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Improvement of Functional Bioactivity in Pear:Blackberry Synergies with Lactic Acid Fermentation for Type 2 Diabetes and Hypertension Management

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IMPROVEMENT OF FUNCTIONAL BIOACTIVITY IN PEAR:BLACKBERRY SYNERGIES WITH LACTIC ACID FERMENTATION FOR TYPE 2 DIABETES AND HYPERTENSION MANAGEMENT

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ABSTRACT

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Type 2 diabetes mellitus (T2DM) is a chronic disease that has a worldwide prevalence which is expected to rise dramatically over the course of the next thirty years. The disease has reached pandemic stages of development in many countries, most notably in developing countries, followed somewhat closely by developed countries with access to an overabundance of refined carbohydrates and fat (refined oils). T2DM is a condition that can be prevented or managed, but not cured; therefore a method of stymieing the development of this disease is paramount to halting its progressively increasing morbidity. An effective method of halting and delaying type 2 diabetes is refining the diet of at-risk people to limit refined carbohydrates and include fresh whole food produce with multiple bioactive factors beyond basic nutrients. In this study, Bartlett pear and Kiowa blackberry were investigated in relation to their potential ability to modify and improve both glucose metabolism and hypertension management with *in vitro* assay models. Effectiveness and bioactive functionality was evaluated by various *in vitro* assays to study the properties of: 100% Bartlett pear juice, 100% Kiowa blackberry juice and a ratio of

70:30 pear: blackberry juice found to have increased phenolic properties due to synergy in previous studies. These *in vitro* assays aimed at determining: alpha-amylase and alpha-glucosidase inhibition, angiotensin converting enzyme inhibition, total soluble phenolic content and antioxidant capabilities. These juices were also fermented with *Lactobacillus helveticus* and *Bifidobacterium longum*, common yogurt culture strains, to investigate if fermentation would improve the bioactive functionality of pear: blackberry synergies. A secondary goal of the experiment was to investigate if these fruit juices could prevent the growth of *Helicobacter pylori,* which is a common bacterium found in the stomach which can lead to ulceration and cancer.

Overall, fermentation increases the stability of phenolic compounds in pear and blackberry juices, as well as the 70:30 pear: blackberry combination. This leads to better stability for the juices in respect to their inhibition of investigated enzymes. Antioxidant activity and phenolic content was also increased overall with fermentation along with the inhibition of alpha-glucosidase. Angiotensin converting enzyme inhibition was increased overall as well, which would alleviate potential hypertension. Fermentation of 100% blackberry with *L. helveticus* and *B. longum* was also shown to inhibit *H. pylori.* Determining the mechanisms in which these enzymes are inhibited *in vitro* will allow research to continue *in vivo* with animal models, with clinical implications.

TABLE OF CONTENTS

Page

ABSTRACT iii

LIST OF FIGURES vii

CHAPTER

1. INTRODUCTION 1

2. REVIEW OF LITERATURE 8

2.1 Type 2 Diabetes: Pathophysiology 8

2.2 Oxidative Stress and Type 2 Diabetes 10

2.3 Antioxidants and their Effects on Stress Induced Metabolic Disorders 12

2.4 Alpha-Amylase and its Role in Glucose Metabolism 13

2.5 Alpha-Glucosidase and its Role in Glucose Metabolism 14

2.6 Phenolic and Glucose Metabolism 15

2.7 Cardiovascular Disease in Relation to Type 2 Diabetes 15

2.8 Angiotensin I Converting Enzyme (ACE) in Relation to Hypertension and Oxidative Stress 17

2.9 Fermentation with Lactic Acid Bacteria 18

2.10 Probiotics and their Benefits to the Digestive System 19

2.11 *Lactobacillus helveticus* as a Fermentative Organism 19

2.12 *Bifidobacterium longum* as a Probiotic Organism 21

3. OBJECTIVES 22

3.1 Main Objectives 22

3.2 Specific Objectives 22

4. IMPROVEMENT OF FUNCTIONAL BIOACTIVITY IN PEAR: BLACKBERRY SYNERGIES FOR TYPE 2 DIABETES AND HYPTERTENSION UTILIZING *LACTOBACILLUS HELVETICUS* FERMENTATION 23

4.1 Abstract 23

4.2 Introduction 24
4.3 Materials and Methods 26
4.3.1 Sample Preparation 27
4.3.2 Bacterial Strains and Other Methods 27
4.3.3 Fermentation with *Lactobacillus helveticus* R0052 27
4.3.4 Absorbance of Samples and Colony Counts 28
4.3.5 Total Phenolics Assay 28
4.3.6 Antioxidant Activity by 1, 1-Diphenyl-2-Picrylhydrazyl (DPPH) Radical Inhibition Assay 29
4.3.7 α-Glucosidase Inhibition Assay 29
4.3.8 α-Amylase Inhibition Assay 30
4.3.9 Angiotensin-1- Converting Enzyme (ACE) Inhibition Assay 31
4.3.10 HPLC Analysis of Phenolic Profiles 31
4.3.11 Preparation of *H. pylori* culture 31
4.3.12 Agar-Diffusion Assay 32
4.3.13 Proline Growth Response Assay 32
4.3.14 Statistical Analysis 32
4.4 Results and Discussion 34
4.4.1 Total Soluble Phenolics 34
4.4.2 DPPH 35
4.4.3 Alpha-Glucosidase 37
4.4.4 α-Amylase 38
4.4.5 Angiotensin Converting Enzyme 40
4.4.6 *H. pylori* 41
4.5 Conclusion 42

5. IMPROVEMENT OF FUNCTIONAL BIOACTIVITY IN PEAR: BLACKBERRY SYNERGIES FOR TYPE 2 DIABETES AND HYPERTENSION UTILIZING *BIFIDOBACTERIUM LONGUM* 42

5.1 Abstract 42

5.2 Introduction 44

5.3 Materials and Methods 46

5.3.1 Sample Preparation 46

5.3.2 Bacterial Strains and Other Methods 47

5.3.3 Fermentation with *Bifidobacterium Longum* 47

5.3.4 Absorbance of Samples and Colony Counts 48

5.3.5 Total Phenolics Assay 48

5.3.6 Antioxidant Activity by 1, 1-Diphenyl-2-Picrylhydrazyl (DPPH) Radical Inhibition Assay 48

5.3.7 α-Glucosidase Inhibition Assay 49

5.3.8 α-Amylase Inhibition Assay 49

5.3.9 Angiotensin-1- Converting Enzyme (ACE) Inhibition Assay 50

5.3.10 HPLC Analysis of Phenolic Profiles 51

5.3.11 Preparation of *H. pylori* culture 52

5.3.12 Agar-Diffusion Assay 52

5.3.13 Proline Growth Response Assay 53

5.3.14 Statistical Analysis 53

5.4 Results and Discussion 54

5.4.1 Total Soluble Phenolics 54

5.4.2 DPPH 55

5.4.3 α-Glucosidase 56

5.4.4 α-Amylase 57

5.4.5 ACE 58

5.4.6 *Helicobacter pylori* 59

5.5 Conclusion 59

BIBLIOGRAPHY 61

LIST OF FIGURES

Figure Page

1: TSP reading for 100% Pear, 70:30 Pear: Blackberry, and 100% Blackberry samples.....35

2: DPPH inhibition results, showing high inhibition for all fruit substrates. 37

3: Alpha-glucosidase inhibition for the substrates with fermentation. Samples were undiluted (ND), 50% diluted (1/2D) and diluted to 1/5 concentration (1/5D).....38

4: Alpha-amylase inhibition by fermented and non-fermented fruit juices. 39

5: ACE inhibition using 100% pear and 70:30 pear: blackberry substrates 40

6: A lawn plate of H, pylori with four sterile filter papers placed on top 41

7: Total soluble phenolics for B. longum fermented substrate and control substrate 54

8: DPPH inhibition assay for the substrates. Samples were fermented with B. longum (BL), fermented and also modified to a steady pH of 6.2 (pH) and left unfermented (Control).....55

9: Alpha-glucosidase inhibition for the substrates with fermentation. Samples were undiluted (ND), 50% diluted (1/2D) and diluted to 1/5 concentration (1/5D) 56

10: Alpha-amylase inhibition for samples with B. longum and control samples without added bacteria 57

11: ACE inhibition using 100% pear and 70:30 pear: blackberry substrates 58

CHAPTER 1

INTRODUCTION

Non-communicable chronic diseases (NCD) are becoming more prevalent every year on a global scale, and one of the most common and quickly growing pandemics is type 2 diabetes (T2DM). The United States has a high prevalence of T2DM, totaling 13.7% in men and 11.7% in women as reported in 2009. The amount of cases varies by state, and have the highest concentration in Southern states (I.E: Mississippi, Louisiana, Texas) and the lowest in Northern states (I.E: Vermont, Minnesota, Montana) (Danaei, 2009). According to the World Health Organization (WHO), 80% of all people suffering from T2DM come from low to middle-income countries. Some notable countries in this category are India and China, who have the two greatest populations and large percentage of diabetes sufferers as well. (WHO, 2011) According to the International Diabetes Foundation, the amount of people diagnosed with T2DM in India is around 62 million, China has about 90 million. The United States has only 21 million citizens suffering; but as a percentage the United States has 9.6% of the population afflicted, while India and China have only 9.2% and 9% respectively (IDF, 2011). Deaths caused by diabetes are expected to double by the year 2030; this is a substantial increase of deaths on a global scale, it requires attention and intervention (WHO, 2011).

