of convenience foods. RS can easily be substituted for starches or flour in baked goods, or as a thickening agent in beverages or gravies. On the other hand, FOS is hydroscopic and water soluble, making it easily incorporated into liquids and a good moistening agent in other food products. Additionally, FOS is sweet in taste, so it could substitute for a portion of caloric sweeteners such as sugar or high-fructose corn syrup. In conclusion, corporations should examine the food applications of RS and FOS, in order to increase the functionality of convenience foods. This will aid in meeting the nutritional needs of today's fast-paced society and reduce the rapidly declining health.

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APPENDIX A: ANIMAL PROTOCOL

REVISED:

APRIL 2002

LSU PROTOCOL FOR ANIMAL CARE AND USE

Instructions for Submission: MUST BE TYPED! (Use additional sheets if necessary and attach to this form or use word processor and add lines). **SUBMIT ORIGINAL plus 13 COPIES** to the IACUC Office (Rm. 1502 School of Veterinary Medicine).

PROTOCOL NUMBER: _____

APPROVAL DATE: _____

SECTION 1: Principal Investigator

Name: Maren Hegsted	Office Phone: 578-1518 Home Phone: 769-3097	E-mail address: mhegsted@lsu.edu
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SECTION 2: Project Title (Enter the name of your project/course number in the block below)

Mineral absorption in rats using a combined dietary supplement of fructooligosaccharide and Aqua-Min

SECTION 3:

Animal Species

Species: Rat		Strain: Sprague-Dawley
Number of animals needed: <u>192</u> Year 1: <u>96</u> Year 2: <u>96</u> TOTAL: <u>192</u>	Maximum number needed at one time: <u>48</u>	Number of animals to be placed in each group: <u>8</u>

REVISED: APRIL 2002

Animal housing and veterinary care have been coordinated with DLAM office OR LSU Agricultural Center Unit.

☒ YES

☐ NO

Name of Animal Housing Representative Contacted (typed): Rick Ramsey

Signature (required): _____

Location of Animals

<input checked="" type="checkbox"/>	DLAM Vivarium	<input checked="" type="checkbox"/>	Life Sciences Vivarium
<input type="checkbox"/>	SVM Barns	<input type="checkbox"/>	SVM Fish Building
<input type="checkbox"/>	LAES (List Site):	<input type="checkbox"/>	Other (List Site):
<input type="checkbox"/>	Field Study (Do not complete sections 6, 11, and 12		

SECTION 4: Abstract Plan of Research/Teaching

Provide a brief layman's description of the project in the block below.

Fructooligosaccharides(FOS) resist digestion in the small intestine similar to fiber and resistant starch. This may aid in the absorption of minerals during fermentation in the colon (and cecum in rats). Aqua-Min (AM) is a dietary supplement produced from seaweed containing Ca, P, S, Mg, Zn, Cu, I, Se, and B. The addition of fructooligosaccharides to the Aqua-Min may increase the amount of mineral absorption into the body. The study will determine the level of mineral absorption in different diet treatment groups. The feces and urine will be collected for 2 weeks at the beginning, midpoint and end of the 12 week study. Mineral levels will be determined for feces, urine, and bone through ICP spectrophotometry. Bone mineral density will be analyzed through peripheral dual energy x-ray absorptiometry (pDexa) *in vivo* and *in vitro*.

SECTION 5: Investigator's Statement. Assurances for the Humane Care and Use of Vertebrate Animals.

By signing this form, we agree to abide by the Policy for the Care and Use of Animals of Louisiana State University, or that of the LSU Agricultural Center. This project will be in accordance with the NIH "Guide for the Care and Use of Laboratory Animals" (except as explained in the accompanying protocol), and the Louisiana State University or the

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LSU Agricultural Center Animal Welfare Assurance on file with the U.S. Public Health Service.

I further assure the Committee that: 1) I will abide by all federal, state, and local laws and regulations governing the use of animals in teaching and research; 2) the investigators and technicians are adequately trained to perform the research techniques required in these studies; and 3) the fewest number of animals required to produce significant results are being used in this study.

