Colonic Fermentation as a Byproduct of Vinegar Consumption: A Parallel Arm
Randomized Control Trial in Adults at Risk for Type 2 Diabetes

by

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ABSTRACT

Many people with or at risk for diabetes have difficulty maintaining normal postprandial blood glucose levels (120-140 mg/dl). Research has shown that vinegar decreases postprandial glycemia. The purpose of this study was to examine a possible mechanism by which vinegar decreases postprandial glycemia, particularly the effect of vinegar ingestion on gut fermentation.

In this parallel arm randomized control trial, the effects of daily ingestion of vinegar on gut fermentation markers were observed among adults at risk for type 2 diabetes in Phoenix, Arizona. Subjects (n=14) were randomly assigned to treatments consisting of a vinegar drink (1.5g acetic acid) or a placebo (2 vinegar pills containing 40mg acetic acid each). All participants were required to consume the vinegar drink (16 oz) or 2 placebo pills every day for 12 weeks. At week 12, participants filled out a questionnaire to report gastrointestinal (GI) symptoms and three consecutive breath samples were taken from each subject to measure fasting breath hydrogen (BH2) with a breath analyzer.

Fasting BH2 measures for the vinegar drink group (16.1±11.8 ppm) were significantly different than those from the pill group (3.6±1.4) with a partial eta squared of 0.39 (p=0.023). After adjusting for age as a confounding factor (r=0.406) and removing an outlier, fasting BH2 measures for the vinegar drink group (4.3±1.1 ppm) were still significantly different than those from the pill group (3.6±1.4) with a partial eta squared of 0.35 (p=0.045). Participants in both groups reported mild changes in GI symptoms. In conclusion, adults at risk for type 2 diabetes that consume 2 tablespoons of vinegar a day may have increased gut fermentation compared to those who do not consume vinegar.
DEDICATION

First, I dedicate this thesis to my father. Thank you for teaching me the value of education ever since I was a young child. Thank you for your constant love and encouragement, your willingness to listen and lend advice, and for the financial and emotional support you have offered me as I have pursued my academic endeavors. I love you and appreciate all that you have done and continue to do for me.

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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>LIST OF TABLES</th>
<th>vi</th>
</tr>
</thead>
<tbody>
<tr>
<td>LIST OF FIGURES</td>
<td>vii</td>
</tr>
</tbody>
</table>

## CHAPTER

1 INTRODUCTION................................................................. 1

- Problem........................................................................ 1
- Proposed Research ...................................................... 3
- Definition of Terms ................................................... 3
- Delimitations and Limitations .................................... 4

2 LITERATURE REVIEW......................................................... 5

- Vinegar Overview....................................................... 5
- Vinegar as a Medicinal Agent ...................................... 8
- Vinegar and Diabetes ............................................... 13
- Colonic Fermentation ............................................... 22

3 METHODS.................................................................. 34

- Participants and Study Design .................................. 34
- Vinegar Treatments.................................................. 35
- Protocol and Procedures ......................................... 36
- Laboratory Analyses ............................................... 37
- Statistical Analyses ............................................... 37

4 RESULTS................................................................ 39

- Recruitment............................................................ 39
- Treatment Adherence ................................................ 39
- Descriptive Statistics .............................................. 40
<table>
<thead>
<tr>
<th>CHAPTER</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fasting BH2 Values</td>
<td>42</td>
</tr>
<tr>
<td>GI Questionnaire</td>
<td>44</td>
</tr>
<tr>
<td>DISCUSSION</td>
<td>47</td>
</tr>
<tr>
<td>Breath Hydrogen</td>
<td>48</td>
</tr>
<tr>
<td>GI Questionnaire</td>
<td>51</td>
</tr>
<tr>
<td>Limitations</td>
<td>52</td>
</tr>
<tr>
<td>CONCLUSION</td>
<td>55</td>
</tr>
<tr>
<td>REFERENCES</td>
<td>56</td>
</tr>
<tr>
<td>APPENDIX</td>
<td></td>
</tr>
<tr>
<td>A METHODS FLOW CHART</td>
<td>65</td>
</tr>
<tr>
<td>B MEDICAL HISTORY QUESTIONNAIRE</td>
<td>67</td>
</tr>
<tr>
<td>C CONSENT FORM</td>
<td>70</td>
</tr>
<tr>
<td>D IRB APPROVAL</td>
<td>73</td>
</tr>
<tr>
<td>E GI QUESTIONNAIRE</td>
<td>77</td>
</tr>
<tr>
<td>F SAMPLE SIZE CALCULATIONS</td>
<td>80</td>
</tr>
<tr>
<td>G QUINTRON ALVEOSAMPLER INSTRUCTIONS</td>
<td>82</td>
</tr>
<tr>
<td>H QUINTRON BREATHTRACKER INSTRUCTIONS</td>
<td>84</td>
</tr>
</tbody>
</table>
LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Descriptive characteristics of all study participants at baseline</td>
<td>41</td>
</tr>
<tr>
<td>2.</td>
<td>Exit fasting breath hydrogen measures of drink group and pill group</td>
<td>42</td>
</tr>
<tr>
<td>3.</td>
<td>Summary of participant responses to questions asked in the GI Questionnaire</td>
<td>46</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Overall Treatment Adherence of Participants</td>
<td>40</td>
</tr>
<tr>
<td>2.</td>
<td>Comparison of Mean Fasting BH2 Values (ppm) for the Drink Group and the Pill Group</td>
<td>43</td>
</tr>
<tr>
<td>3.</td>
<td>Individual Fasting BH2 Values (ppm)</td>
<td>43</td>
</tr>
<tr>
<td>4.</td>
<td>Individual Fasting Breath Methane Values (ppm)</td>
<td>44</td>
</tr>
</tbody>
</table>
Chapter 1

INTRODUCTION

Problem

Seventy-nine million people in the United States are believed to have prediabetes, a condition that precedes type 2 diabetes (1). Prediabetes is a condition that consists of blood glucose concentrations that are above normal (>100 ml/dL), but do not quite reach those of diagnosed type 2 diabetics (>126 mg/dL) (1).

Type 2 diabetes is characterized by a decreased production or use of insulin, or both. Risk factors associated with diabetes include impaired glucose tolerance, hypertension, low HDL cholesterol, and high plasma triglycerides (2). Populations that may be at a higher risk for developing type 2 diabetes include older individuals, African-Americans, Latinos, Native Americans, Asian Americans, Native Hawaiians and other Pacific Islanders (3). Serious complications from diabetes include heart disease, stroke, hypertension, neuropathy, nephropathy, and retinopathy (4,5).

According to the American Diabetes Association, long-term cardiovascular damage is already in progress in prediabetics (1). The goal for treating prediabetes is to stop or slow progression so it does not advance to full-fledged type 2 diabetes, which results in serious health complications. This can be accomplished with medical therapy and healthy lifestyle changes, such as modifying diet and exercise. Due to the increasing prevalence of prediabetes and diabetes, there is an increased demand for the discovery of viable methods to delay the onset of pre and type 2 diabetes.
Studies that have addressed the increasing prevalence of diabetes have found that lifestyle modifications are effective to prevent, delay onset, and aid in the treatment of diabetes (6-8). The benefits of lifestyle modifications to prevent and delay the onset of chronic disease are evident, however, there remains a steady increase in obesity and diabetes (9).

Studies report that lifestyle interventions for prevention of diabetes are cost-effective, so cost is likely not the central problem (10-12). Perhaps trends are showing an increase in diabetes due to poor patient adherence to lifestyle modifications that are meant to reduce risk factors associated with diabetes (13,14). The number one barrier to controlling hypertension—a risk factor for diabetes—is making lifestyle changes such as eating a healthy diet and increasing physical activity to lose weight (15). Barriers to diet modification adherence include unwillingness to change dietary habits, difficulties consuming a diet different from family and friends, and attending social gatherings. Barriers to compliance with exercise recommendations included lack of time, limitations due to other illness or disease, and poor weather conditions (16). There is a constant need for preventative options that are easier for patients to implement and maintain.

Recent studies have shown that consumption of vinegar decreases postprandial glycemia and improves postprandial insulin sensitivity (17-20). Previous data also suggest that vinegar may potentially decrease blood pressure and hemoglobin A1C (HbA1c) values and increase satiety (19,21-23). Vinegar is inexpensive, has a long shelf life, and is an appealing option for prevention and treatment of type 2 diabetes.
A few studies have explored delayed gastric emptying as a potential mechanism by which vinegar decreases postprandial glycemia, however, it appears that there remains a deficiency of studies in the literature exploring the glucose lowering actions of vinegar (24-26).

**Proposed Research**

Studies have shown that vinegar decreases postprandial glycemia and improves risks associated with type 2 diabetes. The importance of this research is to better understand how this phenomenon happens. The purpose of this parallel arm randomized control trial is to observe the effects of daily vinegar ingestion before meals on fermentation markers among adults at risk for type 2 diabetes in Phoenix, Arizona. The aim of this study is to examine a possible mechanism by which vinegar decreases postprandial glycemia, specifically the impact of vinegar ingestion on gut fermentation. This will be explored to ascertain whether vinegar inhibits the digestion of starch in the small intestine. It was hypothesized that the ingestion of 2 tablespoons of vinegar each day would have no effect on fermentation markers in adults at risk for type 2 diabetes in Phoenix, Arizona.

**Definition of Terms**

**Dawn effect**: A rise in blood glucose in the morning in diabetics

**Disaccharide**: A simple sugar consisting of two monosaccharides.

E.g. sucrose, lactose, and maltose

**Disaccharidase**: The digestive enzyme that breaks disaccharides down to simple molecules of sugar such as monosaccharides.

**Glycosidase**: Cleaves β-linked sugar residues from glycosides

**Monosaccharide**: The most basic carbohydrate unit.
E.g. glucose, fructose, and galactose

**Polysaccharide depolymerase**: An enzyme that breaks down polysaccharides to simpler molecules, such as disaccharides and monosaccharides.

**Postprandial glycemia**: A rise in blood glucose after eating

**Prediabetes**: Individuals with a fasting blood glucose of 100-125mg/dL

**Delimitations and Limitations**

Subjects are adults from Phoenix, Arizona who are at risk for type 2 diabetes and have been told by their doctor that they have prediabetes. Therefore the findings of this study cannot generalize beyond this population group. Fermentation markers were measured using a hydrogen breath test. Any self-reported data may not be accurately recorded, and there is no guarantee that subjects are maintaining their exercise and eating habits for the duration of the study. There is also no guarantee that subjects will consume the vinegar pill or drink each day during the 12-week trial as requested.
Chapter 2

LITERATURE REVIEW

Vinegar Overview

Production

Vinegar is derived from the Latin based word “vinaigre,” which means eager wine. The word eager comes from the French word “aigre,” meaning sour or sharp. Therefore, vinegar literally means sour or sharp wine (27).

Vinegars that are widely used in commercially prepared and homemade foods are produced by enzymatic fermentation of carbohydrate sources, such as fruit and grain, to alcohol. Yeast, namely Saccharomyces cerevisiae, is primarily responsible for the conversion of sugars to alcohol (27). Microorganisms called acetic acid bacteria are involved in the transformation of alcohol to acetic acid through oxidation. Acetobacter, a form of acetic acid bacteria, combines with oxygen to oxidize alcohol to acetic acid (vinegar) and water. This process is represented in the following equation:

Step 1: Conversion of sugar to alcohol

\[
\text{Glucose} \xrightarrow{\text{Yeast}} \text{Ethanol} \quad \text{C}_6\text{H}_{12}\text{O}_6 \rightarrow 2(\text{C}_2\text{H}_5\text{OH}) + 2(\text{CO}_2)
\]

Step 2: Fermentation of alcohol to acetic acid

\[
\text{Ethanol} \xrightarrow{\text{Acetobacter}} \text{Acetic Acid (Vinegar)} \quad \text{CH}_3\text{CH}_2\text{OH} + \text{O}_2 \rightarrow \text{CH}_3\text{COOH} + \text{H}_2\text{O}
\]

To complete this process, vinegar manufacturers require phage resistant acetic acid bacteria that can withstand high acetate concentrations, have high production rates, require minimal nutrients for growth, and do not overoxidize the acetic acid they produce. Overoxidation occurs when there is a lack of ethanol, which causes acetic acid to be further oxidized to CO\textsubscript{2} and H\textsubscript{2}O (28).
Commercial vinegar can be made by fast or slow fermentation. Fast fermentation methods, also known as submerged vinegar fermentation, require rapid mixing. In this process, the acetic acid bacteria dwell under the surface, allowing for quick fermentation of sugar-containing liquids. When desired acetic acid concentrations are reached, vinegar is released from the fermenter and replaced with alcohol-rich liquid to create more vinegar product (28). The slow fermentation process, also known as surface fermentation, is used to make traditional wine vinegars and is said to produce the best flavor (28, 29). In this method, acetic acid bacteria remains on the liquid’s surface, resulting in a slower fermentation process that can last weeks, sometimes months. Extended periods of fermentation allow for the growth of a nontoxic slime composed of yeast and acetic acid bacteria. This substance is known as mother of vinegar (29). Vinegar is filtered and pasteurized before bottling in order to prevent the formation and growth of these organisms (28, 29).