T2DM and all of its associated illnesses can be directly caused by oxidative stress, which mainly takes place intracellularly. Mitochondria, which are present in every cell in the oxygen-dependent eukaryotic organisms, are the greatest source of oxidants. Some of mitochondrial oxidants which are produced during respiration are: hydrogen peroxide, hydroxyl radicals, and superoxide (Shigenaga et al., 1994; Paliyath, 2011).There are natural antioxidant counters to these oxidative

compounds that exist within the cell, but these can potentially be overwhelmed and evaded by the oxidants. These escaped oxidative entities can potentially affect nucleic acids which in turn can cause a breakdown of the cell wall and cellular organelles (Ames et al. 1993; Paliyath, 2011).

There have not been any direct genetic ties found for the cause of T2DM, but there are other genetic issues that can increase the chance of developing the disease. Having a genetic predisposition to obesity, especially intra-abdominal fat accumulation, is one of the most recognized causes of T2DM. Intra-abdominal fat has been shown to decrease insulin sensitivity in the body, which can eventually lead to development of T2DM (Kahn, 2003).

Insulin resistance is one of the main causes of T2DM, along with pancreatic beta-cell dysfunction. When these two issues combine, they render the body unable to regulate sugar by itself. The typical response of the pancreas to increased insulin resistance will be to increase the production of beta-cells; therefore allowing for more insulin to be created to compensate for the resistance. Increased production of beta-cells and insulin then causes additional stress on the pancreas, which can ultimately lead to beta-cell dysfunction. Should the increased beta-cell production falter, the result will be the inevitable development of T2DM (Hajer et. al. 2008). Blood glucose is a critical balance in the body as well, and insulin plays a major role in the glucose homeostasis. Hyperglycemia is defined as having $\geq$ 100mg glucose per dL of blood (Wilson, 2005).

One of the most common occurrences of hyperglycemia is postprandial increase of the blood glucose level, which occurs directly after a meal is consumed. After eating foods, the sugars are metabolized and directed into the blood for distribution throughout the body. Enzymes in the saliva and intestines, notably

alpha-amylase and alpha-glucosidase, break down soluble carbohydrates and rapidly increase sugar levels in the blood. Insulin helps regulate this blood sugar, but the effect will be insufficient if there is an insulin deficiency (van der Berghe, 2003). Hyperglycemia can cause several imbalance and leads to physiological disorders like loss of bowel motility (Björnsson, 1994), microvascular diseases, macrovascular diseases, and blood gas imbalances (Marfella, 2000). In essence, the ability of the body to control sugar in the blood stream has a direct impact on a person's overall health in both acute and chronic ways.

The methods in which sugars are absorbed into the bloodstream within the intestines should also be touched upon, as it relates directly to the research being presented in this thesis. Carbohydrates that are ingested inevitably end up in the intestines, where the majority of absorption into the bloodstream occurs. The intestines contain pancreatic enzymes that break down these complex carbohydrates into simple monosaccharides that can be easily taken into the bloodstream through the epithelial wall. Multiple enzymes contribute to the complete breakdown of carbohydrates, with two of the most important being alpha-amylase and alpha-glucosidase which break down amylose and glycogen, respectively. The action of these two enzymes is critical to the absorption of glucose into the body, inhibiting them can improve post-prandial blood sugar levels. Alpha amylase works by cleaving alpha 1,4 bonded glucose molecules within the starch structure, acting as an endo-amylase (van der Maarel, 2002) This in turn breaks the large, branched amylose into smaller sections which are easier for other enzymes to handle. Alpha-glucosidase, on the other hand, breaks maltose in half – which is a disaccharide comprised of two glucose molecules. In essence, the alpha-glucosidase can break two glucose molecules apart in the middle of an amylose chain or break apart the maltose disaccharide itself. This leads to either the formation of two

smaller amylose chains or to the creation two new glucose molecules, which can rapidly increase blood sugar levels through faster absorption into the intestine.

Pharmaceutical drugs can be utilized to prevent sugar uptake into the bloodstream, but it is not always the best option. Many pharmaceutical options for sugar blocking can inhibit alpha-amylase and alpha-glucosidase entirely. While this may seem like an extremely effective method for preventing sugar uptake, it has many side-effects in the body. Not only do the calories from the sugars get blocked; but they also end up being shunted through the small intestine and into the large, where gut bacteria will utilize them for fermentation. This fermentation can have negative repercussions on the body, including gastric distress and irritable bowels. In order to curb but not eliminate the uptake of sugars into the blood, alpha-amylase and alpha glucosidase can be inhibited using natural compounds from plant sources, especially fruits and vegetables.

Cardiovascular diseases (CVD) are often associated with diabetes and the cost of treating heart problems exceeds $100 billion dollars every year (DeFronzo, 1999). This leads researchers to believe that each of the diseases can actually cause the other to occur in patients. In a study, it was shown that using ACE blockers in patients with hypertension, the rate that the patient developed T2DM was lowered from 34% to 11% (Sowers, 2001). The rate of developing T2DM from having hypertension is very large, having one out of three patients develop it from this study. Inhibiting ACE seems like a significant step to prevent the development, reducing the occurrence of diabetes in this example by about 66%.

Many plant-sourced compounds have been studied for use in dietary management of carbohydrate-based calories, their metabolism and for general health benefits. Phenolic compounds from fruits and vegetables play a very

important role in management of blood sugar by their inhibitive properties on gut enzyme activity. For this specific study, pears and blackberries were chosen as the source of phenolic compounds. Phenolics from the Bartlett pear and the Kiowa blackberry have been shown to dramatically inhibit the activities of alpha-amylase, alpha-glucosidase and angiotensin converting enzyme *in vitro*. With a ratio of 70% Bartlett pear to 30% Kiowa blackberry, there has been an impressive level of inhibition in regards to alpha-amylase and alpha-glucosidase, along with a low to moderate decrease in the activity of angiotensin converting enzyme II (ACE) (Warner, 2012). ACE activity can lead to cardiovascular issues and chronic heart disease by causing hypertension and other related effects via the renin-angiotensin-aldosterone pathway (Weir, 1999). Phenolics have been shown to limit the effects of these enzymes by binding to their active sites, therefore preventing enzymatic action (Matsui et al., 2001).

By inhibiting ACE to prevent hypertension and inhibiting alpha-amylase and alpha-glucosidase to prevent expedited sugar uptake, there is potential to prevent the prevalence of chronic diseases. Pear has a high level of ACE inhibition, but low alpha-amylase and alpha-glucosidase inhibitory activity. Blackberry, on the other hand, has a high amount of alpha-amylase and alpha-glucosidase inhibition but no ACE inhibition (Warner, 2012). These fruits can supplement each other and cause a synergistic effect to occur when combined.

Fermentation of foods has been used for thousands of years for a variety of reasons; the most important being for preservation of the foods, but fermentation is also important for flavor and health benefits. Multiple beneficial nutrients such as vitamins or phenolic compounds can be added or made available by different types

of microorganisms. There is a notable increase in vitamin B content during most fermentation processes (Tongnual and Fields, 1979; Paliyath, 2011).

Fermentation can be utilized to further alter the phenolic profile of the fruit juices. The bacteria used to ferment the pear and blackberry used in this study is a lactic acid bacterium (LAB); *Lactobacillus helveticus*. This bacterium, as its name suggests, produces lactic acid from sugars but can also produce other organic compounds. These acids and compounds can interfere with enzymatic activity, but can also be beneficial. This particular strain of bacteria is often found in fermented dairy products used in the United States.

A second LAB has also been investigated in this study in order to determine if different effects would occur regarding enzymatic inhibition if the fermentative organism was altered. The second bacterium, *Bifidobacterium longum,* is another commonly used culture for yogurt production. It ferments sugars into lactic acid in a similar manner to *Lactobacillus helveticus*, but as a different organism it will react differently to the provided environment and conditions. Having a second LAB allows comparison between the two and to check for consistencies. The second bacterium can also open up the possibilities of having multiple bacteria with different properties being combined for their collective health benefits and potential symbiotic relationships.