Principal Investigator Signature	Professor Title/Rank	02/04/2003 Date
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Maren Hegsted
(Type Name of Principal Investigator)

Co-Investigator Signature	Research Associate Title/Rank	02/04/2003 Date
---------------------------	----------------------------------	--------------------

Kathleen L McCutcheon
(Type Name of Co-Investigator)

Co-Investigator Signature	Research Associate Title/Rank	02/04/2003 Date
---------------------------	----------------------------------	--------------------

Anne R Francis
(Type Name of Co-Investigator)

SECTION 6: Special Husbandry Requirements

Do your animals have special needs to be addressed by DLAM?

☒ YES

TEMPERATURE RANGE	(F) 70-72	Humidity:	(%)
LIGHT CYCLE (hours light/hours dark)			
CAGING	Type: metabolic and SS wire	Size: single	Filter tops required? Yes during transport to lab
BEDDING/LITTER	Type: corncob or absorbent paper weigh backs	Autoclaved? No	Changes/week: by investigators

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WATER	Sterile:	De-ionized: yes	Acidified:	Tap:	Other:
DIET	Special Feeding Requirements: special diet provided / we will feed				
OTHER SPECIAL NEEDS	investigators will feed, water, and change caging				

☐ **NO** (If you indicate 'No', your animals will be cared for according to standard operating procedures of DLAM)

☐ **Not Applicable**

SECTION 7: Hazardous Materials

Will zoonotic or recombinant, radioactive, or hazardous chemical agents be **PRESENT IN THE ANIMAL ROOM?**

Zoonotic/Recombinant Agents? <input type="checkbox"/> YES <input checked="" type="checkbox"/> NO Agent(s): <input type="checkbox"/> EXEMPT IBRDS Chairman Signature:	Radioisotopes? <input type="checkbox"/> YES <input checked="" type="checkbox"/> NO Isotope(s): Are you certified by the Radiation Safety Committee? <input type="checkbox"/> YES <input type="checkbox"/> NO	Hazardous Chemicals? <input type="checkbox"/> YES <input checked="" type="checkbox"/> NO Compound(s):
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Note: If zoonotic (infectious to humans) or recombinant organisms are to be used, this protocol request must be submitted to the Biohazardous Materials Safety Committee for approval **PRIOR TO CONSIDERATION** by the IACUC. Similarly, if hazardous chemicals are to be used in the animal room, submit the proposal to the Chemical Safety Committee for prior approval. **P.I. MUST PROVIDE** health and safety measures for animal technicians and facility maintenance personnel. In Standard Operating Procedure (SOP) form, describe any precautions, procedures, or personal protection required in handling animals or waste containing listed agents or compounds, or in working in or around the animal room (including air handling system), and **attach a copy of your SOP(s) to this protocol proposal.**

SECTION 8: Summary of Procedures

Your response in this section should provide the reader with a complete description of how every animal to be used in this project is to be treated during every phase of the study. Your target audience is a faculty member from a discipline unrelated to yours. Do not use jargon. Please answer questions 1-6 in consecutive order (outline format):

- 1) The rationale for using animals. Why should this study be done? What hypothesis will be tested?
- 2) How and/or why you selected the animal species indicated.
- 3) How you arrived at the number of animals to be used.
- 4) A complete description of the proposed use of the animals. Describe the experimental design of the

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study. Include a list of any physical, chemical or biological agents (**name, dose, volume, route, frequency**) that may be administered. Tables and outlines are helpful to indicate group assignments and study progression.

- 5) A description of procedures designed to assure that discomfort and injury to animals will be limited to that which is unavoidable in the conduct of scientifically valuable research. Indicate how analgesic, anesthetic, and tranquilizing agents will be used where appropriate, to minimize discomfort and pain to the animals. It is advisable that you obtain input from LSU's Attending Veterinarian (Dr. David Baker) or from another veterinarian familiar with the species to be used.
- 6) A description of any euthanasia method to be used.