The final product of the vinegar production process is composed of acetic acid, ethanol, vitamins, inorganic salts, amino acids, polyphenols, and organic acids (29, 28). While acetic acid is a key component of vinegar, it only accounts for 4% of the final product. Federal regulation defines vinegar as the product made by alcoholic fermentation of sugar, containing no less than 4 grams of acetic acid per 100 cubic centimeters (27). Although vinegar is only required to contain 4% acetic acid, typically white vinegars contain 4% to 7% acetic acid and cider and wine vinegars contain 5% to 6% acetic acid (29).

Vinegars can be classified as herbal or fruit vinegar depending on what ingredients are added to wine vinegars or white distilled vinegars. Herbs and spices such as basil, cinnamon, clove, garlic, nutmeg and tarragon can be added
to make herbal vinegars. Fruit or fruit juice may be added to vinegar to produce fruit vinegars. These vinegars typically generate a sweet-sour taste. The sour taste for fruit vinegars can be attributed to the acetic acid, while the fruit juices are responsible for its sweetness. Vinegars may also be classified as traditional vinegar, depending on where and how they are produced (29). For example, balsamic vinegar from Modena, Italy is made from Trebbiano grape juices. It is fermented in a series of barrels made from various woods. This process can take anywhere from 3 to 12 years. Traditional vinegars also include rice wine and coconut vinegars from Asia, cane vinegar from the Philippines, and raisin vinegar from the Middle East (27). Vinegar has had many uses throughout history, especially within the fields of the culinary arts, agriculture, and medicine.

**History of Use**

Medicinal use of vinegar can be traced back to the time of ‘the father of modern medicine,’ or Hippocrates (460-370 BC), when it was used as a means of fighting infection and treating other acute conditions. Hippocrates suggested the use of vinegar to clean and treat wounds and sores. He also recommended honey and vinegar for the treatment of persistent coughs, as many physicians still advise today. This medicine was called oxymel, and is a mixture of virgin honey and white wine vinegar 4 parts to 1 (29, 30).

Many others followed after Hippocrates, using vinegar to treat various conditions. Paulus Aegineta (607-690 AD), a Greek physician, used vinegar as an astringent and as a treatment for wounds (30). In the 10th century, Sung Tse, the founder of forensic medicine, professed that infection from autopsies could be avoided by washing the hands with a sulfur and vinegar solution (29).
Legend purports that vinegar was used in the Middle Ages (1348-1350 AD), to prevent infection from the black plague. Four thieves who robbed the homes of French plague victims and never contracted the black plague themselves were put on trial. They claimed they had used a concoction of garlic and vinegar to ward off infection in the presence of the plague victims. Following this incident, vinegar from this region of France became known as “le vinaigre de Quatre Voleurs” or four thieves vinegar, and was used to prevent infection from the bubonic plague (31).

**Vinegar as a Medicinal Agent**

**Infectious Disease**

Historically, vinegar was believed to prevent infection. Medical professionals have used it to treat numerous illnesses including dropsy, croup, cough, fever, wounds, burns, ulcers, and the common cold (29,32). Vinegar is commonly used for household cleaning purposes, however, research shows that chemical products are more effective disinfectants and should be used in place of vinegar (29,33).

Evidence clearly shows that vinegar is a successful antimicrobial agent in food preparation settings, however, its use as an antimicrobial agent for the treatment of wounds is not recommended (34-37). The use of vinegar to prevent bacterial growth in wound care has been ineffective. It has not been shown to prevent the growth of *Escherichia coli*, *Exterococcus*, or bacteroides fragilis, and its ability to inhibit growth of *Staphylococcus aureus* and *Pseudomonas aeruginosa* has only been found mildly effective (37).

Although it is not the most effective antimicrobial treatment, vinegar has shown beneficial effects in the treatment of ear infections and jellyfish stings.
Research shows that diluted vinegar solutions (2% acetic acid) with a low acidity (a pH of 2) may effectively treat ear infections such as otitis media and granular myringitis. However, vinegar concentrations with this level of acidity may damage cochlear hair cells and irritate the skin (29,38,39). Therefore, in the treatment of ear infections, antibiotics are preferred to acetic acid solutions (39). Vinegar is commonly used to treat acute jellyfish stings because it deactivates venom-filled nematocysts. However, immersing an acute jellyfish sting in hot water (40°C) is the most effective treatment for jellyfish stings because heat quickly deactivates the venom (40,41).

Contrary to popular belief, vinegar is not an effective treatment for head lice, as shown in scientific research. Compared to five other home-remedies for head lice, vinegar was the least successful treatment to kill lice and prevent the laying and hatching of eggs. Stronger chemicals are recommended for proper louse removal (42).

**Chronic Disease**

*Cardiovascular Health*

Many studies show that vinegar may have benefit those at risk for cardiovascular health. In a follow-up of the Nurses' Health Study, food frequency questionnaires completed by 76,283 women in 1984 were reviewed to examine associations between fatal ischemic heart disease and consumption of foods containing alpha-linolenic acid. Investigators found that women who consumed oil and vinegar dressing more than 5 times a week had a 54% reduction in risk for fatal ischemic heart disease compared to those who did not consume oil and vinegar dressing (95% CI: 0.27-0.76 p for trend=0.001). The authors of this study speculated that the effects of the dressing could be attributed to its alpha-
linolenic acid content. However, these benefits were not seen with the consumption of mayonnaise, which is also high in alpha-linolenic acid. This suggests that the fatal ischemic heart disease risk reduction seen with the consumption of oil and vinegar dressing might be attributable to other potentially beneficial nutrients, such as acetic acid contained in the vinegar (43).

A 5-week study on male Sprague-Dawley rats explored the relationship between acetic acid and blood lipid concentrations. Rats were put on diets that were either cholesterol-free, cholesterol, or cholesterol plus acetic acid. The rats given acetic acid had significantly lower serum cholesterol concentrations in comparison to both the cholesterol and cholesterol-free groups (p<0.05). They also showed lower triglyceride concentrations compared to the cholesterol free group (p<0.05). This study suggests that acetic acid may lower cholesterol and triglyceride concentrations (44).

A 12-week study in 155 obese Japanese men and women explored the effects of vinegar on blood lipids. Subjects were randomly assigned to drink a 500ml daily beverage containing either 15ml vinegar (750 mg acetic acid), 30ml of vinegar (1,500 mg acetic acid), or 0ml vinegar (placebo). Subjects who consumed vinegar had significantly lower triglyceride concentrations compared to baseline (p<0.001) and the placebo group (p<0.05). No significant effect was observed in total, LDL, or HDL cholesterol values. This study also suggests that vinegar may have a triglyceride lowering effect (45).

In addition to affecting cholesterol and triglyceride levels, vinegar may improve cardiovascular health by lowering blood pressure. A study in non-hypertensive male Sprague-Dawley rats explored the effects of acetic acid consumption on blood pressure. Rats were given a red wine vinegar and grape
juice beverage containing 0.57 mmol of acetic acid a day, distilled water, or temocapril hydrochloride, an angiotensin I-converting enzyme (ACE) inhibitor. No significant differences were seen in baseline values between groups. After treatment, a significant decrease in angiotensin I-induced pressor response was observed among the temocapril and vinegar beverage groups at 10 minutes (p<0.01) and 60 minutes (p<0.05). No effects were seen in the distilled water group (46). This study suggests that vinegar may have hypotensive effects, however, the vinegar beverage used in this study also contained polyphenol antioxidants, which have been shown to have a beneficial effect on blood pressure themselves (46,47).

The effects of long-term vinegar consumption on blood pressure were studied in spontaneously hypertensive rats. Rats were put on a diet with deionized water (control), vinegar, or an acetic acid solution for 6 weeks. The vinegar and acetic acid treatments provided 0.86 mmol acetic acid each day. Systolic blood pressure was significantly reduced in the acetic acid and vinegar groups (20 mm Hg) compared to the control (p<0.05). The vinegar and acetic acid groups also showed significant reductions in plasma rennin activity (35% to 40%) and plasma aldosterone concentrations (15% to 25%) compared to the control group (p<0.05). This suggests that reduced rennin activity and aldosterone concentrations may be responsible for the observed reduction in systolic blood pressure. This study suggests that acetic acid is largely responsible for the antihypertensive actions of vinegar (48). More research is required in addition to these studies to further understand the effects of vinegar consumption on cholesterol and blood pressure in humans.
**Dietary Control**

In addition to cardiovascular benefits, research indicates that vinegar consumption may aid in weight management (19,45). A 12-week study in 155 obese Japanese men and women explored the effects of vinegar on body weight and fat mass. Subjects were randomly assigned to drink a 500ml daily beverage containing 0ml vinegar (placebo), 15ml vinegar (750 mg acetic acid), or 30ml of vinegar (1,500 mg acetic acid). Subjects who consumed vinegar had significantly lower body weight, BMI, visceral fat area, and waist circumference measures compared to baseline and the placebo group (p<0.001). This study suggests that vinegar may aid in weight management, which could reduce obesity and help prevent the onset of chronic disease, such as metabolic syndrome, cardiovascular disease, and diabetes (45).

Östman and colleagues suggest that vinegar may aid in weight management by increasing satiety. Twelve healthy volunteers were randomly assigned to consume 0, 18, 23, or 28g of vinegar (6% acetic acid) with white bread following an overnight fast. Subjects consumed each of the four meals on separate occasions, one week apart. Satiety was measured with a rating scale. Following the 28g vinegar treatment, subjects experienced significantly greater satiation for a longer period of time compared to the control group (p<0.05). They also showed a 22% decrease in glycemic index of the test meal (19). Other research has shown that consumption of lower glycemic index foods is connected to decreased hunger cues compared to foods with a higher glycemic index (49). Vinegar studies have shown an inverse relationship between vinegar and glycemic index. This study suggests that vinegar plays a role in appetite regulation by lowering the glycemic index of foods (19).
Vinegar and Diabetes

Glycemic Control

Diabetics generally have poor glycemic control. Studies have shown that consumption of low glycemic index foods aid in the prevention of type 2 diabetes (50,51). The glycemia of a carbohydrate food can be altered by ingestion of foods that decrease the delivery rate of glucose into the bloodstream (52). Many studies that have explored vinegar consumption and glycemia show that vinegar can lower postprandial glycemia to help delay the onset of type 2 diabetes and aid in treatment once diagnosed. In fact, many diabetics consumed vinegar tea to aid in the management of diabetes before the development of hypoglycemic agents (29).

In 1988, Ebihara and Nakajima were among the first to discover the antiglycemic effect of vinegar and suggest its use to treat type 2 diabetes. In their study, rats given 7% vinegar solution for 10 weeks had significantly lower postprandial blood glucose concentrations (53). In 1995, Brighenti and colleagues examined this effect in humans. In a randomized crossover trial, they assigned 5 healthy subjects to consume lettuce dressed with 10g olive oil (control), 10g olive oil and 20ml vinegar (5% acetic acid), or 10g olive oil and 20 ml neutralized vinegar (sodium acetate, pH=6.0) in random order on three separate occasions. Meals were followed by a glucose challenge test immediately after each treatment, using white bread (50g carbohydrate) as a reference. Blood glucose was reduced 31.4% (p=0.02) over 95 minutes after consumption of acetic acid compared to other groups. This suggests that vinegar containing acetic acid significantly improved glycemic responses to a mixed meal in healthy subjects (24).
Östman and colleagues conducted a study in 2005, to explore whether acetic acid supplementation could lower the glycemic index of a meal consisting of bread. They measured postprandial glucose and insulin concentrations in 12 healthy subjects after consumption of 50g of white bread with 0, 18, 23, and 28 g of white vinegar containing 6% acetic acid. After an overnight fast, subjects underwent each vinegar treatment in random order, on four separate days with a 1-week washout period between treatments. There was a negative linear relationship between 30-minute postprandial blood glucose concentrations and the amount of vinegar consumed with the test meal ($r=-0.47$, $p=0.001$). There was also a negative linear relationship between 30-minute insulin responses and vinegar content of the test meal ($r=-0.44$, $p=0.002$). The glycemic index of the test meal decreased 22% in the group that consumed 28g of vinegar and white bread compared to the group that consumed white bread alone ($p<0.05$). This study suggests that vinegar may lower the glycemic index of bread and decrease postprandial glycemia and insulinemia (19).