Probiotics have become more and more popular over the past few years, and their potential to be utilized by food and health industry is growing along with their popularity. Good probiotic bacteria will colonize the intestinal tract and help increase gut health. The increases in health are usually due to an increase in metabolic stability and increased resistance to pathogen uptake and colonization (Holzapfel, 1998). *Lactobacilli* have often been used as a probiotic addition to foods,

and show very promising results when used as such. *Lactobacilli* are acid tolerant bacteria, and can therefore easily survive the early stages of digestion prior to colonization. These bacteria can ferment foods and provide additional benefits upon consumption besides being purely probiotic. In a study conducted upon rats, the ingestion of lactobacillus containing fermented milk both decreased cardiovascular disease and increased the time it took the rats to develop glucose intolerance (Holzapfel, 1998).

Helicobacter pylori is a harmful bacterium that is commonly found in the stomach and duodenum of humans. This bacterium can cause ulceration and, if left unchecked, can lead to stomach or duodenal cancers via the aggravation of cellular breakdown. Along with the health benefits of these fruits being studied in respect to type 2 diabetes mellitus, the potential of the fruit juices in regards to inhibiting *H. pylori* was also examined; with and without the fermentation by *L. helveticus* (Montecucco, 2001).

CHAPTER 2

REVIEW OF LITERATURE

2.1: Type 2 Diabetes: Pathophysiology

Beta-cell dysfunction and insulin resistance have been attributed to the two major causes of developing T2DM. As insulin resistance increases, insulin sensitivity decreases; which in turn causes insulin to become less effective. Beta-cells in the pancreas begin to dysfunction as this process occurs with the insulin due to the increased insulin production that is signaled for by the body, which leads to the end result: the development of T2DM. Beta-cell dysfunction occurs prior to the development of T2DM and not during the onset of the disease. This was discovered via oral glucose tests on both diabetic and non-diabetic subjects. This indicates that beta-cell dysfunction could be a primary cause of T2DM and not just an aftereffect (Kahn, 2003).

To truly understand how T2DM begins in the human body, one must first understand how insulin functions. Another way to look at the development of T2DM is to understand how insulin deficiencies occur – and how they affect the body. Hyperglycemia is one of the ways that insulin resistance develops, and hyperglycemia can be caused by a multitude of factors. Hyperglycemia can simply occur due to the consumption of food, but can also be caused by injuries or acute trauma and its effects have been coined as the “diabetes of injury”. Insulin administered after injury helps prevent glucose toxicity and further damage to the critically injured. Excessive glucose can cause oxidative stress due to extreme amounts of phosphorylation and glycolysis at the cellular level. Insulin resistance is detrimental to the well-being of a person, and is a strong symptom of diabetes. The

cause of the resistance can reach beyond just nutrition, it is important to study these other causes as well as the main diet related ones (van der Berghe, 2003) .

Postprandial hyperglycemia can lead to many issues and complications beyond insulin resistance. Atherosclerosis is a common disease caused by postprandial which leads to oxidative stress and redox imbalance. Cardiovascular diseases are a very common cause of death and disability in the United States. Postprandial hyperglycemia is common, but controlling the diet and caloric intake by practicing moderation can help with preventing complications due to this form of hyperglycemia. Oxidative stress can also occur due to these metabolic effects on the mitochondria within cells which cannot refuse the ample sugar of the blood to create reactive oxygen species. This oxidative stress can lead to further complications and health risks along with the increased chance of developing T2DM (Ceriello, 2000).

Wilson (2005) took a deeper look into the metabolic syndromes in relation to T2DM and cardiovascular disease. The study focused on five main traits: abdominal adiposity, low HDL (high-density lipoprotein) cholesterol, high triglycerides, hypertension and impaired fasting glucose. This study followed over 3000 middle-aged adults, the adults that developed at least three of the five traits showed conclusive results. The prevalence of at least three out of the five previously mentioned metabolic syndromes in these adults was 26.8% for men and 16.6% in women. This shows that metabolic syndromes are very common in adults and therefore the prevalence of cardiovascular disease and T2DM is likewise as common or at least a high-risk pair of diseases for the average adult (Wilson, 2005) .

Studies have found that adipose tissue in the body can actually affect glucose and lipid metabolism rates. These tissues can also release hormones which can

further affect body functions. Increases in the prevalence of adipose tissue have also increased the prevalence of T2DM and cardiovascular diseases. Studies by Hajer (2008) have shown that these observations are accurate, and that these accumulations of intra-abdominal fat have adverse effects on the body as a whole. Adipose tissue was shown to act as an actual endocrine organ which plays a very important role in the metabolism of lipids and glucose. The hormones that adipose tissue can produce are directly correlated to the development of T2DM and cardiovascular diseases. Managing obesity rates can help manage these dysfunctions if only due to the management of the adipose tissue itself (Hajer et. al. 2008).

2.2: Oxidative Stress and Type 2 Diabetes

Oxidative stress is an extremely damaging event that occurs naturally in cells due to their natural respiration processes, which can lead to detrimental health consequences such as CVD and T2DM. The source of most oxidative compounds is the mitochondria, which is a critical organelle that is used for cellular respiration, and also the oxidation reduction reactions which occur during respiration. About 85% of the oxygen used in the cell is due to the mitochondrial electron transport system. The mitochondria produce ATP for energy through oxidative phosphorylation, which is an oxidation reduction reaction. Over time, the oxidation caused by the by-products of the mitochondrial electron transport chain can damage cells and cellular organelles and lead to cellular dysfunction. Oxidants like superoxide, peroxides, singlet oxygen, and hydroxyl radicals can be produced. This is actually the main cause of aging. All of these by products must be dealt with using antioxidants within the cell, or else the damage caused can lead to metabolic breakdown. It is paramount to the health of the cell to contain enough antioxidants

in order to quench all oxidants that are present. Balancing oxidants and antioxidants will lead to cellular homeostasis, a requirement for cell health and longevity.

Oxidative stress can increase the damage done by obesity by both having an increased prevalence in the body and by occurring in adipose tissue. Obesity causes an increase in free fatty acids (FFA) within the body. FFA has the ability to impair glutathione, a common and effective intracellular antioxidant inhibition of glutathione by FFA increases oxidative stress at an intracellular level. These FFA can also damage mitochondrial function such as uncoupling oxidative phosphorylation or by causing the production of superoxide and other reactive oxygen species (ROS) (Evans, 2003). ROS have also been shown to be produced in adipose tissue, which can lead to more oxidative stress on the body. Higher levels of adipose tissues have also been shown by Furukawa (2004) to cause reduced antioxidative enzyme production and increased expression of NADPH oxidase which is an oxidizing enzyme. The combination of increased ROS production and reduced antioxidants can lead to the development of the metabolic syndrome (Furukawa, 2004).

Shingenaga et al. (1994) found that oxidative damage could occur over time in a variety of cells after exposure to oxygen, and could be caused by a variety of oxidative compounds. The researchers looked for lipofuscin, which is a marker of oxidative damage. When lipofuscin is present, it is determined that oxidative processes have occurred in the cell and have resulted in damage to the organelles, nucleus or membrane. Electron transport is also damaged by oxidation, and this can cause further production of both hydrogen peroxide and superoxide. This is a chain reaction effect that ends up causing extra production of oxidants. These oxidants may increase the rate of metabolic breakdown which leads to T2DM, along with increasing oxidative stress on the body. Mitochondrial damage can also lead to

immune system decay and nuclear damage, which can lead to genetic mutations and can progress the metabolic syndrome (Shigenaga et al., 1994; Paliyath, 2011).

2.3: Antioxidants and their Effects on Stress Induced Metabolic Disorders

Using natural antioxidants provided by fruits and vegetables is an efficient method to counter oxidative stress-induced diseases such as T2DM. It was shown by Soobrattee (2005) that many natural compounds, including phenols and vitamins, scavenged oxidants and exhibited protective and beneficial effects against oxidation. In the research, it was shown that protection of the cardiovascular system by the proanthocyanidin rich extract included: (1) potent hydroxyl and other free radical scavenging abilities; (2) antiapoptotic, antinecrotic and antiendonucleolytic potentials; (3) modulation of apoptotic regulatory bcl-XL, p53 and c-myc genes; (4) cytochrome P450 2E1 inhibition; (5) inhibition of proapoptotic, cardioregulatory genes c-JUN and JNK-1 and (6) the inhibition of constriction of smooth heart muscles and the endothelium (Soobrattee, 2005).