- 1) Rats used to determine the absorption of minerals using the combination of fructooligosaccharide (FOS) and Aqua-Min(AM) to be used as a dietary supplement for human use. An additional diet treatment group using a 60% amylopectin resistant corn starch (RS) will be included to determine absorption differences between RS and FOS. A control diet (C) will follow the recommended AIN-93 G.
- 2) Spague-Dawley rats will be used as the animal model to replicate the variations in human populations and provide information without risk to humans.
- 3) 8 rats per treatment group will allow for statistical data analysis.
- 4) A 2 x 3 design will be used to determine the differences in mineral absorption of each diet treatment group.

		C	FOS	RS
C	AM	C n=8	F n=8	R n=8
		A n=8	AF n=8	RA n=8

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The powdered diets will be provided ad libitum as a modified AIN-93 G formulation containing the supplements listed above. The study will comprise of 12 weeks divided into 3 treatment periods. Feces and urine will be collected in metabolic cages for two weeks during each period. A pDexa will be performed on each rat at the beginning of each period.

PERIOD	week 1	week 2	week 3	week 4
1	p-dexa : day 1 metabolic cages		SS wire cages	
2	p-dexa : day 1 metabolic cages		SS wire cages	
3	p-dexa : day 1 metabolic cages		SS wire cages	

total 12 Weeks

- 5) The animals will be transported in shoe-boxes with micro-isolator lids to room 433 Life Sciences building for each of the 3 pdexa scans. They will be fasted overnight and anesthetized with isoflurane gas prior to scanning and allowed to recover before returning to the 6th floor vivarium.
- 6) At the end of the 12 week treatment period, the rats will be transported individually in shoe boxes with micro-isolator lids to room 433 Life Sciences Building. They will be anesthetized with isoflurane gas prior to a final scan using the pDexa. After scanning while still under anesthesia, they will be euthanized through cardiac puncture. Up to 10cc of blood will be withdrawn with a syringe resulting in termination. Cervical dislocation will assure death prior to dissection. The gastrointestinal tract will be removed and weighed to determine the affects of the FOS and RS on the GI tract. Femur, Humerus and vertebrae will be isolated for analysis.

SECTION 9: Type of Project

Type designation will not affect evaluation or approval of this protocol.

X	TYPE A - Pain or distress will not be induced; animals will only be used for injections, collections, or procedures causing nothing more than minor discomfort; or will be humanely euthanized prior to induction of pain or distress.
	TYPE B - Pain or distress will be relieved by appropriate therapy.
	TYPE C - Drug intervention for pain or distress would interfere with the protocol. (If this block is checked, specific justification MUST be provided.)

SECTION 10: Check "Yes" or "No" to each of the following questions. On a separate page, provide an explanation for any "Yes" answers that are not included in the above summary.

- 7) Provide justification for **why** the action is needed, and include information in Section 8 above, such as **who** will perform procedures, **how** they will be performed, frequency, duration, drugs to be used, dosages, routes of administration, etc. Not all of this information may be needed for every "Yes" answer. The information you provide in this section is very important in highlighting specific points of your study that are important considerations for the IACUC in their review process.

YES	NO		Individual(s) Responsible
	x	Will animals be restrained? (<i>Restraint refers to immobilization or other restrictions to normal movement beyond momentary holding for injections, etc.</i>)	Not Applicable
x		Will animals be fasted?	Not Applicable
x		Are any ANESTHETICS, ANALGESICS, or TRANQUILIZERS to be used? Who will administer?.....	Hegsted, Francis, McCutcheon
	x	Are neuromuscular blocking agents to be used? Who will administer agents?..... How will animals be monitored?	