White and Johnston studied the influence of vinegar ingestion at bedtime on the dawn effect. In a randomized crossover trial, 11 type 2 diabetics followed the same 2-day meal plan consuming either 2 tbsp of apple cider vinegar or water (placebo) at bedtime. Participants measured their fasting blood glucose at 7:00am each morning with a glucometer and underwent a 3-5 day washout period between treatments. Fasting glucose concentrations were reduced 2% (0.15mmol/l) in the placebo group and 4% (0.26mmol/l) in the vinegar group ($p=0.033$). The results of this study suggest that vinegar consumption before bedtime may have a favorable impact on the dawn effect in type 2 diabetics (54).
In 2009, Johnston and colleagues showed that vinegar ingestion improved glycemic control in individuals with type 2 diabetes. Twenty-four healthy subjects were randomly assigned to consume a vinegar pill (15mg acetic acid), a pickle (700mg acetic acid) or 2 tbsp of vinegar (1400mg acetic acid) twice a day for 12 weeks. The vinegar pill served as the control because 15mg of acetic acid is so miniscule it is without effects. Fasting blood samples were taken at weeks 0, 6, and 12. HbA1c decreased 0.16% in the vinegar group over the 12 weeks while values increased in the vinegar pill and pickle groups (p=0.018). The observed decrease in HbA1c in the vinegar group indicates an improvement in glycemic control with the ingestion of 2 tbsp of vinegar a day (23).

Johnston and colleagues explored the effectiveness of various vinegar doses (10g or 20g), ingestion times (during meal or 5h before meal), forms (acetic acid from vinegar or sodium acetate), and carbohydrate sources (dextrose or complex carbohydrate) on the reduction of postprandial glycemia in a series of four randomized crossover trials. All trials consisted of a standardized meal the night before testing, an overnight fast, and a 2-hour glucose test after consumption of either a dextrose solution or a bagel and juice meal. Postprandial glucose concentrations were compared between treatments for up to 120 minutes post meal. All treatments within each trial were separated by a 1-week wash-out period. Results showed that 10g of vinegar (5% acetic acid) decreased postprandial glycemia by 23-28% (p=0.05) compared to the placebo whereas the 20g vinegar dose only decreased postprandial glycemia by 6-12% compared to the placebo group. Vinegar ingestion at the same time as the test meal decreased postprandial glycemia by 19% (p=0.169) but had no impact on postprandial glycemia when ingested 5 hours before the test meal. Postprandial
glycemia was reduced 90% more with ingestion of vinegar with a bagel compared to vinegar ingested with a dextrose drink (p=0.059). Acetic acid from vinegar decreased postprandial glycemia by 13-17% compared to the sodium acetate and placebo treatments (p=0.097). This study suggests that 10g of a 5% acetic acid vinegar solution, ingested with complex carbohydrates, will decrease postprandial glycemia in healthy individuals (20).

Similar effects of vinegar on glucose and insulin responses were also seen in other studies (55, 18, 17, 22). The evidence shows that vinegar has a beneficial effect on glycemia. The next step in research is to understand what causes the antiglycemic effect of vinegar. There are many possible mechanisms that may explain this phenomenon. Potential mechanisms explored in current research include delayed gastric emptying, inactivation of digestive enzymes, and enhanced tissue uptake.

**Glycemic Control Mechanisms**

**Delayed Gastric Emptying**

The idea that acids can slow gastric emptying rates has been around since the late 1800’s. Results were mainly shown using hydrochloric acid. In 1972, Hunt and Knox studied the effects of 9 different acids (hydrochloric, acetic, lactic, tartaric, phosphoric, citric, propionic, butyric, and hexanoic) on gastric emptying rates in 20 healthy subjects. They found that acids with lower molecular weights had more potential to delay gastric emptying than those with higher molecular weights. Since acetic acid (60g/mol) has a lower molecular weight than the other acids in this study, it should be the most effective in slowing gastric emptying rates (56).
Gastric emptying rates can be determined by measuring antrum diameter. Pressure in the antrum, the area just inside the pylorus of the stomach, builds from peristaltic contraction. High pressures in the antrum lead to more rapid gastric emptying (57). Food consumption stimulates chemosensitive receptors, which lead to a decrease in antral pressure and pyloric contraction, thus slowing gastric emptying. By measuring the diameter of the antrum, this pressure can be measured to determine gastric emptying rates (58).

In the 1995 study by Brighenti and colleagues that was previously outlined in this text, no effect of various vinegar treatments was seen on gastric emptying rates. Gastric emptying was determined in 5 healthy subjects by gastric antrum diameter measures attained by real time ultrasonography (24). Brighenti and colleagues measured antrum diameter just prior to each meal and every 15 minutes post-meal until values fell back to baseline. No differences in gastric emptying rates were observed after the white vinegar (5% acetic acid) and neutralized vinegar (sodium acetate with a pH of 6.0) treatments, suggesting that vinegar is working to decrease glycemia by some other mechanism (24).

In 1998 Liljeberg and Björck examined the effects of acetic acid (in the form of vinegar) on gastric emptying rates. They used paracetamol, an over-the-counter pain reliever and fever reducer, as an indirect marker for gastric emptying. They incorporated paracetamol (1.0g per test meal) into the white bread. Ten healthy subjects were served two test meals after an overnight fast on separate days, in random order, with 1 week between tests. The reference meal consisted of 122g white bread (50g starch) dipped in 8g olive oil and was eaten with 23g cheese (providing 10% fat). The test meal consisted of the reference meal plus 20g white vinegar to dip the bread in. Capillary blood
samples were taken at 0, 15, 30, 45, 70, and 95 minutes post meal to measure paracetamol concentrations using an enzyme kit. Postprandial glucose, insulin, and paracetamol responses were significantly lower in the test meal compared to the reference meal (p<0.05). The area under the curve for paracetamol was approximately 20% lower for the test meal compared to the reference meal (p<0.05). This study suggests that vinegar decreases postprandial glucose and insulin responses by delaying gastric emptying in healthy individuals (59).

Research indicates that vinegar ingestion prior to a meal has a beneficial effect on gastric emptying rates. However, the studies that show these results measured blood paracetamol concentrations as an indirect marker for gastric emptying. Concentrations of paracetamol in the blood can be altered by other factors such as food components and gastrointestinal events, so these results should be carefully considered (29). In addition, hormones such as cholecystokinin (CCK), amylin, and glucagon-like peptide-1 (GLP-1), also influence gastric emptying rates. Therefore, it is possible that vinegar or other food components are acting on one of these hormones to influence gastric emptying (60-62). While reduced gastric emptying rates may help explain how vinegar decreases postprandial glycemia, other mechanisms may be driving this response.

**Digestive Enzyme Inactivation**

In addition to studying the effects of vinegar on gastric emptying rates, scientists have also researched the influence of vinegar on digestive enzyme activation. Salivary amylase and pancreatic enzymes initiate carbohydrate digestion. Once carbohydrates are broken down, disaccharides are hydrolyzed by enzymes in the intestinal brush border, and monosaccharides are absorbed
through sugar transport systems. Research shows that vinegar may interfere with disaccharidase activity to decrease postprandial glycemia (63).

Ogawa and colleagues found that acetic acid suppresses disaccharidase activity, but has no effect on glucose transport in vitro. Caco-2 cells (human colonic carcinoma cells) were used in this study because they resemble human intestinal epithelial cells. They serve as a good model to study the physiological function of the small intestine because they express glucose transporters and disaccharidases similar to human small intestinal cells. Caco-2 cells were cultured on media containing either no acetic acid (control), or various amounts of acetic acid for 15 days. When exposed to up to 2.5 mmol/L of acetic acid, there was no significant increase in glucose uptake. However, when cells were exposed to 5 mmol/L acetic acid, sucrase activity was suppressed to 43% of the control, maltose activity to 39% of the control, and trehalase and lactase activity to less than 8% of the control (p<0.01). Supression of disaccharidase activity was seen with acetic acid but not with other organic acids (citric, succinic, L-malic, L-lactic, L-tartaric, and itoconic acids). This study suggests that acetic acid (vinegar) decreases disaccharidase activity in vitro (63).

In 2010, Johnston and colleagues found similar results in humans. Postprandial glycemia was decreased when vinegar was ingested with complex carbohydrates, but not when ingested with monosaccharides. Two test meals were administered one week apart, on separate occasions to 10 healthy adults. The test meals consisted of 75g of dextrose consumed with vinegar drink containing 40g water, 1tsp saccharine, and 20g vinegar (1g acetic acid) or placebo drink containing 40g water and 1tsp saccharine alone (0g acetic acid). Results were not significant; however mean postprandial glycemia was 90%
greater after the vinegar treatment compared to the placebo treatment \((p=0.059)\). This suggests that vinegar only lowers postprandial glycemia when ingested with complex carbohydrates, but has no effect when ingested with simple carbohydrates (20).

Since disaccharidase activity was suppressed in the presence of vinegar, it is possible that vinegar decreases postprandial glycemia by interfering with the catabolism of disaccharides to monosaccharides, such as glucose. If there is less glucose in the intestinal lumen, then less glucose will be absorbed into the bloodstream through sugar transport systems, which will result in decreased postprandial glycemia (63).

*Enhanced Tissue Uptake*

In addition to gastric emptying rates and digestive enzyme inactivation, scientists have explored the effects of acetic acid on enhanced tissue uptake. Historically, athletes consumed pickle juice after physical activity (65). It is possible that this practice also contributes to repletion of glycogen stores. Research has shows that acetic acid activates gluconeogenesis and glycogenesis in rats (65-67).

After acetic acid is absorbed and taken up into the liver and tissues, it feeds into the Krebs cycle as acetyl-CoA and is metabolized to create ATP energy. When this happens, glucose is spared and stored as glycogen. Fushimi and colleagues explored the influence of acetic acid on glycogenesis in the liver of male Sprague-Dawley rats. The rats were randomized to receive the AIN-76 diet (69) with the replacement of sucrose with glucose and 0 (control), 4, 8, or 16g of acetic acid. Glycogen concentrations were determined by measures of glucose-6-phosphate and fructose-1,6-phosphate in the liver and muscles.
Concentrations of fructose-1,6-biphosphate, a substrate in glycolysis, were measured to determine the effect of vinegar consumption on glycolysis. Citrate concentrations were also measured as increased levels of citrate have been shown to inhibit the activity of phosphofructokinase, a rate-limiting enzyme in glycolysis, in vitro. The authors of this study hypothesized that acetic acid may increase levels of citrate, which would lead to decreased phosphofructokinase activity, cause a downregulation of glycolysis, and ultimately spare glucose 6-phosphate for glycogen synthesis. Glycogen concentrations from this study were approximately 60% higher in the soleus muscle of the groups fed acetic acid compared to the control (p<0.05). Concentrations of citrate and fructose 6-phosphate were elevated >1.3 fold in the groups fed acetic acid (p<0.1) These results suggest that as acetic acid is metabolized in the Krebs cycle, negative feedback causes glucose (from glycolysis) to be spared and stored as glycogen in the muscle and liver (65).

Similar results were seen in a study that examined glycogen repletion in male Wistar rats following exhaustive exercise. After an overnight fast, one group of rats was sacrificed and measured for baseline glycogen stores. In the remaining rats, glycogen stores were depleted by exhaustive swimming and rats were randomized to receive water, glucose, acetic acid, acetic acid and glucose, citric acid, or citric acid and glucose treatment immediately following exhaustion. Two hours after treatment, rats were sacrificed and glycogen was measured in liver and muscle tissues. Results showed that glycogen stores were significantly higher in those treated with acetic acid and glucose compared baseline (p<0.05) (66).
Another study was conducted in 2002 that examined the effects of acetic acid on glycogen repletion after exercise in male Sprague-Dawley rats. Immediately following 2 hours of exhaustive swimming exercises, rats were randomized to be sacrificed or receive glucose or glucose with acetic acid. Rats were sacrificed 2 hours after feeding and muscle and liver glycogen stores were measured. Glycogen stores were significantly depleted after extreme exercise and were increased in all tissues 2 hours after both glucose and acetic acid treatments. However, muscle glycogen was significantly higher in the acetic acid group compared to the glucose group (p<0.05). This suggests that consumption of glucose with acetic acid following exercise may accelerate glycogen repletion in muscle more than glucose alone (67).

Studies in rats have shown potential for acetic acid to enhance uptake of glucose into muscle tissues, however more research is needed to understand this more clearly. Also, these results are from animal studies alone; no studies have explored these effects in humans.

Many studies have shown that vinegar has a beneficial influence on glycemia, but the exact mechanisms by which this happens remain unclear. Researchers have explored several options, many of which have yielded promising results. However, additional studies are required to more completely understand the physiological mechanisms driving the antiglycemic effect of vinegar. For example, fermentation is a possible mechanism that has not been explored in the research.

**Colonic Fermentation**

Food that is not digested in the stomach or the small intestine passes into the large intestine, through the caecum, into the colon, where it is fermented by
the microflora in the colon. In healthy individuals, approximately 1.5kg of material passes through the colon each day. Here, microorganisms break down material that has not been degraded in previous parts of the digestive system (69).