Phenolic compounds and other plant-based antioxidants have been shown to be an effective means of alleviating metabolic disorder through improved redox balance. Rahimi (2005) has noted that flavonoids from plants are adept at scavenging free radicals in the body. Compounds like quercetin have been shown to be effective ion chelators as well. Reactive metals can increase the rate of oxidation within the body, so chelating compounds are a welcome source of relief to this issue. Plants are also a great source of antioxidative vitamins, such as beta-carotene (as provitamin A), ascorbic acid (vitamin C) and alpha-tocopherol (vitamin E). Increasing the dietary intake of plant based phenolics and antioxidants is a proven diet-based preventative measure in combating T2DM. These same phenolics have many effects on the glucose metabolism, which include the inhibition of key starch-

digesting enzymes. Inhibiting these enzymes helps lowering sugar uptake into the bloodstream and can therefore help with regulating insulin production in the pancreas and reduce the system shock factor from quickly absorbed refined sugars. One of the most important enzymes pertaining to the digestion of starch is alpha-amylase, which quickly breaks amylose chains into smaller and more easily dissolved fractions (Rahimi 2005).

2.4: Alpha-Amylase and its Role in Glucose Metabolism

In a review by van der Maarel (2002), alpha-amylase and its applications were explored. As the name implies, the alpha-amylase enzymes attack alpha bonds on amylose structures. These alpha bonds connect glucose residues together; when they are broken the glucose can then be taken away from the larger amylose for use by organisms. Van der Maarel (2002) noted at least 21 different bond specificities in the alpha-amylase enzyme family. This means that there are over 21 different types of enzyme, each specialized in breaking a specific type of glucose bonding. There are two main varieties of alpha-amylase; one being starch-hydrolyzing enzymes which can break apart the amylose, while the other type is starch-modifying enzymes that will simply change conformations within the amylose. Each different alpha-amylase could be used for various applications, but they are all useful for starch modification and digestion. (van der Maarel, 2002)

There are multiple forms of alpha-amylase enzyme that exist in the human body. Humans use these to digest carbohydrates and extract the glucose and other sugars from them. The enzymes exist in both salivary fluids within the mouth and pancreatic fluids within the intestines. Both alpha-amylase sources produce different enzymes varieties pertaining to various bond structures which need to be broken down within the food which humans consume. In a study by Hagele (1982),

it was found that the pancreatic alpha-amylase enzyme cleaved 9% of amylose between the second and third glucose unit, 31% between the third and fourth, and 60% between the fourth and fifth. The corresponding results for the salivary alpha-amylase enzyme are 10, 26, and 64% respectively. These two enzymes both function in a similar method, but at slightly different ratios. It was also shown that the salivary enzyme worked around twice as fast as the pancreatic enzyme did. This may be due to the fact that since the food spends such a long time in the intestines that it would be more beneficial to the body to prevent a quick sugar uptake at the start of digestion. Alpha-amylase does not fulfill the entire role of starch digestion since it has a difficult time of breaking the starch down into single glucose molecules. In this respect, alpha-amylase requires a co-enzyme to complete starch breakdown, namely alpha-glucosidase. (Hagele 1982)

2.5: Alpha-Glucosidase and its Role in Glucose Metabolism

Alpha-glucosidase is an oligosaccharide-hydrolase that can break down both carbohydrates and glycogen in lysosomes. This enzyme can produce the alpha form of glucose out of the more complex substrates which humans consume. It completes this reaction by hydrolyzing bonds within the carbohydrate. Chiba (1997) has found that alpha-glucosidase hydrates the double bond of D-glucose to produce 2-deoxy-D-glucose. The enzyme protonates the C2 position of D-glucose from different directions, being either above or below the plane (alpha or beta). Alpha-glucosidase works alongside with alpha-amylase to fully break down starches; since each enzyme can attack different parts of the substrate, the combined efforts of both can complete a more full degradation of the carbohydrate. Alpha-glucosidase also facilitates glucose absorption in the intestines. Inhibiting these enzymes will be paramount in mediating glucose absorption rate to alleviate postprandial

hyperglycemia. An effective way to do so is by utilizing plant-based phytochemicals, particularly phenolics through consumption of edible plants. **(**Chiba, 1997)

2.6: Phenolics and Glucose Metabolism

Phenolic compounds are a diverse species of secondary metabolites of plants that range in size, function and composition. There are many of these in fruits and vegetables that work as antioxidants or enzyme inhibitors. Humans can reap the beneficial properties of the plant-based phenolic compounds by utilizing them as diet to tolerate biotic and abiotic stresses. These phenolics have been shown to be able to inhibit alpha-amylase and alpha-glucosidase, which for the plant would act as a pest deterrent to prevent a pest from digesting the sugars and controlling overall metabolism in relation to environmental adaptation. In a human body when consumed, phenolic inhibitors act to slow down postprandial hyperglycemia and delay the time it would take to digest and absorb sugars into the bloodstream. High alpha-glucosidase and moderate alpha-amylase inhibition has been shown to potentially have the best postprandial hyperglycemia control by Cheplick (2010) in a phenolic rich strawberry. Excessive alpha-amylase inhibition has been shown to lead to undigested starch in the intestines, which can lead to an irritated feeling in the gut. This is due to the fact that starch will not be broken down sufficiently in the small intestine, which leads it to be broken down by bacteria in the large intestine. When bacteria break down starch, they create gases which can lead to bloating and other discomforts (Cheplick et. al. 2010).

Phenolics from plants have been shown to inhibit glucose absorption in studies by McCue et al (2004). The study done by McCue et al (2004) alluded to the fact that phenolic extracts, in this case rosmarinic acid, from plants can inhibit alpha-amylase *in vitro* anywhere from around 50% (natural source) or up to around

90% (concentrated extract). Slowing alpha-amylase potentially slows the entire glucose absorption pathway in the digestive tract by limiting the amount of areas that alpha-glucosidase can affect. Limiting alpha-glucosidase activity effectively slows the rate that single glucose molecules will be pulled from the starch chain. The chain-reaction from limiting enzyme activity can drastically decrease sugar absorption into the blood and therefore decrease the effects of postprandial hyperglycemia which can lead to diabetes, the metabolic syndrome and potential cardiovascular issues (McCue et al 2004). Phenolic compounds also play an important role in glucose absorption by affecting enzymatic activity and oxidation-reduction reactions. This in turn alters beta-cell function as insulin is produced at more controlled intervals and at lower quantities (Hannineva et. al, 2010)

2.7: Cardiovascular Disease in Relation to Type 2 Diabetes

T2DM can lead to many complications which can become life-threatening. The cardiovascular system suffers greatly due to T2DM, and people afflicted by T2DM often develop dyslipidemia, hypertension, obesity, clotting abnormalities, microalbuminuria, and accelerated atherosclerosis (Defronzo, 1999). These cardiovascular issues can be deadly and devastating and the chance to develop them is greater due to suffering from T2DM. These complications are all coupled with the insulin resistance syndrome, also called the metabolic syndrome or syndrome X. Cardiovascular disease accounts for the most deaths due to T2DM, which are often due to the increased levels of sugars and lipids that are present in the bloodstream (DeFronzo, 1999).

Diabetes mellitus is often referred to as a comorbid disease due to the multitude of metabolic breakdowns and complications that it causes, and can also be caused by. In a study that was touched upon by Sowers, 2001 the chance of

developing T2DM was 2.5 times as likely to occur within patients suffering from hypertension than those without the complication. T2DM, likewise, also greatly increases the chance of developing hypertension. Cardiovascular disease accounts for around 80% of the deaths in humans with T2DM, and mortality is 7.5 times greater within the T2DM afflicted population without a previous myocardial infarction than those without the disease. The presence of hypertension and T2DM greatly increases the chance of developing serious cardiovascular complications (Sowers, 2001).

2.8: Angiotensin I Converting Enzyme (ACE) in Relation to Hypertension and Oxidative Stress

One way to control hypertension is by utilizing ACE inhibiting compounds. ACE, or angiotensin I converting enzyme, converts inactive angiotensin I in the body into angiotensin II. Angiotensin II stimulates the synthesis and release of aldosterone from the adrenal cortex, which then increases blood pressure via promoting sodium retention. This is called the renin-angiotensin-aldosterone pathway, and it naturally helps regulate blood pressure. The issue lies in when there is too much production of ACE. In past investigations, it has also shown that angiotensin II also stimulates the production of superoxide anion and hydrogen peroxide in the polymorphonuclear leucocytes, which in turn causes oxidative stress on the system. There are many stressors associated with ACE, inhibiting the enzyme will reduce a significant amount of hypertensive stress and oxidative damage (Barbosa-Filho et al., 2006; Nielsen, 1991).