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	x	<p>Will surgical procedures be employed? Are they:</p> <p>Survival Multiple Terminal</p> <p>Who will perform surgery?.....</p> <p>If survival:</p> <p>1) Who will be responsible for recovery of the animals?.....</p> <p>...</p> <p>2) Who will maintain post-operative records?.....</p> <p>3) Where will records be maintained?</p> <p>4) Who will provide post-op analgesics?.....</p> <p>Note: <i>Survival surgeries must be conducted aseptically, and major surgical procedures performed on non-rodent species must be conducted in a dedicated surgical facility.</i></p>	
	x	Do you anticipate any adverse effects of the experimental procedures on the animals (e.g., pain, discomfort, reduced growth, fever, anemia, etc)?	Not Applicable
	x	Is death an endpoint in your experimental procedure? Note: <i>Death as an endpoint refers to acute toxicity testing, assessment of virulence of pathogens, neutralization tests for toxins, and other studies in which animals are not euthanized, but die as a direct result of the experimental manipulation.</i>	Not Applicable
x		Are there emergency treatments by the DLAM veterinary staff that would not be allowed?	Not Applicable
	x	Are you using wild or exotic species for which permits are necessary? (ATTACH COPY) Note: <i>A copy of permit must be received before animal use begins.</i>	Not Applicable

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x		Will animals be euthanized during or at the close of the study? Who will perform euthanasia?.....	Hegsted, Francis, McCutcheon
	x	Will animals be used for antibody production?	Not Applicable
	x	Will Complete Freund's Adjuvant be used? If yes, please justify based on scientific reasons.	Not Applicable
	x	Will other adjuvants be used? If yes, please specify.	Not Applicable
	x	<p>Will blood be collected?</p> <p>How often?</p> <p>Volume?</p> <p>Who will collect blood?.....</p> <p>Note: Blood equal to 1.5% of the animal's body weight per 2 weeks represents the upper approvable limit, unless scientific justification is provided.</p>	

SECTION 11: Animal Management

Individual (or groups of) animals are identified by (i.e. tag, tattoo):

Check all applicable below:

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CARE OF SICK ANIMALS	DISPOSAL OF DEAD ANIMALS	PEST CONTROL
<input checked="" type="checkbox"/> Call Investigator <input type="checkbox"/> Clinician to Treat <input type="checkbox"/> Euthanasia no antibiotics given due to gut microflora	<input checked="" type="checkbox"/> Call Investigator <input type="checkbox"/> Necropsy <input type="checkbox"/> Disposal. List any special requirements for disposal?	<input checked="" type="checkbox"/> Call Investigator <input type="checkbox"/> Pesticides OK <input type="checkbox"/> No Pesticides

SECTION 12: Disposition of Animals

What will be done with any animals at the conclusion of the project?

x	Animals will be euthanized.
	DLAM/LAES has permission to REASSIGN animals to another IACUC-approved protocol.
	TRANSFER animals to the following IACUC-approved protocol(s). Please list Protocol Number:
	OTHER (Please state)

SECTION 13: Narrative Statement

Federal regulations mandate that you provide **written, narrative statements** for all projects:

- 1) that the activities do not unnecessarily duplicate previous experiments. In this statement, include sources used to make such a determination (e.g., Databases, workshops, expertise in the field, etc.) If an electronic database was used, include database, years and words searched, and date of search.

Note: Address the following items only if you indicated project Type B or C in SECTION 9.

- 2) that you have considered alternatives to procedures producing more than momentary or slight pain or distress. Indicate what those alternatives were and why they are not appropriate.
- 3) describing the methods you used to determine that alternatives to such procedures were not available (Databases, years and words searched, date of search etc.). Put your statements in the block below.

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SECTION 14: Investigator Training

In accordance with IACUC policy, all personnel conducting animal-based research must attend a Rules and Regulations Course and verify their training, experience and skills in the care and use of the animals and techniques they are responsible for.

List all persons involved in animal care and use for this study below.