A healthy colon contains over 400 species of anaerobic bacteria that are either directly or indirectly involved in human metabolism. Colonic bacteria are responsible for the fermentation and degradation of complex carbohydrates, proteins, peptides, amino acids, hydrogen, lactate, succinate, and ethanol. The major species of colonic bacteria responsible for the breakdown of complex carbohydrates, or polysaccharides, are gram-negative anaerobes, including bacteriosides, bifidobacteria, and ruminococci. These bacteria produce polysaccharide depolymerases and glycosidases that degrade polysaccharides. The growth of these bacteria in the colon is largely dependent upon the amount of fermentable carbohydrates available (69). Polysaccharide degrading colonic bacteria ferment sugar and water to produce hydrogen gas, carbon dioxide gas, ATP energy, and short-chain fatty acids (SCFAs). The SCFAs produced in this process are acetate, propionate, and butyrate (70). This process is represented in the equation shown below:

$$\text{Glucose} \quad 57.5(C_6H_{12}O_6) + 45(H_2O) \quad \overset{\text{Microbes}}{\rightarrow} \quad 65(CH_3COO^-) + 20(C_2H_5COO^-) + \quad n\text{-butyrate}$$

$$15 \quad (CH_3CH_2CH_2COOH) +$$

$$140 \quad (H_2) + 95 \quad (CO_2) + 288 \quad \text{ATP}$$

Carbohydrate fermentation is most prevalent in the ascending colon. Bacterial growth rates are highest in this portion of the colon since food substrates for bacterial fermentation are more prevalent. This increase in
carbohydrate fermentation leads to higher SCFA production (127 mmol/L) in this region. The ascending colon has the lowest pH (5.4 to 5.9) found in the large intestine, which accommodates the growth of the bacteria in this region (69).

As material moves through the transverse colon, bacterial activity is reduced, resulting in decreased fermentation and reduced SCFA production (117 mmol/L). The environment in this part of the colon is more basic (pH = 6.2) compared to the ascending colon (69).

Materials proceed to the descending colon where the most basic environment in the entire colon is found (pH = 6.6-6.9). Little carbohydrate fermentation takes place in this region since bacteria do not thrive in this environment. The lack of carbohydrate fermentation in this area results in the lowest rate of SCFA production (90 mmol/L) in the colon. However, protein fermentation flourishes in this section of the colon and produces materials such as phenols, indoles, and ammonia. Once materials leave this region, they are excreted from the body through the rectum as fecal matter (69).

**Fermentation of Undigested Carbohydrates**

Individuals who are lactase-deficient or have carbohydrate maldigestion, have decreased catabolism of carbohydrates in the stomach and small intestines. As a result, more carbohydrate sources pass into the colon where they are broken down by fermentation. Increased colonic fermentation results in increased production of hydrogen gas, which diffuses into the blood and is exchanged into the breath where it is excreted (71). This causes higher breath hydrogen concentrations in these individuals.

In one study, nine healthy lactase-deficient individuals showed at least a 20 ppm rise in breath hydrogen after the ingestion of 20 g of lactose, indicating a
significant increase in colonic fermentation. The mean increase in peak breath hydrogen concentrations was 40ppm among the participants in this study. Eighty-eight percent of these individuals reported diarrhea, excessive flatulence, and abdominal discomfort after milk consumption (72). These symptoms are associated with colonic fermentation, as fermentation produces gas and increases peristaltic contractions (73). This reaffirms the notion that undigested carbohydrates that pass into the colon are degraded by fermentation.

In another study, 15 healthy individuals with lactose maldigestion showed at least a 10ppm rise in breath hydrogen after the ingestion of milk containing 20g of lactose. Breath hydrogen concentrations rose approximately 49ppm in lactose-intolerant individuals after the consumption of 20g lactose in the form of milk. These individuals also reported increased flatulence following ingestion of milk containing 20g lactose (74).

In 2006, Brighenti and colleagues measured breath hydrogen as a marker of fermentation in those consuming meals containing various amounts of indigestible carbohydrates. In a crossover trial, 10 healthy volunteers were given test meals for breakfast on 3 separate occasions, in random order. The meals consisted of a low glycemic index (LGI) meal, high glycemic index (HGI) meal, and a high glycemic index meal plus lactulose (HGI-lac). Lactulose is a highly fermentable and indigestible disaccharide; therefore it should increase hydrogen excretion. Subjects were served a uniform lunch. Breath hydrogen was measured immediately after each test meal and at 1-hour intervals for 10 hours thereafter. There was no peak in breath hydrogen in the HGI meal, however the LGI meal showed a significant increase in breath hydrogen measures 6 hours post-ingestion and remained high throughout the following 4 hours (p<0.001).
The same effect that was seen with the LGI meal was seen with the HGI-lac meal, however hydrogen measures were significantly higher in this group compared to both the LGI and HGI meals (p<0.001). The average peak breath hydrogen concentration was 18ppm after the LGI test meal and 26ppm after the HGI-lac test meal. This suggests that colonic fermentation increases with an increased supply of indigestible carbohydrate sources (75).

Nilsson and colleagues conducted a study exploring indigestible carbohydrate content of cereal foods (wheat, rye, oat, and barley) and their ability to influence glycemia. They measured breath hydrogen to determine fermentation of these foods in the colon, and blood glucose to monitor glycemia. They found a negative correlation between postprandial breath hydrogen and blood glucose concentrations at lunch (r=-0.33 p<0.05) and dinner (r=-0.22 p<0.05). Compared to white bread, consumption of barley kernels resulted in the highest breath hydrogen concentrations (17.2±2.6ppm and 41.0±5.1ppm respectively p<0.001) and lowest blood glucose area under the curve (698.5±70.7mmol/L and 485.5±40.1 respectively <0.05). This is likely because barley kernels have the highest content of dietary fiber among the cereal foods tested, and fiber consumption contributes to colonic fermentation (76).

These studies show that as indigestible carbohydrate sources pass into the colon, colonic fermentation increases. This results in an increased production of hydrogen gas, a byproduct of fermentation, which diffuses into the blood, and is excreted in the breath (75).

**Colonic Health**

Colonic microorganisms pose many benefits to human health. Growth of colonic microflora can be affected by factors such as diet, stress, infection,
medication, ageing, and genetics (77). Bacteria in the colon are responsible for fermentation of dietary fibers and other fermentable sources, producing metabolites, such as SCFAs, that are beneficial to colonic health (78). One of the most studied colonic bacteria is bifidobacteria. Increased levels of this bacterium have been associated with increased immune function and reduced risk for colon cancer and gastrointestinal disease due to improved bowel function. These benefits are likely caused by a decrease in inflammation. Studies have shown that acetic acid is associated with decreased markers of inflammation, such as tumor necrosis factor (TNF-α) and other inflammatory cytokines (79).

Studies show that supplementation of isomalto-oligosaccharides (IO), increases concentrations of bifidobacteria in healthy adults. IOs are oligomers that naturally occur in fermented foods such as miso and soy sauce. IOs such as isomaltose, isomaltotriose, isomaltotetraose, and panose have α 1→6 glucosidic linkages which resist digestion. IOs act similarly to dietary fiber and are fermented by colonic bacteria (77).

In a double-blind, placebo controlled study, the effects of IO supplementation on fecal microflora, bowel function, and fecal SCFAs in 13 constipated elderly subjects, resulted in improved bowel function. Subjects underwent a run-in period followed by a 4-week period in which they drank a placebo drink containing 100mL of water and 4mL of fructose syrup every afternoon. For the next 4 weeks (IO1) subjects ingested an IO drink that contained 11g IO every afternoon. After the first week of this period, the IO drink was increased to 22g a day for the remainder of the time. This treatment was repeated for an additional 4-week period (IO2). The IO supplementation period
was followed by a 4-week post period. Fecal characteristics and microflora were determined from fecal stool samples taken at the last week of each period (78).

Results showed that concentrations of bifidobacteria, lactobacilli, bacteroides, and total bacteria were significantly increased after IO supplementation and remained higher than in the post period compared to the placebo period. Fecal bifidobacteria content increased approximately 5%, fecal lactobacilli content doubled, and stool output increased 24% from the end of the placebo period to the end of the IO2 period. There was an increase in SCFAs in the feces following IO supplementation. Acetate concentrations increased 42.6% after IO1, 89.2% after the IO2, and remained 32% higher in the post period compared to the placebo. Total SCFAs (acetate, propionate, and butyrate) increased in both IO periods and remained high in the post period (75.9%, 134.0%, and 36.1% respectively) compared to the placebo period. This study showed that increased fermentation in the colon lead to improved fecal microflora, SCFAs, and bowel function, suggesting an improvement in colonic health (78).

**Short Chain Fatty Acids and Colonic Health**

Research shows SCFAs contribute to good colonic health. SCFAs such as n-butyrate, propionate, and acetate are produced by colonic fermentation and absorbed in the colon through passive transport (80). These fatty acids improve colonic health because they promote normal turnover of colon cells (79). SCFAs may also help reduce the risk for chronic disease, including type 2 diabetes, by improving appetite regulation and inflammatory responses (81).

A study conducted by Tarini and Wolever in 2010 found that SCFAs reduce concentrations of free-fatty acids and ghrelin, an appetite-regulating
hormone. Twelve healthy subjects (age 26±1.8) came in on 3 separate occasions following an overnight fast. Subjects consumed a test drink containing either 80g high fructose corn syrup, 56g of high fructose corn syrup plus 24 g oligofibre instant inulin, or 56g of high fructose corn syrup dissolved in water (control) with a 1-week washout between treatments. A fasting blood sample was taken before each test drink and subsequent blood samples were taken at various intervals up to 4 hours post ingestion. A lunch consisting of a cheese and tomato sandwich, apple juice, water and 2 chocolate cookies was provided after the 4-hour blood sample was taken. Additional blood samples were taken after lunch at various intervals over the next 2 hours. After the inulin treatment, serum acetate levels were 90% higher at 4-6 hours than the other treatments (p<0.05). Propionate and butyrate concentrations were 50% higher 4-6 hours after inulin ingestion compared to the other treatments (p<0.05). Concentrations of free fatty acids (FFAs) decreased with all treatments, but were much lower after the inulin treatment at 4 hours compared to the 56g of high fructose corn syrup (-0.11 mmol/L p<0.05). Serum gastric inhibitory polypeptide (GIP) was significantly decreased 30 minutes after the inulin treatment and ghrelin was significantly lower at 4.5 and 6 hours (p<0.05). The results from this study suggest that there may be a link between SCFAs and reduced concentrations of ghrelin and FFAs. The authors allude to the fact that SCFAs produced by colonic fermentation may protect against diabetes. They suggest that SCFAs increase insulin sensitivity by decreasing concentrations of postprandial FFAs and decrease food intake by beneficially influencing appetite-regulating hormones, such as ghrelin (81).

Aside from improving appetite regulation, SCFAs may also reduce chronic inflammation associated with disease. Chronic inflammation is a result of
recruitment and activation of immune cells that release pro-inflammatory cytokines into the circulation. These cytokines include interleukins (IL) and TNF-α (82). High levels of IL-6 and TNFα have been associated with obesity. Since obesity is a major risk factor for prediabetes and type 2 diabetes, circulating concentrations of IL-6 and TNFα are a concern (83).

In a crossover study, 10 healthy men consumed a meal of white bread (105g) or cooked barley kernels (86g dry kernels cooked in 250g water with 1g salt) the evening prior to testing. Each meal provided subjects with 50g of carbohydrates and was consumed with 250 ml of tap water. The white bread contained 2g nondigestible carbohydrates while the barley kernels contained 15g. Venous blood was collected at -90, -60, -30, -15, and 0 minutes prior to an oral glucose tolerance test (OGTT) and breath samples were collected at -120, -90, -60, -30, -15, and 0 minutes prior to an OGTT. An OGTT was started after the 0 min measurements and consisted of 55 g glucose in 250 mL water. Blood samples were taken every 15 minutes for 2 hours and then every 30 minutes for another 2 hours. Plasma IL-6, TNFα, SCFAs, and breath hydrogen were measured. The mean postprandial 4-hour IL-6 concentrations were 74% (p=0.024) higher and TNFα concentrations were 32% (p=0.008) higher after the white bread meal compared to the barley kernel meal. There was no significant difference found in plasma acetate and propionate concentrations between treatments. However, butyrate was significantly higher 0-2 hours after glucose ingestion the morning following the barley meal compared to the white bread meal (p=0.041). Breath hydrogen measures were higher 0-2 and 0-4 hours after glucose ingestion the morning following the barley evening meal (40.0±6.5 and 39.5±7.0ppm) compared to the white bread meal (15.2±1.4 and 14.2±1.2).
respectively (p<0.05). This suggests that higher consumption of indigestible carbohydrates at an evening meal may increase colonic fermentation, which increases SCFAs and leads to lower concentrations of proinflammatory cytokines in the plasma (84).