Utilizing natural ACE inhibitors from plant-based sources are more attractive, due to the fact that most pharmaceutical drugs fully inhibit many other digestive enzymes as well as ACE. With this in mind, research has been done on utilizing food

sources to inhibit ACE. In studies performed by Kwon (2006), it was shown that many edible plants have the ability to safely inhibit ACE. In fact, this research showed that some plant phenolic extracts can inhibit ACE by up to 90% *in vitro.* Dietary management of hypertension is a natural and safe method to utilize in contrast to over-the-counter drugs which can end up causing intestinal discomfort (Kwon, 2006; Barbosa-Filho et. al, 2006)

2.9: Fermentation with Lactic Acid Bacteria

Fermentation of foods using lactic acid bacteria can improve the antioxidant capacity of phenolic compounds. It was shown by Rodriguez (2009) that by using lactic acid bacteria in food, *L. plantarum* specifically, the amount of tannins and phenolic acids decreased significantly. This was due to the activity of tannases and phenolic acid decarboxylases, two types of enzymes utilized by the bacteria. These broken down tannins and phenolic compounds can be subsequently converted to antioxidant compounds (Rodriguez et. al. 2009).

Lactic acid bacteria are the most commonly used traditional starter cultures by humans for fermentation processes. Their ability to be used as a probiotic adds an additional health benefit to utilizing LAB as a fermenting organism. The term “probiotic” is sourced from the Greek language, where it translates to “life”. The term has recently been used to describe a microbe that, when ingested in sufficient numbers, can help regulate an animal's intestinal microflora. This regulation is achieved by the production of compounds by the probiotic bacteria that are harmful to pathogenic organisms and prevention of pathogen attachment to the epithelial wall of the intestines. Other probiotic benefits of note are their potential to metabolize nutrients for the host and alleviation of bowel disorders. (Ankolekar, 2011)

2.10: Probiotics and their Benefits to the Digestive System

Lactic acid bacteria consumed in the diet can act as a probiotic. Probiotics are important for both maintaining gut health and sustaining healthy microflora in the intestines. Holzapfel (1998) has described multiple benefits in having strong gut microflora, including: prevention of pathogenic adherence to the intestinal walls, modification of dietary proteins, modification of bacterial enzyme capacity, and influence of gut mucosal permeability. Gut microflora make pathogens to have a more difficult time of infecting the host, help with monitoring the diffusion of compounds into the bloodstream, and help digesting foods via enzyme bolstering. *Lactobacillus helveticus* is useful for improving gut microflora as a probiotic when consumed. When a fruit juice is inoculated with *L. helveticus,* it would retain the beneficial effects of the fruit and gain the beneficial probiotic attributes of a lactic acid bacterium (Holzapfel, 1998).

2.11: *Lactobacillus helveticus* as a Fermentative Organism

Previous work by LeBlanc (2002) has shown that sarcoma occurrences can be lessened when milk which has been fermented with *L. helveticus* is ingested to mice. This study shows that multiple immune response abilities are heightened by the presence of *L. helveticus* metabolites in fermented dairy products. This seems to be relative to the phagocytic index of the fermented product, or the ability of the food to increase phagocytosis in the host. Phagocytosis in this case refers to the enveloping of pathogenic organisms via immune system cells such as IgA (LeBlanc, 2002).

In a study by Johnson-Henry (2006), protein extracts that were taken from *L. helveticus* were shown to inhibit the adhesion of pathogenic *E. Coli* O157:H7 onto the epithelial wall of the intestinal tract. Adhesion is one of the most important steps

of bacterial infection in the digestive system, inhibiting the ability of pathogens to attach to intestine cells can greatly reduce the chance of contracting a disease. The mechanism for the inhibition of adhesion seems to simply be competition for binding sites on the epithelial wall. When *L. heleveticus* is consumed, it will block off the sites of the epithelial cell prior to the ingestion of *E. Coli* O157:H7 (Johnson-Henry, 2006).

Lactobacillus helveticus is a lactic acid bacterium that is often used in the fermentation of milk products. This bacterium can also be used with fruit, and has shown positive results for antioxidant activity in previous studies. In research done by Ahire et al (2011), oxidation of ascorbate was reduced by 27.5±3.7%. *L. helveticus* exudates also scavenged 20.8±0.9% of hydroxyl radicals. These results are promising for the effectiveness of lactic acid fermentation, and especially for *L. helveticus* (Ahire, 2011).

Studies by Mousavi (2010) have shown that probiotic lactic acid bacteria can survive in refrigerated temperatures when introduced to fruit juices. Lactic acid bacteria were viable for up to three weeks in refrigerated temperatures; ergo the drink would be perishable in regards to the probiotic benefit. Mousavi (2010) found that the bacteria utilize citric acid as their main source of carbon during fermentation, which is in abundance for most fruits. Lactic acid bacteria also metabolized both glucose and fructose within the fruit juice, which means that the bacteria are not overly selective when it comes to sugar substrates. This study lends viability to the idea of a probiotic fruit drink utilizing *Lactobacillus helveticus* and *Bifidobacterium longum* (Mousavi, 2010).

In previous research, *L. helveticus* has been found to be a beneficial organism when placed into fruit juices. Fermentation with *L. helveticus* has been shown to

increase functionalities in potentially managing the metabolic syndrome during early stages of Type 2 diabetes. In the previous studies, apple and blueberry juice combinations along with cherry and pear juices were used and the bacteria successfully grew to a high enough levels to function as a probiotic when ingested (Augustinah, 2012; Ankolekar, 2011; Apostolidis et al., 2006)

2.12 *Bifidobacterium longum* as a Probiotic Organism

Bifidobacterium longum is a lactic acid bacterium that is commonly utilized as a live culture probiotic in foods, much like *L. helveticus*. This organism has been shown by Harmsen (2002) to work as a probiotic in human subjects, and has also been demonstrated to be GRAS, or "generally regarded as safe" (Harmsen, 2002). In regards to the bacterium and the effect it has on diabetes symptoms, Chen, 2008 has shown that the presence of this microorganism in the digestive tract can lead to improved insulin resistance and the lowering of metabolic inflammation. Rats were fed a high-fat diet and then either fed *B. longum* or not fed any probiotic at all. Rats which were fed the *B. longum* had lower incidences of the metabolic syndrome (Chen, 2008). While the testing by Chen (2008) was performed on rats, the results are likely a close analog to the effects on a human digestive system.

In previous research, *B. longum* has been found to be a beneficial organism when combined with fruit juices. Fermentation with *B. longum* has been shown to increase functionalities in potentially managing the metabolic syndrome during early stages of Type 2 diabetes. In the previous studies, apple and blueberry juices were used and the bacteria successfully grew to a high enough level to function as a probiotic when ingested (Augustinah, 2012).

CHAPTER 3

OBJECTIVES

3.1: Main Objectives

The major goals of this thesis research are as follows:

To evaluate using *in vitro* assays the change in ability of Bartlett pear extract, Kiowa blackberry extract, and a ratio 70:30 pear/blackberry extracts to potentially manage early stages of T2DM and CVD through the diet after fermentation by various strains of bacteria.

A second major objective was to determine if these same fermented juices inhibit *H.pylori* more than their unfermented counterparts.

3.2: Specific Objectives

The specific goals derived from the main objectives are as follows:

1) To evaluate the synergistic effect of pear and blackberry on *in vitro* enzyme inhibition relevant to early stages of type 2 diabetes and hypertension management after fermentation with *Lactobacillus helveticus* and also with a separate fermentation by *Bifidobacterium longum.*

2) To determine if these same fermented juices inhibit *H.pylori* more than their unfermented counterparts and improve gut health.

CHAPTER 4

IMPROVMENT OF FUNCTIONAL BIOACTIVITY IN PEAR: BLACKBERRY SYNERGIES FOR TYPE 2 DIABETES AND HYPERTENSION UTILIZING *LACTOBACILLUS HELVETICUS* FERMENTATION

4.1: Abstract

Bartlett pear and Kiowa blackberries have been shown to exhibit bioactive beneficial potential in regards to the management of Type 2 diabetes mellitus and hypertension, a common Type 2 diabetes co-factor based on *in vitro* assays. Combining these juices at a ratio of 70:30 pear:blackberry has been shown in previous studies to contain the highest amount of phenolic-linked activity due to synergistic effects. The focus of this study was to determine the effectiveness of lactic acid fermentation, specifically with *Lactobacillus helveticus*, in regards to preserving phenolic-linked activity over time and enhancing the health benefits of the juices. The secondary goal of the experiment is to validate if any fruit juice, fermented or not, can inhibit the growth of *Helicobacter pylori*, a common bacterium found in the gut which can cause ulceration of the stomach and duodenum and can subsequently lead to potential cancer development in the aforementioned organs.