RULES AND REGULATIONS TRAINING COURSES					
Name	Rules/Regulations Training Course	Date Attended	Species Wet Lab*	Date Attended	Training and Experience**
Maren Hegsted	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	3/8/00	<input type="checkbox"/> YES <input type="checkbox"/> NO		<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO
Anne Francis	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	11/6/01	<input type="checkbox"/> YES <input type="checkbox"/> NO		<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO
Kathy McCutcheon	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	11/6/01	<input type="checkbox"/> YES <input type="checkbox"/> NO		<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO
	<input type="checkbox"/> YES <input type="checkbox"/> NO		<input type="checkbox"/> YES <input type="checkbox"/> NO		<input type="checkbox"/> YES <input type="checkbox"/> NO
	<input type="checkbox"/> YES <input type="checkbox"/> NO		<input type="checkbox"/> YES <input type="checkbox"/> NO		<input type="checkbox"/> YES <input type="checkbox"/> NO

* Exemption based on previous experience with the study species may be obtained by written request to the IACUC.

** Training/Experience in assigned procedures for this protocol.

Who will train individuals for participation in protocol procedures?

Personnel participating in the project that have not attended the Rules and Regulations Course or the applicable Species Wet Lab, will have **six (6) months** from the approval date of the project to complete them.

Rules and Regulations Courses will be held the first Tuesday of every month from 11:00 a.m. until Noon, in room 1212C, School of Veterinary Medicine. The Wet Labs will be

held on the same day beginning at 1:00 p.m. in the DLAM facility. Please call Ms. Dawn Best-Desjardins at 578-9643 to sign up for these courses.

SECTION 15: Occupational Health and Safety

It is the responsibility of the principal investigator to conduct a hazard analysis and risk assessment to determine if personnel involved in the proposed study should participate in the Occupational Health Program administered through DLAM and the Student Health Center. Currently, there is **no direct cost** for participation in the program. **Please have all persons listed in Section 14 read the following and indicate level of participation with their signature:**

The Division of Laboratory Animal Medicine operates an Occupational Health Program (OHP). Participation is voluntary, and is open to all personnel with direct or indirect contact with animals used in teaching or research, their bodily products, or materials to which they may be exposed, as described in this protocol. Eligible persons include facility services personnel, animal caretakers, principal investigators, technical staff, graduate and other student workers, and post-doctoral and visiting scientists. All medical information is kept confidential, and is retained by the Student Health Center. You have the right to refuse any and all procedures recommended.

To determine the extent of your participation in the OHP, discuss with the principal investigator named on this protocol, and/or your health professional, any potential physical, chemical, or infectious hazards to which you may be exposed while working on the project. Whether or not you participate, questions related to health risks should be directed to Dr. Tim Honigman, Campus Physician, at the Student Health Center. To participate in the OHP, please contact Mr. Rick Ramsey at 578-9644 for information.

If you are at increased risk of illness or injury due to drug-related immune suppression, HIV infection, pregnancy, concurrent illness, musculoskeletal problems, etc., you are advised to discuss your risks with Dr. Honigman, your personal physician, or another health professional.

Persons listed in Section 14 above:

Printed Name	Signature	<input type="checkbox"/> I choose to participate <input type="checkbox"/> I choose NOT to participate
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Printed Name	Signature	<input type="checkbox"/> I choose to participate <input type="checkbox"/> I choose NOT to participate
--------------	-----------	--

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Printed Name

Signature

☐ I choose to participate
☐ I choose NOT to participate

Printed Name

Signature

☐ I choose to participate
☐ I choose NOT to participate

Printed Name

Signature

☐ I choose to participate
☐ I choose NOT to participate

APPENDIX B: IACUC APPROVAL

LOUISIANA STATE UNIVERSITY
Institutional Animal Care and Use Committee
Protocol Checklist
February 2002

Dear Investigator:

The LSU IACUC has agreed that the following items must be completed on your IACUC form before the IACUC coordinator, Ms. Dawn Best-Desjardins, can accept it. I will review this completed checklist before the next IACUC agenda is set. If you have a question concerning anything on this checklist or your IACUC form, please feel free to contact me at 578-4763 or via email at pelzer@lsu.edu.

Please note that Ms. Best is not responsible for obtaining the information to make your protocol complete, and that the committee has charged her not to take any protocols that do not comply with the list below.