Tedelind and colleagues showed that SCFAs may decrease inflammatory markers. Human neutrophils were stimulated with lipopolysaccharide (LPS) to induce the release of inflammatory markers, such as TNF-α, in cell culture. The cells were exposed with 30mmol/L concentrations of acetate, propionate, or butyrate. Release of TNF-α was decreased 33% by acetate (p<0.01), 67% by propionate (p<0.01), and 75% by butyrate (p<0.01). These SCFAs also decreased IL-6 protein release in colon organ cultures obtained from mice. Organ cultures were treated to induce production of IL-6 and exposed to 30mmol/L of acetate, propionate, or butyrate. Levels of IL-6 decreased 67% when exposed to acetate, 92% when exposed to butyrate, and 83% when exposed to propionate (p<0.01). This study shows that SCFAs have the ability to decrease release of cytokines, TNF-α and IL-6, in vitro (82). However, these data should be considered carefully since the concentrations of SCFAs are higher than physiological levels (85).

Usami and colleagues showed that acetate, propionate, and butyrate decrease TNF-α. Blood samples were collected from five healthy subjects, and mononuclear cells were isolated and incubated with various concentrations (0.5 to 10 mM) of acetate, propionate, and butyrate. Inflammation was induced by LPS in a portion of the samples. TNF-α was measured by enzyme-linked immunosorbent assay (ELISA). LPS-induced TNF-α secretion was 58% lower after exposure to 2mM of butyrate than in the control containing no SCFAs.
(p<0.01). TNF-α secretions were approximately 44% lower after exposure to 2mM propionate and 16% lower after exposure to 30mM propionate (p<0.01). A
dose-dependent decrease was seen in secretion of TNF-α with acetate, with a
30% decrease after exposure to 5mM acetate (p<0.01). These results suggest
that acetate, propionate, and butyrate suppress secretion of LPS-induced TNF-α,
which should result in anti-inflammatory effects (86).

Another study investigated the effects of SCFAs on inflammatory markers
in vitro and in vivo. Neutrophils and monocytes were isolated from human blood
samples. Cells were incubated overnight with various concentrations of acetate,
propionate, and butyrate (0, 0.2, 2, and 20nmol/L) in the presence and absence
of LPS to induce pro-inflammatory markers. Cytokines, and chemokines were
measured by ELISA. Results showed that SCFAs inhibited production of TNF-α
and IL-6 induced by LPS in a dose-dependent manner. SCFAs also inhibited
monocyte chemotactic protein-1 (MCP-1) production in both cells exposed and
not exposed to LPS. MCP-1 recruits monocytes, which release cytokines, and
induce inflammation. Therefore, a decrease in MCP-1, TNF-α, and IL-6 would
decrease inflammation (87).

Research shows that SCFAs may have beneficial effects on health by
regulating inflammatory and appetite responses in the body. These SCFAs can
be produced from colonic fermentation; however, SCFAs can be produced in the
body by other means as well.

When acetic acid is ingested in the form of vinegar, it does not require
fermentation to exert its health benefits in the colon. The acetic acid is
metabolized to acetate, a SCFA that may directly and indirectly contribute to
overall colonic health (88).
If vinegar increases fermentation in the colon, SCFAs will be produced in amounts that are beneficial to overall and colonic health. These health benefits may aid in the prevention and possible treatment of chronic disease, specifically pre- and type 2 diabetes (86).
Chapter 3

METHODS

Participants and study design:

**Subject Selection:**

Subjects were considered eligible for the study if they were nonsmokers, > 20 years of age, not taking insulin, had stable medication use, no unresolved medical conditions, and were told by their doctor that they were prediabetic. Subjects were excluded if they had any known food intolerances (i.e. carbohydrate malabsorption or lactose intolerance). Subjects with known food intolerances were excluded from this study because some forms of food intolerance cause colonic fermentation. The intention was to only include subjects who were told by their doctor that they had prediabetes, however, recruiting was not robust so recruitment was expanded to adults at risk for type 2 diabetes.

**Recruitment**

Prediabetic men and women ≥ 20 years of age were recruited by list serves and flyers for participation in this research. List serves were obtained from the Arizona State University campus community and local businesses and hospitals. Interested parties were directed to Surveymonkey to complete an online medical history questionnaire (see appendix B). Those who qualified and still expressed interest to participate in the study were contacted and recruited. IRB approval and written consent were obtained (see appendices C and D).

This study was a 12-week parallel arm randomized control trial examining breath hydrogen as a fermentation marker in adults at risk for type 2 diabetes. Weight, height, body fat percentage, and waist circumference measurements
were taken 1 week prior to the study, 0, 6 and 12 weeks. Weight and body fat percentage were measured using a Tanita scale. Fasting venous blood samples were taken at weeks 6 and 12 to determine glucose and insulin values. Exit fasting breath samples were taken at week 12 to measure subject breath hydrogen values and participants were also asked to complete a gastrointestinal (GI) questionnaire at this visit to determine if they experienced any gastrointestinal changes throughout the study (see appendix E). Subjects used glucometers to record daily postprandial glycemia throughout the entire 12-week study.

**Sample Size:**

Preliminary studies examining breath hydrogen concentrations in subjects with carbohydrate malabsorption provided data for sample size calculations (72, 74-76). See appendix F. The alpha error level for the outcomes of this study was set at 0.05 and the beta error level at 0.2 (a power of 80% that a difference will be seen in fermentation markers due to the intervention). A 20% dropout rate was anticipated. The estimated sample size was 40. The assumption was made that breath hydrogen would increase approximately 33.5±18.1 ppm in the vinegar group and would have little to no change in the placebo group (72, 74-76). Participants were stratified by gender, age, and body mass prior to group assignment.

**Vinegar Treatments:**

Subjects enrolled in this study were randomly assigned to receive either a vinegar drink or a placebo. The vinegar drink (Bragg Apple Cider Vinegar Drink, Bragg Live Foods Inc., Santa Barbara, CA) was 16 fluid oz and contained 2 tablespoons of apple cider vinegar (1.5g acetic acid). The placebo pill (apple
cider vinegar pills, General Nutrition Center (GNC) Pittsburgh, PA) contained 40mg of acetic acid from vinegar. This pill was used as the placebo treatment because research has shown that the amount of acetic acid contained in it are miniscule and without effect (23). All participants were required to consume 16 oz of the vinegar drink or 2 placebo pills every day for 12 weeks.

Participant recorded daily pill or vinegar drink consumption on a calendar to measure adherence to the prescribed vinegar treatment regimen. Participants were emailed once a week to ensure the treatments went as prescribed, ask if they had questions about the study, and remind them to mark daily treatment completion on their calendars.

**Protocol and Procedures**

Subjects were screened for inclusion and exclusion criteria through an online medical history questionnaire (see appendix B). Those who fit the subject criteria were asked to visit the test site one week prior to the start of the trial. After visit 1, participants were randomly assigned a treatment. At week 0, participants came in for a second visit. At this time they were informed of the treatment protocol and provided with the corresponding vinegar drinks or pills and glucometers. Subjects also provided written informed consent at this time. Baseline measures of height, weight, waist circumference, glucose, and insulin were collected at week 0.

Participants were asked to record their blood glucose values daily for one week prior to starting treatment to collect baseline data. Participants in the vinegar drink group were directed to consume 8oz of the vinegar drink prior to lunch and dinner, every day, for 12 weeks (16oz total per day). Placebo group participants were directed to take 2 placebo pills prior to lunch and dinner with
8oz of water, every day for 12 weeks. All participants were asked to maintain their usual diet and activity patterns for the duration of the trial.

**Laboratory Analyses**

**Breath Hydrogen Measures**

Three consecutive breath samples were taken from each subject at week 12 of the study. Hydrogen gas was measured by a hydrogen breath analyzer (BreathTracker SC model QTL0054 REV E and QuinTron AlveoSampler bags modelQT00842-P, QuinTron, Milwaukee, WI). The investigator used the protocol outlined in the AlveoSampler instructions (see appendix G) and the QuinTron BreathTracker SC Instrument instructions (see appendix H).

**Blood Glucose and Insulin**

Fasting blood draws (<1/2 tbsp/day) were taken at weeks 0 and 12. Capillary blood glucose was also measured and recorded daily from glucometers for one week prior to the start of the trial and for the entire 12-week trial. Each subject was assigned a glucometer (ACCU-CHEK, Avia meter system) to measure and report daily blood glucose concentrations at 2-hours post-meal ingestion. Glucometers were collected from subjects at weeks 6 and 12 to download daily glucose measures. The results pertaining to blood glucose and insulin data were part of a companion study and will not be reported in this paper.

**Statistical Analyses**

Anthropometric data, descriptive data, glucose, insulin, and breath hydrogen measures were analyzed using SPSS v.19 Statistical Analysis System (SAS, Chicago, IL) to run descriptive and inferential statistical analysis. Outcome variables were tested for normality. Skewed data was log-transformed for analysis. All values are expressed as mean ± SEM, unless otherwise indicated.
Data change was calculated (week 12 to baseline) and compared using independent t-tests for glucose and insulin. If data was normally distributed the Pearson correlation was used. A Spearman correlation was run on all non-normal data. Exit breath hydrogen measures were statistically analyzed using univariate analysis. Data was considered statistically significant at $p < 0.05$. 
Recruitment

A total of 56 people completed the questionnaire on SurveyMonkey. Of these people, 48 qualified to participate in this study and were solicited. A total of 23 participants were enrolled in the study. Two people were excluded from the study because they were taking diabetic medications to control blood glucose and 7 others dropped from the study for various reasons. Of the 7 subjects who dropped, two acquired sickness and/or injuries unrelated to the study, two did not have enough time to participate, one did not believe sufficient compensation was offered, one reported that the vinegar made her nauseous, and one stopped responding. It was originally planned to recruit subjects who were told by their doctor that they had prediabetes, however, recruiting was not robust so the study was expanded to include people at risk for type 2 diabetes and one vegetarian to participate in the study.

Treatment Adherence

Participant adherences to prescribed treatments were measured by self-reported treatment completion on a calendar provided to each participant at the beginning of the study. Each treatment was to be taken twice a day and participants were to mark completion of these treatments on a calendar each day for the entire 12-week study. The participants returned these calendars at the duration of the study.

Adherence was determined by the percentage of days each participant reported taking their treatment 0, 1, or 2 times each day. Individual percentages for each group were averaged to give a mean group percentage of the amount of
days each participant reported completing their treatment 0, 1, or 2 times each day. Three participants from the pill group did not return their adherence calendars and were excluded from the analysis of participant adherence. Overall, the drink group (n=7) reported that they did not complete any of their treatments 7% of the days, completed their treatment once a day 4% of the days, and completed their treatment twice a day 89% of the days. The pill group (n=4) reported that they did not complete any of their treatments 7% of the days, completed their treatment once a day 16% of the days, and completed their treatment twice a day 77% of the days. See Figure 1.

**Figure 1.** Comparison of group adherence to prescribed treatment. Each treatment was to be taken twice a day. This figure shows a comparison of the adherence reported by each subject on his or her daily treatment calendar. Adherence for each particular group is represented as a percentage of the amount of days the participants of that particular group reported completing their treatment 0, 1, or 2 times a day. Three participants from the Pill Group did not return their adherence calendars and were excluded from the analysis shown above. Drink Group n=7 and Pill Group n=4.

**Descriptive Statistics**

All statistical tests used to determine outcome and descriptive measures were run as non-parametric tests due to the small sample size of the study. Chi-
Square tests were used to determine the amount of males, females, and prediabetics involved in the study. All data was reported as mean ± SE and Mann-Whitney tests were run to compare means of the variables and provide p-values for these comparisons.

Of the 14 participants that completed the study, 50% were in the drink group and the remaining 50% were in the pill group. Only one participant involved in the study was male and he was in the pill group. Forty-three percent of total participants were told by their doctors that they were pre-diabetic. Of the participants in the drink group, 29% were prediabetic and 71% were not. Of the participants in the pill group, 57% were prediabetic and 43% were not. There were no significant differences in descriptive characteristics of the study participants in the drink group and the pill group at baseline. See Table 1.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Drink Group (n=7)</th>
<th>Pill Group (n=7)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>0/7</td>
<td>1/7</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>7/7</td>
<td>6/7</td>
<td></td>
</tr>
<tr>
<td>Prediabetic</td>
<td>2/7</td>
<td>4/7</td>
<td></td>
</tr>
<tr>
<td>Age (yrs)</td>
<td>48±5</td>
<td>44±6</td>
<td>0.898</td>
</tr>
<tr>
<td>Weight (lbs)</td>
<td>168±14</td>
<td>169±16</td>
<td>0.949</td>
</tr>
<tr>
<td>Body Mass Index (kg/m²)</td>
<td>29±2</td>
<td>28±2</td>
<td>0.655</td>
</tr>
<tr>
<td>% Body Fat</td>
<td>34±5</td>
<td>34±4</td>
<td>0.749</td>
</tr>
<tr>
<td>Waist Circumference (in)</td>
<td>36±2</td>
<td>36±2</td>
<td>0.798</td>
</tr>
<tr>
<td>Venous Fasting Glucose (mg/dL)</td>
<td>101±5</td>
<td>96±6</td>
<td>0.810</td>
</tr>
<tr>
<td>Venous Fasting Insulin (uU/mL)</td>
<td>17±3</td>
<td>21±5</td>
<td>0.873</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>4±1</td>
<td>5±1</td>
<td>0.910</td>
</tr>
</tbody>
</table>

All values are represented as mean ± SE. All p-values were determined using Mann-Whitney non-parametric tests. HOMA-IR is the homeostasis model assessment estimated insulin resistance index. HOMA-IR was determined by multiplying fasting glucose (mmol/L) by fasting insulin (mU/L) and dividing by 22.5. No significant difference between means at the .05 level.