Over the course of the study, results showed that the 70:30 combinations had good synergy, the highest content of soluble phenolics, higher antioxidant activity, and the fermentation with *L. helveticus* lead to higher phenolic activity retention over time. Fermentation increased the ability over time of phenolic compounds in regards to the inhibition of the studied enzymes relevant to glucose metabolism, showing a stabilization effect of the fermentation on the phenolics. A 100% Kiowa

blackberry juice was shown to inhibit the growth of *H. pylori,* but only after fermentation. The control showed no inhibition whatsoever, nor did any 70:30 combination or 100% Bartlett pear juice show any inhibition of the bacterium either. The 70:30 combination is the most cost-effective way to reap the greatest benefits of the fruits studied. Fermentation with *L. helveticus* increases the overall effectiveness by adding bioactive stability and additional health benefits.

4.2: Introduction

Lactic acid bacteria have long been used to preserve foods and prevent spoilage since before constant refrigeration was possible. Investigating one step further than spoilage prevention would be to examine the change of certain nutritional benefits and attributes of the fermented product. In this study, *Lactobacillus helveticus* was utilized in order to compare phenolic content and capabilities of a fermented fruit juice and the non-fermented counterpart. The aim was to increase the ability of the juice to inhibit the alpha-amylase, alpha-glucosidase and angiotensin converting enzymes while also evaluating if the antioxidant functions are affected by the fermentation. The main focus of the experiment is to develop a beverage with bioactive benefits for managing early stages of Type 2 diabetes mellitus by dietary management and supplementation with various fruit juices. Phenolic compounds which naturally occur in fruits have the ability to slow the action of metabolic enzymes which digest simple carbohydrates such as amylose and amylopectin, which are a common and plentiful source of glucose. These carbohydrates are often found in inexpensive processed foods, which are widely consumed by the populace of the United States and the BRIC countries (Brazil, Russia, India, and China). Altering and slowing glucose

metabolism is a key approach to avoid sugar spikes in the blood, which can lead to Type 2 diabetes mellitus or postprandial blood sugar.

Glucose metabolism is the key to diabetic development; since insulin management is one of the most important aspects of preventing the development of Type 2 diabetes mellitus. When glucose is absorbed into the bloodstream, insulin is produced by the pancreatic beta-cells to keep blood sugar regulated. When too much glucose is absorbed, insulin production must also increase. The increased production of insulin can lead to insulin resistance, and then ultimately beta-cell dysfunction as the beta-cells are overworked (Ceriello, 2000).

Fruit juices can also inhibit the angiotensin converting enzyme (ACE), which can cause oxidative stress and hypertension when produced in abundance by the body. Hypertension is a co-factor for the development of Type 2 diabetes mellitus, and is often seen as a comorbid disease due to the increased chances of developing one of the conditions when the person already has the other. One way of developing hypertension is through the renin-angiotensin-aldosterone pathway which occurs naturally to regulate blood pressure. The angiotensin converting enzyme in the body converts angiotensin I, a harmless compound, to angiotensin II which increases blood pressure and can lead to hypertension. Managing this enzyme can therefore help alleviate hypertension (DeFronzo, 1999) and associated problem of type 2 diabetes.

Bartlett pear and Kiowa blackberries have been studied previously and have been shown to inhibit enzyme activity related to glucose metabolism (Warner, 2012). As a support to the preservation aspect of lactic acid fermentation, another benefit that was examined is the change in total soluble phenolic content over time. Phenolic compounds inhibit the key dietary metabolism regulating enzymes and are

also partly responsible for the antioxidant properties of the fruit juices. The preservation of stability of phenolic activity over time would increase the shelf life of a product with regard to the nutritional properties it exhibits. This preservation technique could also be utilized to repurpose fruits that may not be consumed before their expiration, which would increase economic value and decrease losses due to spoilage.

Two different fruit juices were examined in three different combinations: 100% Bartlett pear, 100% Kiowa blackberry, and a combination of the two at a ratio of 70:30 pear:blackberry. All three varieties of juice were tested in the exact same manner: total soluble phenolic content, 1, 1-Diphenyl-2-Picrylhydrazyl (DPPH) radical inhibition assay, and for the inhibition of: alpha-amylase, alpha-glucosidase and angiotensin converting enzyme. These juices were fermented for 48 hours total and examined at the zero hour, 24 hours and 48 hours for changes in bioactive attributes. The juices were also tested as pH adjusted to 6 (with inoculum) and as a control with no inoculation in tandem. Juices will also be plated after the main experiment on a bacterial lawn of *Helicobacter pylori* in order to perform an agar diffusion assay to determine if any fermented or non-fermented juice has the ability to inhibit the growth of this pathogenic organism. Inhibition of *H. pylori* is a secondary objective in the study, but positive results would prove to be an additional boon to the already nutritionally beneficial fruit juices.

4.3: Materials and Methods

The enzymes, α-glucosidase from yeast *S. cerevisiae* (EC 3.2.1.20), porcine pancreatic α-amylase (EC 3.2.1.1) and angiotensin-1-converting enzyme from rabbit lung (EC 3.4.15.1) were purchased from Sigma Chemical Co. (St. Louis, MO). Unless noted, all chemicals were also purchased from Sigma Chemical Co. (St. Louis, MO).

The Bartlett pear was sourced from a local Big Y supermarket (Hadley, MA), while the Kiowa Blackberries were from the Auburn University (Auburn, Alabama) and the cultivar developed by Arkansas Agricultural Experiment Station breeding program.

4.3.1: Sample Preparation

Fresh Bartlett pears were homogenized using a Waring blender for 3 min and subsequently the supernatant of the pear was collected following centrifugation at 15,000g for 15 min. Thawed blackberry fruit was homogenized for 3 min using a Waring blender and then centrifuged two times at 15,000g for 15 min each to produce a clear juice. Supernatants were collected and stored at -20 °C during the period of study. The pear and blackberry supernatants were either used as 100% pure samples, or combined at a ratio of 70/30. Pear and blackberry juice alone were used as controls. Some combinations and controls were prepared in the adjusted pH 6 (with several drops of 0.1N NaOH) while some were used with natural acidic pH conditions. The juices were stored at 4 °C during their analysis.

4.3.2: Bacterial Strains and Other Materials

Strains of microorganisms used in this study were *L. helveticus* R0052 that was supplied by Rosell Institute Inc., Montreal, Canada (Lot# XA 0145, Seq# 00014160) and *H. pylori* ATCC 43579 of human gastric origin that was provided by American Type Culture Collection (Rockville, MD).).

4.3.3: Fermentation with *Lactobacillus helveticus* R0052

Initially, 100 μL of *L. helveticus* R0052 frozen stock were inoculated into 10 mL of MRS broth (Difco) and incubated at 37 ˚C for 16 h. A hundred microliter of the overnight grown strain were sub cultured to 10 mL of MRS broth, incubated at 37 ˚C

for 16 h and used as an inoculum. An inoculum size of 10% (v/v) was added aseptically to the 100% Bartlett pear juice, 100% Kiowa blackberry juice, or the 70:30 P:BB combination to achieve a total volume of 90 mL in a 125-mL sterile Erlenmeyer flask. Initial pH was measured and adjusted to 6 using 1N NaOH at 0h fermentation. Fermentation was performed at 37 ˚C and 13 mL of samples were taken out at 0, 24 and 48 h. At every time point, the inoculated sample was placed into 2 different tubes. Prior to any assays, one tube was adjusted to pH 6 using 1N NaOH, while the pH of another tube was not adjusted (labeled as fermented acidic pH) and added with distilled water to keep the volume same. All samples were then centrifuged at 15,000g for 15 min and used for the assays.

4.3.4: Absorbance of Samples and Colony Counts

These measurements were done before the final pH treatment. Turbidity of the fermented samples at every time point as an indicator of bacterial growth was estimated using absorbance at 600 nm. More accurate estimation of living cells were made by plate count technique and expressed as CFU/mL. At 0, 24 and 48 h, 100 µL of the appropriate dilution were plated on MRS Agar and incubated at 37 ˚C for 24 h. Individual colonies were counted in the valid range of 25-250 to determine the CFU/mL of each sample.

4.3.5: Total Phenolics Assay

Total phenolic content of pear, blackberry, and pear/blackberry juice combinations were determined using an assay modified by Shetty et al. (1995). A volume of 500 microliters of sample and 500 microliters of distilled water were transferred into a test tube and added with 1 mL of 95% ethanol, 5 mL of distilled water and 0.5 mL of 50% (v/v) Folin-Ciocalteau reagent, respectively. The mixture was left to incubate for 5 min, followed by the addition of 1 mL of 5% Na_2CO_3. After

thorough mixing, the reaction mixture was incubated in the dark for 60 min and the absorbance was read at 725 nm after this time had elapsed. Standard curves were generated using increasing concentrations of gallic acid in 95% ethanol. Absorbance values were converted to total phenolics and expressed as mg of gallic acid equivalent (GAE) per mL volume of the pear and blackberry combination.