Thank you,

Philip H. Elzer, Ph.D., Chairman
Institutional Animal Care and Use Committee

CHECKLIST:

- ☐ A. 13 copies, plus original.
- ☐ B. SECTION 3: Signature of animal housing representative on the original form.

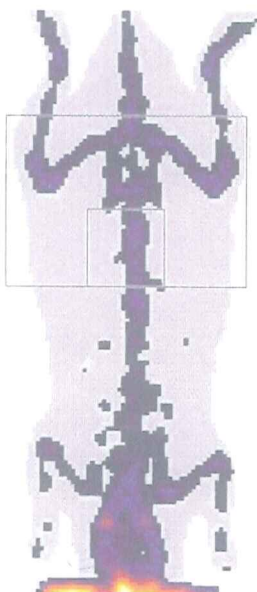
DLAM: Rick Ramsey, Cecelia (CK) Koon, Laurie Henderson, or Dr. David Baker (Ms. Best is not an animal housing representative)
AgCenter: Gill Barker, Chip Lemieux, Trey Harding, Mike Canal, or Joel Walker
- ☐ C. SECTION 5: Signature of PI, CoPI, and Surgeon on original form.
- ☐ D. SECTION 7: Hazardous Material Information section filled out properly with signatures if applicable.
- ☐ E. SECTION 9: Type of project must be checked. This will not influence approval of your protocol.
- ☐ F. SECTION 13: Complete based on Section 9.
- ☐ G. SECTIONS 8 and 13: Answer all questions. Do not attach inserts from your grant application. This protocol form serves as a "stand alone" document.
- ☐ H. Proposal Routing and Approval Form (PRAF): Office of Sponsored Programs

NOTE: IF ANY OF THE ITEMS ABOVE ARE MISSING, YOUR PROTOCOL WILL NOT BE ON THE AGENDA OF THE NEXT MEETING.

APPENDIX C: Initial pDEXA of R6

Name	R6	Ethnic	SD
ID	R6 5-8-03	Height	10.0
Age	0	Sex	Male
		Weight	127

L  H Research on 05/02/03 7:09



05/02/03 0.075

Bone image not for diagnosis
Histogram Averaging Width: 0.0300 g/cm²

MD RES Scan

	BMD	BMC	AREA	LENGTH	WIDTH
	g/cm ²	g	cm ²	cm	cm
VERT	0.0750	0.1207	1.609	2.00	2.00
AB FAT	0.0703	0.6311	8.970	4.40	6.20

See Operator's Guide for information on CVs.

1.0 x 1.0 mm, 40 mm/s, 11.40 cm Rev. 3.9.4/1.1.1 Calib. 05/02/03

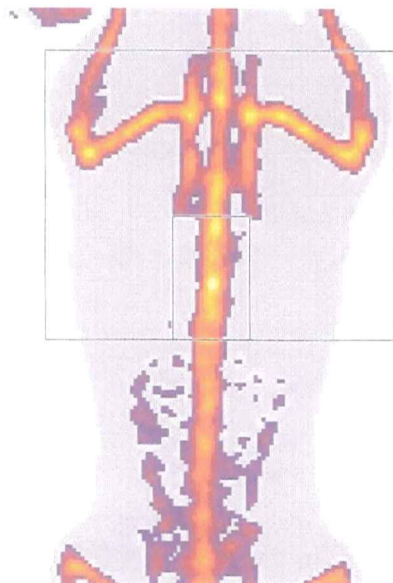
COMMENTS

 NORLAND

APPENDIX D: Midpoint pDEXA of R6

Name	R6	Ethnic	SD
ID	R6	Height	16
Age	0	Sex	Male
		Weight	350

L  H Research on 06/13/03 2:49



06/13/03 0.155

Bone image not for diagnosis
Histogram Averaging Width: 0.0600 g/cm²

MD RES Scan

	BMD g/cm ²	BMC g	AREA cm ²	LENGTH cm	WIDTH cm
VERT	0.1547	0.5371	3.472	3.20	2.00
AB FAT	0.1341	2.741	20.44	7.50	9.10

See Operator's Guide for information on CVs.