**Fasting BH2 Values**

Fasting breath hydrogen (BH2) values were log-transformed to normalize the data (Shapiro-Wilk test of normality p = 0.058). Age was correlated to BH2
(r = .406). The univariate analysis was used to examine differences between groups using the log of BH2 and controlling for age. The mean fasting BH2 values were 0.76±0.21ppm for the drink group (n=7) and 0.38±0.16ppm for the pill group (n=7) with a partial eta squared of 0.387 (p=0.023). This suggests that 39% of the BH2 variance can be explained by the vinegar treatment (p=.023).

Although logBH2 values were normal, one subject who was considered an outlier was excluded from analysis (this subject was a vegetarian and had much higher fasting BH2 values compared to the other subjects in the study). After removing the outlier, another univariate analysis of logBH2 was run controlling for age. With this outlier removed, the mean fasting BH2 values were 0.57±0.10 for the drink group (n=6) and 0.38±0.16 for the pill group (n=7) with a partial eta squared of 0.345 (p=0.045). This shows that 35% of the BH2 variance can be explained by the vinegar treatment. The mean fasting BH2 values found for the placebo pill group (3.6±1.4) were consistent with fasting BH2 values from other studies in healthy controls (89, 90). See Table 2 and Figures 2 and 3.

Breath methane results were also collected, however, it appeared that most participants in this study were not methane producers. See Figure 4.

**Table 2**

<table>
<thead>
<tr>
<th>Exit fasting breath hydrogen measures of drink group and pill group</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Drink Group</strong></td>
</tr>
<tr>
<td>BH2 (ppm)^a</td>
</tr>
<tr>
<td>BH2 (ppm)^b</td>
</tr>
</tbody>
</table>

*All values are represented in ppm as Mean±SE. BH2 was log transformed to normalize data prior to analyses. We controlled for age because it was correlated with BH2 (r=0.406).*  
^a. All subjects (n=14)  
^b. Outlier removed (n=13)  
*Significant difference in mean BH2 values between the drink group and the pill group were determined at the .05 level
Figure 2. Comparison of the effects of daily vinegar consumption on mean fasting BH2 measures (ppm) for participants of the drink group (n=6) compared to participants of the pill group (n=7). Values represented as Mean±SE.

Figure 3. Individual fasting BH2 values (ppm) of the drink group (n=6) and the pill group (n=7) reported according to age, which correlated with fasting BH2 values (r=0.406). One subject in the drink group (age 30) was an outlier with a fasting BH2 of 86.7 ppm and was excluded for analysis.

*Male subject
**Figure 4.** Individual fasting breath methane values (ppm) of the drink group (n=7) and the pill group (n=7) reported according to age.

*Male subject

**GI Questionnaire**

At the duration of the study, all participants filled out a questionnaire that reported information about their experiences with the vinegar and its effects on their GI system.

Six out of seven of the participants in the drink group reported that the vinegar source was easy to incorporate into their diet and that they consumed it everyday. The remaining participant reported that the vinegar drink was not easily incorporated into her diet and she did not consume the vinegar source daily because it made her nauseous. All participants of the vinegar pill group reported that the vinegar source was easy to incorporate into their diet and they consumed their vinegar source daily. Three of seven of participants in the vinegar group and Three of seven participants in the drink group reported that they consumed other vinegar containing foods during the study (salad dressing and pickles). Four of seven participants in the vinegar group and four of seven
participants in the drink group reported that they did not consume vinegar from any additional sources.

Participants were asked if they experienced any change in flatulence, stool frequency, stool consistency, or bloating during the study. If participants reported a change in any of these areas, they rated that change on a scale of 1-5 with 1 being “little change” and 5 being “a lot of change.” None of the participants in the pill group reported a change in flatulence and 5 out of 7 participants in the drink group reported no change in flatulence. The remaining 2 participants in the drink group reported a 2.5 average change in flatulence. Six out of 7 of the drink group participants reported no change in stool frequency while the remaining participant reported a change in stool frequency which she rated a change of 2. The pill group also reported that 6 out of 7 participants reported no change in stool frequency. However, the participant from the pill that reported a change in stool frequency rated her change as a 4. All participants in the drink group and 6 out of 7 participants from the pill group reported no change in stool consistency throughout the study. The participant from the pill group that reported a change in stool consistency rated her change as a 1 and reported that her stool was more firm. Six out of 7 of the drink group participants reported no change in feelings of bloating and 1 participant reported feelings of bloating which she rated a 2. Five of 7 participants in the pill group reported no change in feelings of bloating and 2 participants reported a change in feelings of bloating with an average rated change of 2.5.

When asked if any of these symptoms interfered with normal daily activities, only 1 of 14 total participants said yes with nausea as her chief complaint. Participants were also asked if any of these symptoms made them
change or avoid any social activities during the study and all reported normal social activities. See Table 3.

Table 3
Summary of participant responses to questions asked in the GI questionnaire (n=14).

<table>
<thead>
<tr>
<th>Question</th>
<th>Drink Group</th>
<th></th>
<th>Pill Group</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Was the vinegar source easy to incorporate into your diet?</td>
<td>6/7 86%</td>
<td></td>
<td>7/7 100%</td>
<td></td>
</tr>
<tr>
<td>Have you eaten any vinegar sources other than treatment?</td>
<td>3/7 43%</td>
<td></td>
<td>3/7 43%</td>
<td></td>
</tr>
<tr>
<td>Have you experienced changes in flatulence (gas) frequency?</td>
<td>2/7 29%</td>
<td></td>
<td>1/7 14%</td>
<td></td>
</tr>
<tr>
<td>Have you experienced any changes in stool frequency?</td>
<td>1/7 14%</td>
<td></td>
<td>1/7 14%</td>
<td></td>
</tr>
<tr>
<td>Have you experienced any changes in stool consistency?</td>
<td>0/7 0%</td>
<td></td>
<td>1/7 14%</td>
<td></td>
</tr>
<tr>
<td>Have you felt bloating since the beginning of the study?</td>
<td>1/7 14%</td>
<td></td>
<td>2/7 29%</td>
<td></td>
</tr>
<tr>
<td>Have symptoms interfered with your normal daily activities?</td>
<td>1/7 14%</td>
<td></td>
<td>0/7 0%</td>
<td></td>
</tr>
<tr>
<td>Have symptoms made you change any social activities?</td>
<td>0/7 0%</td>
<td></td>
<td>0/7 0%</td>
<td></td>
</tr>
</tbody>
</table>

Complete questions are represented in the GI Questionnaire in the appendix. For the drink group n=7 and for the pill group n=7. All participant responses are represented as ratios and percentages of the treatment group to which they belong.
Chapter 5
DISCUSSION

People with prediabetes and type 2 diabetes often find it difficult to keep fasting and postprandial blood glucose levels within normal ranges (fasting blood glucose, 80-110 mg/dl and postprandial blood glucose 120-140 mg/dl), therefore, various suggestions of methods to help control blood glucose levels and prevent or delay the onset of type 2 diabetes are consistently found in the literature (6-8). Among these methods, the ingestion of vinegar prior to mealtime has been shown to decrease postprandial glycemia in healthy subjects, prediabetics, and type 2 diabetics (17-20, 22-24, 54, 55). Additional research has been conducted to explore the mechanisms of vinegar action; however, the mechanism(s) remain obscure (20, 24, 56, 59, 63, 65-67). Therefore, this study examined a potential mechanism for the antiglycemic property of vinegar, namely the effect of vinegar ingestion on gut fermentation. This was explored to ascertain whether vinegar increased fermentation markers, which would indicate an increase in gut fermentation itself. An increase in gut fermentation would support the notion that vinegar inhibits the digestion of starch in the small intestine.

In this randomized, placebo-controlled trial, subjects consumed 16 oz of a vinegar drink (1.5 g acetic acid) everyday or a placebo (2 vinegar pills containing 40 mg acetic acid each) everyday for 12-weeks. Subjects consumed 8 oz (1 tablespoon of vinegar) of the vinegar drink or one vinegar pill (40 mg acetic acid) immediately prior to lunch and dinner meals. Subjects filled out a GI Questionnaire and BH2 was measured from fasting breath samples collected at week 12 of the study.
Breath Hydrogen

After controlling for age as a confounding factor (r=0.406), and removing an outlier, fasting BH2 measures for the vinegar drink group were significantly greater than those for the pill group (4.3±1.1 ppm and 3.6±1.4 ppm respectively) with a partial eta squared of 0.35 (p=0.045). Thus, 35% of the variance in fasting BH2 values could be attributed to the vinegar treatment. According to these results it appears that those who consume vinegar daily may have higher fasting BH2 values compared to those who do not. It is possible that the vinegar may inhibit the digestion of starch in the small intestine, causing it to pass into the colon and increase fermentation, which would ultimately raise BH2 values. A rise in BH2 values should indicate an increase in fermentation because once hydrogen, a major end product of fermentation, is produced in the colon, it diffuses into the blood, and is exchanged into the breath. This hydrogen is excreted in the breath where it can be measured as a marker of colonic fermentation (71).

Studies suggest that undigested and/or partially digested carbohydrates that pass into the colon are broken down further by fermentation. In a crossover trial of 10 healthy volunteers, subjects consumed breakfasts consisting of various amounts of indigestible carbohydrates on three separate occasions in random order followed by a standard meal five hours after breakfast. BH2 was measured every 30 minutes for 2 hours after breakfast, then every hour until lunch. After lunch, BH2 was measured every 30 minutes for 2 hours and then every hour until the total study had reached 10 hours. BH2 was significantly higher in subjects after they consumed the meals that were higher in indigestible carbohydrates (p<0.001). The average peak breath hydrogen concentration at 7 hours (2 hours
after lunch) was 18 ppm after consuming a low glycemic index meal and 26 ppm after consuming a high glycemic index meal with lactulose, a highly fermentable and indigestible disaccharide. This suggests that the indigestible carbohydrates passed into the colon where they were broken down by fermentation (75).

Another study that explored indigestible carbohydrate content of foods measured BH2 to determine the amount of fermentation that took place in the colon after consumption of these indigestible carbohydrates. The largest difference in postprandial BH2 was found between the consumption of white bread (17.2±2.6 ppm) and barley kernels that are high in dietary fiber (41.0±5.1 ppm) (p<0.001). These results suggest that BH2 values will be higher after the consumption of foods containing high amounts of indigestible carbohydrates (76).

These studies show that as indigestible carbohydrate sources pass into the colon, colonic fermentation increases resulting in an increased production of hydrogen gas that diffuses into the blood, and is excreted in the breath where it can be measured (71, 75). Therefore, since this study observed a significantly higher fasting BH2 measure in the vinegar group compared to the placebo pill group, it is possible that this was observed because the vinegar caused undigested starch to pass into the colon, where it was fermented, leading to increased presence of hydrogen gas in the breath. It is expected that acute testing for postprandial BH2 values may show a greater difference in BH2 after consumption of a vinegar test meal compared to a control. This would provide additional data to determine if vinegar is responsible for a rise in BH2. Therefore, future studies should examine the acute effect of vinegar on BH2 to further explore this link between vinegar and the presence of hydrogen in the breath.
Validity and Reliability

The investigator measuring breath samples attended a training meeting with the staff at QuinTron to learn how to appropriately obtain the samples and use the BreathTracker SC digital microlyzer to ensure measurement validity of BH2. When the breath samples were collected, all subjects were asked to take a normal breath, breathing in through his or her nose and out through his or her mouth for the breath sample collection. Definition of a normal breath can be subjective, so subjects were observed for signs of abnormal breathing such as gasping or wheezing sounds, forced inspiration or expiration, lifting of the shoulders to expand the rib cage and fill the lungs, or subjective chest expansion. To ensure subjects took a normal breath to collect the breath sample, all participants were asked to focus on their normal breathing prior to collection of the breath sample. They were also asked to take a practice breath into the breath collection bag and were observed for signs of abnormal breathing as described above. If subjects appeared to exhibit any of these signs they were asked to take another breath for collection. Following these procedures, the fasting BH2 measures of the placebo control group (3.6±1.4 ppm) were consistent with those found in other studies, which showed fasting BH2 measures in healthy controls to be about 3-4 ppm (89, 90).

Reliability was also carefully assessed during BH2 measurements. Three consecutive breath samples were taken from each subject for BH2 measurement. The three BH2 measures were averaged for the recorded value. The intraclass correlation coefficient (ICC=0.946) showed that these measures
were strongly correlated (p<0.001). This analysis indicates that the BH2 measures from the breath samples were consistent and reliable.