4.3.6: Antioxidant Activity by 1, 1-Diphenyl-2-Picrylhydrazyl (DPPH) Radical Inhibition Assay

Antioxidant activities of pear and blackberry juices and their 70/30 combination were measured using a modified DPPH radical inhibition assay (Cervato et al., 2000). In a microcentrifuge tube, a volume of 0.25 mL sample mixture was added to 1.25 mL of 60 µM DPPH in 95% ethanol. During 5 min of incubation, the samples were vortexed and then centrifuged at 15,000g for 1 min. The absorbance (*A*) was read at 517 nm. As a control, 0.25 mL of 95% ethanol was used instead of a sample mixture. The antioxidant activity was expressed as % inhibition of DPPH radical formation and calculated using the following formula:

$$\%\ Inhibition = \frac{(A_{517}\ control - A_{517}\ sample)}{A_{517}\ control}\ x\ 100$$

4.3.7: α-Glucosidase Inhibition Assay

The α-glucosidase inhibition assay was performed by mixing 50 µL of sample and 100 µL of 0.1 M phosphate buffer containing (pH 6.9) containing α-glucosidase enzyme solution (1.0 U/mL) in 96-well plates. The mixture solutions were incubated at 25 °C for 10 min. After incubation, 50 µL of 5 mM p-nitrophenyl-α-D-glucopyranoside solution in 0.1 M phosphate buffer (pH 6.9) was added to each well at timed intervals. The reaction mixtures were incubated at 25 °C for 5 min. Before and after that 5 min incubation, absorbance (*A*) reading were recorded at 405 nm by

micro plate reader (Thermomax, Molecular Device Co., VA, USA) and the difference between 0 and 5 min readings were noted as ΔA. For the control, 50 µL of buffer solution was added instead of sample. The result was expressed as % inhibition of α-glucosidase and calculated using the following formula:

$$\%\ Inhibition = \frac{(A_{517}\ control - A_{517}\ sample)}{A_{517}\ control}\ x\ 100$$

4.3.8: α-Amylase Inhibition Assay

Two doses were tested in this assay, a total volume of 500 µL comprised of 100 µL of sample and 400 µL of distilled water (1/5 concentration) or 50 µL of sample combined with 450 µL of distilled water (1/10 concentration). The water and sample mixtures were combined with 500 µL of 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M sodium chloride) containing α-amylase enzyme solution (0.5 mg/mL) were incubated at 25 °C for 10 min. After incubation, 500 µL of 1% (w/v) starch solution in 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M sodium chloride) was added to each tube at timed intervals and then incubated at 25 °C for 10 min. The reaction was stopped with the addition of 1.0 mL of dinitrosalicylic acid color reagent. The test tubes were incubated in a boiling water bath for 10 min and then cooled down to room temperature. The reaction mixture was then diluted with 10 mL of distilled water and the absorbance (A) was read at 540 nm. The result was expressed as % inhibition of α-amylase and calculated using the following formula:

$$\%\ Inhibition = \frac{(A_{517}\ control - A_{517}\ sample)}{A_{517}\ control}\ x\ 100$$

4.3.9: Angiotensin-1- Converting Enzyme (ACE) Inhibition Assay

The ACE inhibition activity was measured using a modified Cushman and Cheung (1971) method. A volume of 50 µL of sample was incubated with 200 µL of 0.1 M NaCl-borate buffer (pH 8.3) containing 2.0 mU ACE-I solution at 25 °C for 10 min. After incubation, 100 µL of 5 mM substrate solution (hippuryl-histidine-leucine, HHL) was added. The reaction mixture was incubated at 37 °C for an hour. The reaction was then stopped with the addition of 150 µL of 0.5 N HCl. The product of ACE reaction, hippuric acid, was detected and quantified using HPLC.

Five microliters of sample was injected using Agilent ALS 1100 autosampler into an Agilent 1100 series HPLC (Agilent Technologies, Palo Alto, CA) equipped with DAD 1100 diode array detector. The solvents used for gradient elution were (A) 10 mM phosphoric acid (pH 2.5) and (B) 100% methanol. The methanol concentration was increased to 60% for the first 8 min and to 100% for the next 5 min, then decreased to 0% for the last 5 min (total run time is 18 min). The analytical column used was Nucleosil 100-5C18, 250x4.6 mm i.d., with packing material of 5 µm particle size at a flow rate 1 mL/min at ambient temperature. During each run, the chromatogram was recorded at 228 nm and integrated using Agilent Chemstation enhanced integrator for detection of liberated hippuric acid. The peak area of hippuric acid (*E*) chromatogram was noted. Pure hippuric acid was used to calibrate the standard curve and retention time. The result was expressed as % inhibition of ACE and calculated using the following formula:

$$\%\ Inhibition = \frac{(A_{517}\ control - A_{517}\ sample)}{A_{517}\ control}\ x\ 100$$

4.3.10: HPLC Analysis of Phenolic Profiles

Two ml of pear, blackberry or the 70/30 juice mixture was filtered through a 0.2 μm filter. A volume of 5 μL sample was injected using Agilent ALS 1100 autosampler into an Agilent 1100 series HPLC (Agilent Technologies, Palo Alto, CA) equipped with DAD 1100 diode array detector. The solvents used for gradient elution were (A) 10 mM phosphoric acid (pH 2.5) and (B) 100% methanol. The methanol concentration was increased to 60% for the first 8 min and to 100% over the next 7 min, then decreased to 0% for the next 3 min and was maintained for the next 7 min (total run time is 25 min). The analytical column used was Agilent Zorbax SB-C18, 250x4.6 mm i.d., with packing material of 5 μm particle size at a flow rate of 1 mL/min at ambient temperature. During each run, the chromatogram was recorded at 225 nm and 306 nm and integrated using Agilent Chemstation enhanced integrator. Pure standards of gallic acid, protocatechuic acid, chlorogenic acid, caffeic acid, resveratrol, rutin, p-coumaric acid, m-coumaric acid and rosmarinic acid in 100% methanol were used to calibrate the standard curves and retention times.

4.3.11 Preparation of *H. pylori* Culture

Culture of *H. pylori* was grown according to Stevenson et al. (2000). Standard plating medium was composed of 10 g of special peptone (Oxoid Ltd, Basing-Stoke, England), 15 g of granulated agar (Difco Laboratories, Becton, Dickinson and Co., Sparks, MD, USA), 5 g of sodium chloride (EM Science, Gibbstown, NJ, USA), 5 g of yeast extract (Difco) and 5 g of beef extract (Difco) per liter of water. Broth media were consisted of 10 g of special peptone (Oxoid Ltd) per liter, 5 g of sodium chloride (EM Science) per liter, 5 g of yeast extract (Difco) per liter and 5 g of beef extract (Difco) per liter of water. One milliliter of *H. pylori* stock culture was inoculated to 10 mL of sterile broth medium and incubated at 37 ˚C for 24 h. The

active culture was then spread on *H. pylori* standard plating agar plates to make bacterial lawn for the agar-diffusion assay.

4.3.12: Agar-Diffusion Assay

The antimicrobial activity of the fermented sample extracts on *H. pylori* was analyzed by agar-diffusion method. Sterile 12.7 mm diameter paper disks (Schleicher & Schuell, Inc., Keene, NH) were placed on the surface of seeded agar plates. The test extracts were sterilized using 0.22 μm Milipore filter membrane (Fisher Scientific, Pittsburgh, PA). One hundred microliters of test extracts were aseptically added onto the paper disks. Distilled water was used as control. Treated plates were incubated at 37 ˚C for 48 h in BBL GasPak jars (Becton, Dickinson and Co., Sparks, MD) with BD GasPak Campy container system sachets (Becton, Dickinson and Co., Sparks, MD). Each experiment was repeated twice and consisted of triplicates (3 disks per sample or treatment in 1 plate). Diameter (D) of clear zone surrounding each disk was measured (in millimeter). The result was expressed as an index of inhibition using the following formula.

4.3.13: Proline Growth Response Assay

The inhibition mechanism of *H. pylori* mediated by phenolic phytochemicals was proposed by Shetty and Wahlqvist (2004). Bacterial lawns of *H. pylori* were prepared as described previously. The standard plating medium was modified by the addition of Proline to a final concentration of 5 mM. A similar protocol as mentioned in the agar-diffusion assay was followed.