1.0 x 1.0 mm, 40 mm/s, 11.40 cm Rev. 3.9.4/1.1.1 Calib. 06/12/03

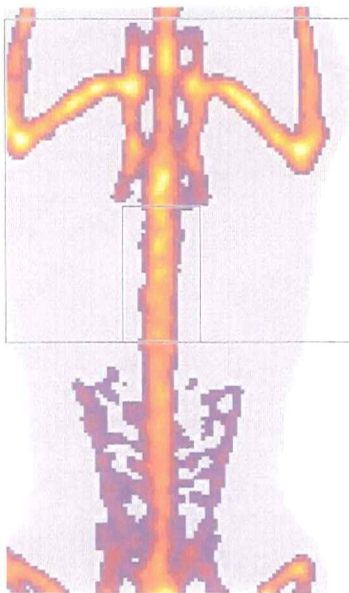
COMMENTS

 NORLAND

APPENDIX E: Final pDEXA of R6

Name	R6	Ethnic	SD
ID	R6 8/13/03	Height	17.5
Age	0	Sex	Male
		Weight	448

L  H Research on 07/22/03 5:20



07/22/03 0.164

Bone image not for diagnosis
Histogram Averaging Width: 0.0600 g/cm2

MD RES Scan

	BMD g/cm ²	BMC g	AREA cm ²	LENGTH cm	WIDTH cm
VERT	0.1641	0.6256	3.811	3.50	2.00
AB FAT	0.1580	3.677	23.27	8.30	8.90

See Operator's Guide for information on CVs.

1.0 x 1.0 mm, 40 mm/s, 11.40 cm Rev. 3.9.4/1.1.1 Calib. 07/22/03

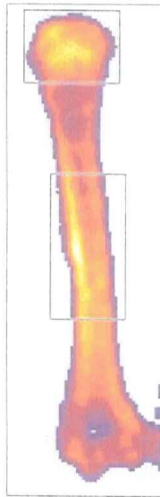
COMMENTS

 NORLAND

APPENDIX F: Humerus pDEXA of N4 (F4)

Name	N4	Ethnic	SD
ID	N4 HUMERUS	Height	
Age	0	Sex	Male
		Weight	

L  H Research on 07/29/03 5:44



07/29/03 0.170

Bone image not for diagnosis
Histogram Averaging Width: 0.0600 g/cm²

MD RES Scan

	BMD g/cm ²	BMC g	AREA cm ²	LENGTH cm	WIDTH cm
HUMERUS	0.1699	0.2370	1.395	3.38	1.08
	0.1973	0.06827	0.3460	1.00	0.52
SHOULDER	0.1969	0.05006	0.2542	0.50	0.66

See Operator's Guide for information on CVs.

0.2 x 0.2 mm, 10 mm/s, 1.30 cm Rev. 3.9.4/1.1.1 Calib. 07/29/03

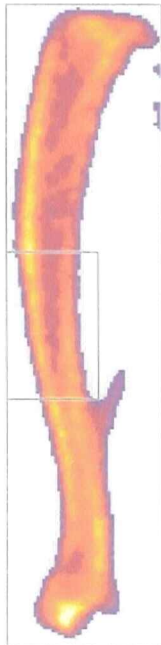
COMMENTS

 NORLAND

APPENDIX G: Tibia pDEXA of N4 (F4)

Name	N4 TIBIA	Ethnic	SD
ID	N4	Height	
Age	0	Sex	Male
		Weight	

 H Research on 07/27/03 9:26



07/27/03 0.171

Bone image not for diagnosis
Histogram Averaging Width: 0.0600 g/cm²

MD RES Scan

	BMD g/cm ²	BMC g	AREA cm ²	LENGTH cm	WIDTH cm
TIBIA	0.1715	0.3530	2.059	4.36	1.06
MIDSHAFT	0.1705	0.06827	0.4005	1.00	0.62

See Operator's Guide for information on CVs.