**GI Questionnaire**

GI symptoms, such as increased gas production, are associated with colonic fermentation (73). Because of this, subjects filled out a questionnaire at the end of the study to report changes in GI symptoms such as flatulence frequency, stool frequency, and stool consistency. It was expected that those in the vinegar group would experience more potential changes in GI symptoms related to increased fermentation than those in the placebo pill group. However, participants in both groups reported mild changes in GI symptoms. Because of this, it does not appear that those in the vinegar group experienced any more GI symptoms than those in the pill group. Since most subjects in the drink group and the placebo pill group did not report much change in these areas, it cannot be determined whether or not these data support the assumption that vinegar consumption inhibits the digestion of starch in the small intestine to increase gut fermentation. However, the sample size for this study is small and changes may not have been noticeable or present with this amount of vinegar ingestion. Results may appear to be more notable with a larger sample size, more frequent measures of subject GI status, or an increased ingestion of vinegar. It is suggested that future studies collect data on a larger sample and consider the use of a daily log of GI symptoms to better track and report the GI status of each subject. It is also possible that the amount of vinegar ingested in this study does not have a large enough effect on gut fermentation to cause noteworthy changes in GI symptoms.
Limitations

A small sample size was used in this study, therefore it is suggested that larger studies are done to explore this phenomena and produce more robust results. It would also be beneficial to conduct short-term studies that measure fasting BH2 values before and after a meal at various time intervals as other researchers studying BH2 and carbohydrate malabsorption have done (75, 76). Studies may also consider using a tool to measure GI symptoms more frequently and more closely.

The breath microlyzer used to run the breath samples in this study reported both BH2 and breath methane values for each sample. No relationship was found between vinegar ingestion and breath methane. Mean BH2 and breath methane values were not correlated. It would be beneficial for future studies to measure other more sensitive markers of fermentation in addition to BH2 and breath methane with closer monitoring of GI symptoms to report better construct validity. This would give stronger data to support whether or not vinegar ingestion increases colonic fermentation.

It does not appear that there is a gold standard against which to compare BH2 measures (91). In fact, it appears that BH2 is the most sensitive and commonly used measure of colonic fermentation in the literature (72, 74-76, 91-93).

BH2 is considered the most promising measure for evaluating colonic fermentation because the method is non-invasive and highly sensitive (92, 94). No known mammalian cell produces hydrogen and methane. The formation of these gases is specific to anaerobic bacteria, which are found in the flora in the colon (94-97). Therefore, the presence of hydrogen and methane in the breath
should be a valid and specific indicator of colonic fermentation (96, 98). BH2 is considered a sensitive measure of fermentation, however, recent research indicates that methane may not be. These findings indicate that some people are naturally methane producers or non-producers, therefore, breath methane is not very sensitive measure of actual colonic fermentation (72, 92-94). The literature states that CO$_2$ produced from colonic fermentation of starch is converted to methane by methanogenic bacteria in the colon. This methane diffuses into the blood, then into the breath where it is excreted. The measurement of breath methane is not considered a very sensitive marker of fermentation because methanogenic bacteria are only present in about 50% of the population. Those who carry methanogenic bacteria are considered methane producers. Those who do not are considered non-producers (70). Breath hydrogen and methane measures can be affected by the presence or absence of hydrogen and methane producing flora in the gut. Gut flora may be altered by various practices, such as the use of antibiotics (98). Despite this, BH2 is still considered the most promising measure of gut fermentation (92, 94).

Subject reported GI symptoms such as discomfort related to flatulence and bloating can serve as a measure for fermentation as the production of gases from the fermentation process can result in these symptoms (99, 100). For example, fermentable fibers lead to increased gas production, flatus frequency, and feelings of bloating in human subjects (99, 101). Since GI symptoms are self-reported and can be related to other health and medical conditions, such as irritable bowel syndrome, these symptoms are not a good measure of fermentation alone. However, they can serve as a good indicator of colonic fermentation in combination with other measures (101).
The intention for this study was to complete pre and post measures of fasting BH2 values for both the vinegar group and the placebo pill group to determine any changes in fasting BH2 values, however, instrumentation was not obtained in time to do a pre and post measure. Because of this an exit fasting BH2 measure was conducted. One might argue that a pretest is not necessary for this type of design as the purpose of a pretest is to determine if groups are different prior to treatment and subjects for this study were stratified and randomly assigned to groups. Using this type of research design, the goal is to determine if the dietary intervention caused a significant change in 2 randomized and largely similar (p>0.05) groups at the end of the treatment period (102).
Chapter 6

CONCLUSION

Data from this research suggests that daily consumption of vinegar may lead to higher fasting BH2 values in individuals at risk for type 2 diabetes. Increased BH2 values are an indicator of increased colonic fermentation, which suggests that vinegar ingestion may increase fermentation in the colon. This may explain why vinegar consumption prior to mealtime is linked to decreased postprandial glycemia (17-20, 22-24, 54, 55). This was the first study to explore the effects of vinegar ingestion on colonic fermentation measuring fasting BH2.

These results may not be completely representative of the population at large because a small sample size (n=14) was used, only post BH2 measures were taken, and not all markers of fermentation measured were considered sensitive. Additional research is needed to determine if vinegar increases colonic fermentation by measuring long-term and acute BH2 and breath methane values and tracking daily GI symptoms. Future studies should use a larger sample size and explore these effects in various independent populations such as healthy, prediabetic, and type 2 diabetic subjects. Short-term studies exploring the effects of vinegar on colonic fermentation, specifically breath hydrogen measures would help to further explore these effects as well. It is suggested that short-term studies measure fasting BH2 values and postprandial BH2 values following a vinegar test meal in several time increments. Past studies observing BH2 as a byproduct of fermentation collected measures in 30-minute increments for 2 or more hours post meal ingestion (75, 76). Future research in this area may lead to additional information about how vinegar works to decrease postprandial glycemia.
REFERENCES


List Serve

Medical History Questionnaire

Visit 1: Screening
• Consented Subjects
• Measured Anthropometrics
• Conducted 24 Hour Recalls
• Provided with glucometers

Visit 2: Week 0
• Measured Anthropometrics
• Given Vinegar Drinks/Pills
• Downloaded glucometers
• Took Fasting Blood Draws

Visit 3: Week 6
• Measured Anthropometrics
• Conducted 24 Hour Recalls
• Downloaded Glucometers
• Gave $10.00 Gift Cards

Visit 4: Week 12
• Measured Anthropometrics
• Downloaded Glucometers
• Took Fasting Blood Draws
• Collected Fasting Breath Samples
• Administered GI Questionnaires
• Gave $15.00 Gift Cards
APPENDIX B

MEDICAL HISTORY QUESTIONNAIRE
MEDICAL HISTORY QUESTIONNAIRE

ID# ___________________________

Height _____ ft. _____ in.  Weight: _____ lbs.  Waist: _____ ins.

Age: ______________

Gender: □ Male □ Female

Smoker: □ Yes □ No

1. Have you been diagnosed with pre-diabetes?  Y  N

2. How long have you had pre-diabetes?  _________

3. Do you take insulin to treat pre-diabetes?  Y  N

4. Do you take any medications regularly?  Y  N
   Please list what kind and how frequently:
   __________________________________________
   __________________________________________
   __________________________________________

5. Do you currently take supplements (vitamins, minerals, herbs, etc.)?  Y  N
   If yes, what supplements and how often?
   __________________________________________
   __________________________________________
   __________________________________________
   __________________________________________

6. Do you have any medical conditions that you see a physician for on a regular basis?  Y  N
   Please explain
   __________________________________________
   __________________________________________
   __________________________________________
   __________________________________________

OVER—»
7. Do you have any food allergies?  

10. Do you follow a special diet? (weight gain/loss, vegetarian, low-fat, etc.)
   If yes, please specify

11. Will you have any problems fasting for 12 hours prior to testing sessions?

12. Do you have dentures?

12. Do you have any swallowing issues?

13. Will you have any problem drinking an apple cider drink (8 oz with meals twice daily)?

14. Will you have any problem swallowing an apple cider vinegar pill (2 per day)?

15. Will you have a problem providing venous blood samples? (3 samples during the study)

16. Will you have a problem pricking your own fingers daily to provide blood drops for analyses? (2 pricks per day)

17. If you drink alcohol or caffeine, will you be able to abstain from these beverages for the 24-hour periods prior to test days?

18. If you exercise regularly, will you be able to not exercise (other than normal activity) for the 24-hour periods prior to testing?

19. Do you consume vinegar (flavorings, dressings, pickled foods) on a regular basis? If yes, please describe how often and in what form:

20. Will you have a problem reducing vinegar consumption to no more than the assigned treatment per week during the study duration (up to 12 weeks)?

21. Please describe any other medical conditions that may affect your participation below (i.e. pregnancy, infections, allergies, etc):
12-WEEK VINEGAR TRIAL IN HEALTHY ADULTS WITH PRE-DIABETES

INTRODUCTION
The purposes of this form are (1) to provide you with information that may affect your decision as to whether or not to participate in this research study, and (2) to record your consent if you choose to be involved in this study.

RESEARCHERS
Dr. Carol Johnston, a Nutrition professor at Arizona State University Downtown Campus, and Samantha Quaglino and Serena Loeb, nutrition graduate students, have requested your participation in a research study.

STUDY PURPOSE
The purpose of the research is to examine the effects of vinegar consumption on blood glucose concentrations.

DESCRIPTION OF RESEARCH STUDY
You have indicated to us that you are minimum age of 20 years of age, a non-smoker, generally healthy, and have stable medication use over the last 3 months. You have been diagnosed with pre-diabetes but you do not take insulin. Participants will be asked to maintain their usual diet and physical activity level throughout the trial. This study will initially involve the completion of a brief medical history questionnaire to demonstrate the absence of medical conditions (aside from pre-diabetes) that may impact the study. Your weight, height, and girth will be measured at the start and at the end of the trial. This first meeting will take ~30 minutes. This research study will last 12 weeks. At the start of the study and at trial weeks 6 and 12, you will travel to ASU (the Nutrition labs at the ASU Downtown campus) early in the morning to meet investigators for some testing: breath sampling at 0, 6, and 12 weeks, and a blood draw (<1/2 Tbsp/day) at 0 and 12 weeks. For the blood draw, you will fast overnight (12 hours). These visits will last <45 minutes.

You will be randomly assigned to the ‘apple cider vinegar drink’ group or to the ‘apple cider vinegar tablet’ group. During the 12-week trial you are asked to consume 8 oz vinegar drink or 1 vinegar pill (depending on group assignment) twice daily with the lunch and dinner meals (a total of 16 oz vinegar drink or 2 vinegar pills per day). You will be given a glucometer to use daily to measure blood glucose at waking and at 2-hours post meal ingestion after your largest meal. You will mark your blood glucose reading on a 12-wk calendar to be posted at home or office. During the 12-week trial, you are asked to not change your typical diet or activity patterns. If you deviate from your routine diet, or if you begin taking medications, at any time between during the 12-wk trial, you are to immediately notify the investigators of the study. About 40 subjects will participate in this study.

A research nurse will draw blood using standard, sterile techniques. Blood samples will be analyzed for biomarkers that are associated with blood glucose control such as glucose, insulin, and hemoglobin A1c.

RISKS
Bruising of the skin or a feeling of faintness is possible during the blood draws. For the at home finger pricks, subjects will be provided with disposable retractable lancets, strips, and glucometers as well as instructions for sterile conditions.

BENEFITS
This study will provide information regarding the usefulness of vinegar for controlling blood glucose and insulin concentrations in individuals with pre-diabetes. If desired, you will be provided with study results and your personal blood data at the end of the study.

NEW INFORMATION
If the researchers find new information during the study that would reasonably change your decision about participating, then they will provide this information to you.

CONFIDENTIALITY
All information obtained in this study is strictly confidential unless disclosure is required by law. The results of this research study may be used in reports, presentations, and publications, but your name or identity will not be revealed. In order to maintain confidentiality of your records, Dr. Johnston will use subject codes on all data
collected, maintain a master list separate and secure from all data collected, and limit access to all confidential information to the study investigators. Plasma from blood samples will be stored for 5 years in freezers in the laboratories of the Nutrition Program at Arizona State University Downtown Campus after which time they will be disposed of as biohazard waste.

WITHDRAWAL PRIVILEGE
You may withdraw from the study at any time for any reason without penalty or prejudice toward you. Your decision will not incur negative treatment to you by the researchers.

COSTS AND PAYMENTS
The test foods (drinks or pills) will be given to you during the study free of charge. You will also receive one $10 and one $15 gift certificate during the study.

COMPENSATION FOR ILLNESS AND INJURY
If you agree to participate in the study, then your consent does not waive any of your legal rights. However, in the event of harm, injury, or illness arising from this study, neither Arizona State University nor the researchers are able to give you any money, insurance coverage, free medical care, or any compensation for such injury. Major injury is not likely but if necessary, a call to 911 will be placed.

VOLUNTARY CONSENT
Any questions you have concerning the research study or your participation in the study, before or after your consent, will be answered by Dr. Carol Johnston; 500 N. 3rd Street Phoenix, AZ 85004; 602-827-2265.

If you have questions about your rights as a subject/participant in this research, or if you feel you have been placed at risk, you can contact the Chair of the Human Subjects Institutional Review Board, through the ASU Research Compliance Office, at 480-965 6788.

This form explains the nature, demands, benefits and any risk of the project. By signing this form you agree knowingly to assume any risks involved. Remember, your participation is voluntary. You may choose not to participate or to withdraw your consent and discontinue participation at any time without penalty or loss of benefit. In signing this consent form, you are not waiving any legal claims, rights, or remedies. A copy of this consent form will be given to you.

Your signature below indicates that you consent to participate in the above study.

_____________________________   ______________________   _______________________
Subject’s Signature            Printed Name            Date

_____________________________
Contact phone number            Email

INVESTIGATOR’S STATEMENT
“[I certify that I have explained to the above individual the nature and purpose, the potential benefits, and possible risks associated with participation in this research study, have answered any questions that have been raised, and have witnessed the above signature. These elements of Informed Consent conform to the Assurance given by Arizona State University to the Office for Human Research Protections to protect the rights of human subjects. I have provided the subject/participant a copy of this signed consent document."

_____________________________   ______________________
Signature of Investigator            Date
APPENDIX D

IRB APPROVAL
To: Carol Johnston
   ABC 132

From: Carol Johnston, Chair
       Biosci IRB

Date: 01/23/2012

Committee Action: Amendment to Approved Protocol

Approval Date: 01/23/2012

Review Type: Expedited F12

IRB Protocol #: 1112007155

Study Title: Therapeutic value of vinegar for adults classified as pre-diabetic.

Expiration Date: 12/06/2012

The amendment to the above-referenced protocol has been APPROVED following Expedited Review by the Institutional Review Board. This approval does not replace any departmental or other approvals that may be required. It is the Principal Investigator’s responsibility to obtain review and continued approval of ongoing research before the expiration noted above. Please allow sufficient time for reapproval. Research activity of any sort may not continue beyond the expiration date without committee approval. Failure to receive approval for continuation before the expiration date will result in the automatic suspension of the approval of this protocol on the expiration date. Information collected following suspension is unapproved research and cannot be reported or published as research data. If you do not wish continued approval, please notify the Committee of the study termination.

This approval by the Biosci IRB does not replace or supersede any departmental or oversight committee review that may be required by institutional policy.

Adverse Reactions: If any untoward incidents or severe reactions should develop as a result of this study, you are required to notify the Biosci IRB immediately. If necessary a member of the IRB will be assigned to look into the matter. If the problem is serious, approval may be withdrawn pending IRB review.

Amendments: If you wish to change any aspect of this study, such as the procedures, the consent forms, or the investigators, please communicate your requested changes to the Biosci IRB. The new procedure is not to be initiated until the IRB approval has been given.

Please retain a copy of this letter with your approved protocol.
12-WEEK VINEGAR TRIAL IN HEALTHY ADULTS WITH PRE-DIABETES

INTRODUCTION
The purposes of this form are (1) to provide you with information that may affect your decision as to whether or not to participate in this research study, and (2) to record your consent if you choose to be involved in this study.

RESEARCHERS
Dr. Carol Johnston, a Nutrition professor at Arizona State University Downtown Campus, and Samantha Quagliano and Serena Loeb, nutrition graduate students, have requested your participation in a research study.

STUDY PURPOSE
The purpose of the research is to examine the effects of vinegar consumption on blood glucose concentrations.

DESCRIPTION OF RESEARCH STUDY
You have indicated to us that you are minimum age of 20 years of age, a non-smoker, generally healthy, and have stable medication use over the last 3 months, and that you have not been diagnosed with diabetes. Participants will be asked to maintain their usual diet and physical activity level throughout the trial. This study will initially involve the completion of a brief medical history questionnaire to demonstrate the absence of medical conditions that may impact the study. Your weight, height, and girth will be measured at the start and at the end of the trial. This first meeting will take ~30 minutes. This research study will last 12 weeks. At the start of the study and at trial weeks 6 and 12, you will travel to ASU (the Nutrition labs at the ASU Downtown campus) early in the morning to meet investigators for some testing: breath sampling at 0, 6, and 12 weeks, and a blood draw (~1/2 Tbsp/day) at 0 and 12 weeks. For the blood draw, you will fast overnight (12 hours). These visits will last <45 minutes.

You will be randomly assigned to the ‘apple cider vinegar drink’ group or to the ‘apple cider vinegar tablet’ group. During the 12-week trial you are asked to consume 8 oz vinegar drink or 1 vinegar pill (depending on group assignment) twice daily with the lunch and dinner meals (a total of 16 oz vinegar drink or 2 vinegar pills per day). You will be given a glucometer to use daily to measure blood glucose at waking and at 2-hours post meal ingestion after your largest meal. You will mark your blood glucose reading on a 12-wk calendar to be posted at home or office. During the 12-week trial, you are asked not to change your typical diet or activity patterns. If you deviate from your routine diet, or if you begin taking medications, at any time between during the 12-wk trial, you are to immediately notify the investigators of the study. About 40 subjects will participate in this study.

A research nurse will draw blood using standard, sterile techniques. Blood samples will be analyzed for biomarkers that are associated with blood glucose control such as glucose, insulin, and hemoglobin A1c.

RISKS
Bruising of the skin or a feeling of faintness is possible during the blood draws. For the at home finger pricks, subjects will be provided with disposable retractable lancets, strips, and glucometers as well as instructions for sterile conditions.

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<thead>
<tr>
<th>Subject’s Signature</th>
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</tr>
</thead>
<tbody>
<tr>
<td></td>
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Contact phone number Email

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</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Questionnaire to assess GI tract change

Subject # _______________ Date: ___/___/___

Vinegar pill
Vinegar drink

As part of the study, we want to know if participants are experiencing any changes in their digestive system by consuming the vinegar products. Please circle your responses to the following questions.

1. Have you been able to easily incorporate the vinegar source into your diet?
   Yes
   No

   IF NO, What difficulties have you had?: ____________________________________________

2. How many days each week did you consumed your vinegar source?
   Everyday
   Less than 7

   If you responded LESS THAN 7, what has kept you from consuming the vinegar product?
   ____________________________________________

3. In the past week, have you eaten any vinegar sources other than the test foods provided to you?
   No
   Yes

   IF YES, how many times per week would you estimate you ate any vinegar sources other than the test foods provided to you? In what amounts?
   ____________________________________________
Questionnaire to assess GI tract change

4. Have you experienced any changes in flatulence (gas) frequency over the past 12 weeks?
   
   Yes
   No

IF YES, how would you rate the amount of change in flatulence on a scale from 1-5 as compared to flatulence prior to the study with 1 being little change and 5 being a lot of change? (please circle a number)

1.……2.……3.……4.……5
Little change Greatest change

5. Have you experienced any changes in stool frequency over the past 12 weeks?
   
   Yes
   No

IF YES, how would you rate the amount of change in stool frequency on a scale from 1-5 as compared to your stool frequency prior to the study with 1 being less frequent and 5 being much more frequent? (please circle a number)

1.……2.……3.……4.……5
Less frequent More frequent

6. Have you experienced any changes in stool consistency such as the stool becoming more ‘soft or loose’ or more ‘hard or firm’?
   
   Yes
   No

IF YES, has the stool consistency been more loose or more firm? ‘more loose’ ‘more firm’ (circle one)

How would you rate the amount of change in stool consistency on a scale from 1-5 as compared to stool consistency prior to the study with 1 being little change and 5 being a lot of change? (please circle a number)

1.……2.……3.……4.……5
Little change Greatest change

7. Have you felt any bloating since the beginning of the study?
   
   Yes
   No
### Sample Size

<table>
<thead>
<tr>
<th>Author</th>
<th>Year</th>
<th>H2 change</th>
<th>n per group</th>
<th>Calculated n per group</th>
<th>Age range</th>
<th>Subject state</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Savaiano et al</td>
<td>1984</td>
<td>40±21</td>
<td>9</td>
<td>12</td>
<td>20-28</td>
<td>Healthy lactose deficient</td>
<td>Milk–Lactose fermentation (parallel arm)</td>
</tr>
<tr>
<td>Hertzler et al</td>
<td>2003</td>
<td>50±17 ppm</td>
<td>15</td>
<td>8</td>
<td>20-28</td>
<td>Healthy lactose deficient</td>
<td>Milk–Lactose fermentation (parallel arm)</td>
</tr>
<tr>
<td>Brighenti et al*</td>
<td>2006</td>
<td>15±12</td>
<td>10</td>
<td>24</td>
<td>30-50</td>
<td>Healthy</td>
<td>High vs. Low glycemic food fermentation (parallel arm)</td>
</tr>
<tr>
<td>Nilsson et al</td>
<td>2008</td>
<td>35±29 ppm</td>
<td>12</td>
<td>22</td>
<td>21-41</td>
<td>Healthy</td>
<td>Barley Kernels -Fiber and colonic fermentation (parallel arm)</td>
</tr>
</tbody>
</table>

**Average** | 33.5±18.1 | 12 | 17 | 20-50 |

All data is represented as means±SD

*Low glycemic foods have fermentable fiber, high glycemic foods have nonfermentable fiber, the addition of lactose to high glycemic foods increases colonic fermentation without altering the GI of the food.
APPENDIX G

QUINTRON ALVEOSAMPLER INSTRUCTIONS
The QuinTron AlveoSampler is a disposable tool that consists of a collection bag, a mouthpiece with an opening to connect a syringe, and a syringe with a stopcock and pull plunger to collect alveolar air. This tool is only meant to be used on a single patient, then discarded to eliminate risks associated with breath sample contamination and inter-patient cross-infection.

AlveoSampler Assembly

1. Remove bag, mouthpiece, and syringe from sealed bag.
2. Put the stopcock on the syringe and open it.
3. Attach the syringe to the AlveoSampler bag by placing the male end of the stopcock into the opening in the side of the plastic mouthpiece.

Breath Sample Collection

1. Have the patient take a normal breath and place the mouthpiece into the mouth at the end of inspiration with his/her lips closed tightly around it. Instruct the patient to exhale normally into the bag so it fills with air. The bag is vented with a small hole to allow the patient to maintain exhalation.
2. Collect 20 ml of alveolar air in the syringe by pulling the plunger out of the syringe while the bag is inflated and the patient is exhaling into the bag.
3. Once 20 ml has been collected, close the stopcock on the syringe to collect the sample. At this point, the patient can stop exhaling and remove the mouthpiece.
4. Remove the syringe filled with the sample from the mouthpiece and analyze the sample within 2 hours. If the sample needs to be held for a longer time a sample holding bag can be obtained.
APPENDIX H

QUINTRON BREATHTRACKER INSTRUCTIONS
Instrument Warm up

Turn on the main power and the pump power. Allow the BreathTracker to warm-up for at least 48 hours before calibrating the machine or analyzing the samples. If the BreathTracker is on standby (main power on and pump power off), allow at least 8 hours for warm-up prior to calibration.

Instrument Calibration

1. The display should read 0 for each gas, if not, push the ZERO (Ø) key.
2. Perform the full calibration. Collect ≥20 ml QuinGas in the calibrationsyringe and close stopcock. Insert syringe into the sample port on the BreathTracker, open the stopcock, and inject ≥ 20 ml QuinGas. Press the START key.
3. Then, perform the half calibration. Collect 15 ml QuinGas and 15 ml room air in the calibration syringe and close the stopcock. Insert syringe into the sample port on the BreathTracker, open the stopcock, and inject the diluted QuinGases. Press the START key.
4. The display will read CAL DONE when the calibration is complete.

Note: Calibration may required again depending on the length of the testing session QuinTron recommends at least two calibrations if analyzing patients over an eight hour time period.

Sample Analysis

1. Review the breathing technique and instructions for breath sample collection with the patient.
2. Collect the patient sample using the collection tool (bag, mouthpiece, and syringe) as outlined in the AlveoSampler instructions.
3. The display should read 0 for each gas, if not, push the ZERO (Ø) key.
4. Insert the sample drying tube into the sample port of the BreathTracker.
5. Attach the syringe containing the patient sample to the sample drying tube, turn the stopcock, and inject ≥ 20 ml of the patient sample into the sample drying tube. Press the START key.
6. Once the sample has been analyzed use the self-correction feature by pressing the DOWN key. The corrected H2 and CH4 values should appear on the display with a correction factor.
7. If the correction factor is above 4.0, the sample is contaminated and another sample should be collected and analyzed to replace the contaminated sample. If the correction factor is below 4.0, record all values (H2, CH4, CO2, and the correction factor).
8. Press the START key and wait for the LED status light to blink green to run the next sample.