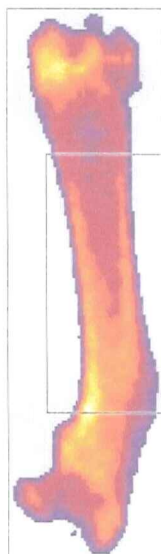
0.2 x 0.2 mm, 10 mm/s, 1.30 cm Rev. 3.9.4/1.1.1 Calib. 07/27/03

COMMENTS

APPENDIX H: Femur pDEXA of N4 (F4)

Name	N4	Ethnic	SD
ID	N4 FEMUR	Height	
Age		Weight	
	Sex Male		

L  H Research on 08/17/03 16:46



08/17/03 0.213

Bone image not for diagnosis
Histogram Averaging Width: 0.0600 g/cm²

MD RES Scan

	BMD g/cm ²	BMC g	AREA cm ²	LENGTH cm	WIDTH cm
FEMUR	0.2128	0.5672	2.665	4.24	1.20
MIDSHAFT	0.2184	0.2479	1.135	2.00	0.90

See Operator's Guide for information on CVs.

0.2 x 0.2 mm, 10 mm/s, 2.00 cm Rev. 3.9.4/1.1.1 Calib. 08/17/03

COMMENTS

 NORLAND

APPENDIX I: Rat Femoral Head Bio-Mechanical Testing Procedure

Developed by Michael Reeves M.S. (68)

Preparing the Femur Mounting Hardware

Materials

- ½ inch I.D. copper pipe
- Mini-pipecutter
- Metallic duct tape
- Hot-glue gun
- H.B. Fuller hot-melt wood glue sticks

Procedure

1. Using the mini-pipecutter, cut the copper pipe into 1" long sections
2. Seal one end of the copper pipe using a 1" square of metallic duct tape
3. Seal the bottom of each pipe section with hot glue

Femur Mounting Procedure

Materials

- Sealed ½ " I.D. copper pipe sections
- Crushed ice
- Metal or plastic container with approximately 12 in² of bottom area
- Hot-glue gun
- H.B. Fuller hot-melt wood glue sticks
- Kimwipes tissues
- Specimen tray
- Plastic-lined paper or plastic wrap
- Dial caliper
- Spray bottle filled with 0.9% saline solution
- Femur specimens stored in 0.9% saline solution

Procedure

1. Label 1" sections of copper pipe to correspond with specimen labels
2. Prepare the ice-water bath and place in refrigerator. Adjust the level of ice/water mixture to approximately 1" deep.
3. Cover the bottom of the specimen tray with either plastic-lined paper or plastic wrap.
4. Label the specimen tray with a waterproof marker to correspond with specimen labels.
5. Plug the glue gun cord into an electrical outlet and place the nozzle over a non-flammable, disposable material.
6. Thaw femurs in their sealed containers under cold running water until free from surrounding ice.
7. Wrap each femur in a Kimwipes ® tissue and place on tray to correspond with appropriate label.
8. Immediately spray each femur with 0.9% saline solution until the Kimwipes ® tissue is moistened to prevent bone drying.
9. Fill the appropriate copper pipe section about 2/3 to ¾ from the top with hot glue.

Note: standardization among samples is necessary in this step.

10. Wait about 15 seconds for the hot glue to begin to gel and insert the distal end of the femur until about 2/3 of the femur is imbedded in the glue. Make sure that the femur is directly perpendicular to the horizontal base.
11. Immediately submerge the copper pipe with the imbedded femur in the ice-water bath for 2 to 3 minutes.
12. Using the dial caliper, make vertical and horizontal measurements at the narrowest part of the femoral neck to get an average neck diameter.
13. Using the dial caliper, measure the distance between the top of the femoral head to the surface of the hardened glue inside the copper pipe to determine the gauge length.
14. After taking the measurements, wrap the exposed femur in the saline-moistened Kimwipes® tissue until time for mechanical testing.