4.3.14: Statistical Analysis

All experiments were performed in triplicate and repeated three times each. Means and standard errors were calculated from the replicates within the experiments and analyzed using Microsoft Excel XP. Significant differences were determined using one way ANOVA, then least significant difference test at $p < 0.05$.

For all assays except the phenolic profiles, the results were compared using a basic mathematical calculation. The results generated by various formulas were dependent on the combination ratios of 100% Kiowa blackberry juice and 100% Bartlett pear juice.

4.4: Results and Discussion

4.4.1: Total Soluble Phenolics

At 100% juice, Kiowa blackberries had the highest total soluble phenolic (TSP) content, and Bartlett pears had the least (Figure 1). While the blackberry control had the highest average findings for TSP, they quickly dissipated over 48 hours. Adjustment to a pH of 6 lowered the overall stability of phenols in solution, while fermentation by lactic acid bacteria increased this stability. The 70:30 combinations showed good phenolic content while also proving to be very stable over time, as opposed to blackberries. The change for the pH adjusted samples increased or decreased over 48 hours, but not to the same extent as the unmodified fermented samples. The lessened changes held true between all pH and non-pH adjusted samples for all substrates. Overall, the pH adjustment lessens the availability of the measurable bioactive phenolic compounds within these fruit samples.

As seen in Figure 1, the amount of soluble phenolics increased steadily as the fermentation time increased in pear. These phenolic compounds are very versatile in their abilities to inhibit metabolic enzymes and quench oxidative species; an increase in phenols is directly proportional to an increase in health benefits for the fruit juices.

Blackberries are naturally high in phenolic compounds, the bacteria decrease the available soluble phenols at 0 hours most likely due to immediate oxidation managing reactions that they create, which would quench the antioxidants. Over time, the amount and availability of antioxidants becomes more stable than the control which is likely due to a combination of pH changes and bacterial metabolic effect.

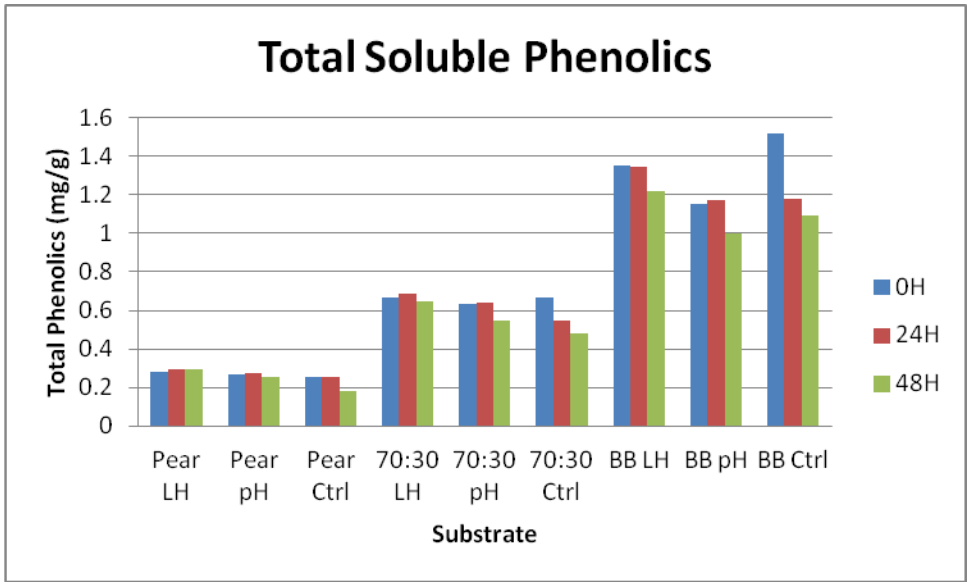

Figure 1: TSP reading for 100% Pear, 70:30 Pear:Blackberry, and 100% Blackberry samples. Blackberry shows the highest amount of soluble phenolic compounds, pear shows the least.

4.4.2: DPPH

Antioxidant activity was high for every combination of fruits, but the fermented 70:30 pear:blackberry combination showed the highest results for the DPPH assay (Figure 2). Fermentation almost always increased the ability of the fruits to improve antioxidant effects. As shown in Figure 2, the control of the 48 hours pear sample far exceeded the 48 hour fermented and pH adjusted samples. When pH was adjusted, it usually lowered the ability of the fermented juice to

function in regards to the antioxidants, with the exception of the 48 hour blackberry sample. This would indicate that modified pH plays an important role in the ability of these enzymes to function.

Antioxidant activities always increased with the addition of lactic acid bacteria. The exact amount of time that it takes to reach peak inhibition changes with each different fruit juice, but the trend upwards with the addition of LAB is consistent. Also of note is the obvious increase of antioxidant activity of the pear and 70:30 control samples over time, where in the blackberry it decreased. Reasons for these changes are likely due to pH modifications and the addition of lactic acid to the juices. Lower pH seems to increase the ability of blackberry phenolics to function against DPPH while hindering the pear phenolic compounds. Even without fermentation, the antioxidant capacity of the control sample increased when pear juice was included in the sample. Prior experiments in our laboratory have led to similar conclusions in respect to the general ability of these fruits to all inhibit DPPH at a moderate to high level. 70:30 pear:blackberry was shown to have improved results over the pure 100% pear and 100% blackberry samples in previous research as well (Warner, 2010).

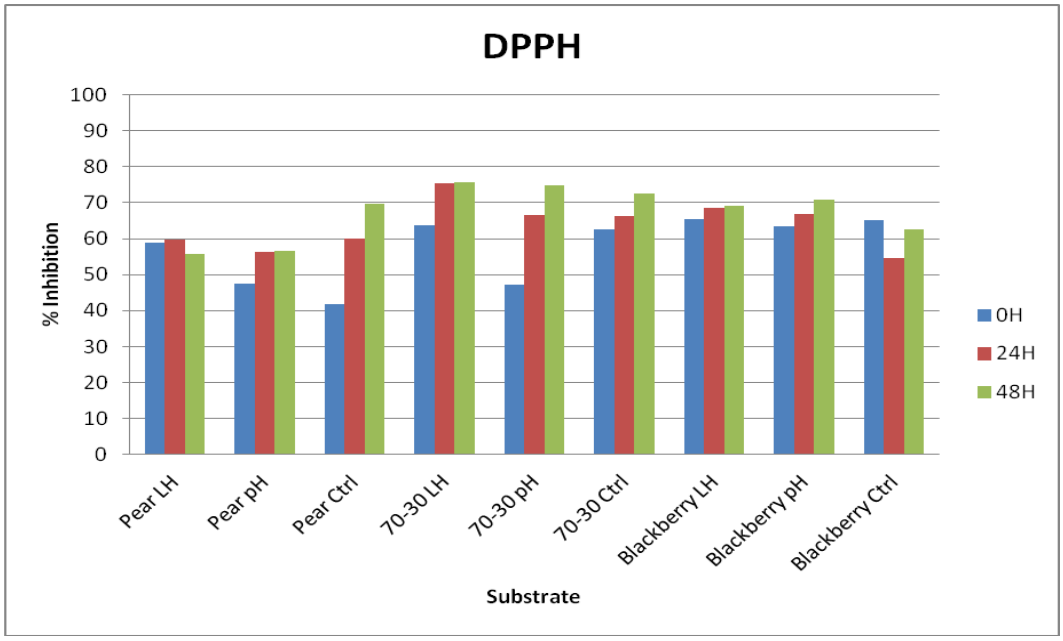

Figure 2: DPPH inhibition results, showing high inhibition for all fruit substrates. 70:30 had the highest results, followed by 100% blackberry. 100% Pear had the lowest readings for DPPH inhibition.

4.4.3: Alpha-Glucosidase

Initially, pear had the highest alpha-glucosidase activity; but when fermented for 24 or 48 hours, the blackberries quickly surpass pear (Figure 3). Pear also exhibited the greatest decrease of activity in regards to dose-response. Blackberry exhibits excellent inhibition when undiluted, but dose-response can quickly drop when dilution occurs. Fermented 70:30 had the highest stability, especially under dilution, after 48 hours. This could be attributed to a synergistic relationship between compounds within pears and blackberries, or perhaps just a more successful fermentation within regard to alpha-glucosidase when there are multiple types of fruit substrates.

Fermentation over longer periods of time benefits the 70:30 combination and 100% blackberry juice the most. Blackberries could therefore be postulated to have

compounds that increase the bioactive benefit of alpha-glucosidase inhibition when exposed to lactic acid fermentation; whereas the initially high alpha-glucosidase inhibition found in pear juice was consumed by the bacteria. Previous studies have had similar results with regard to pH and fermentation time versus inhibition (Augustinah, 2012).

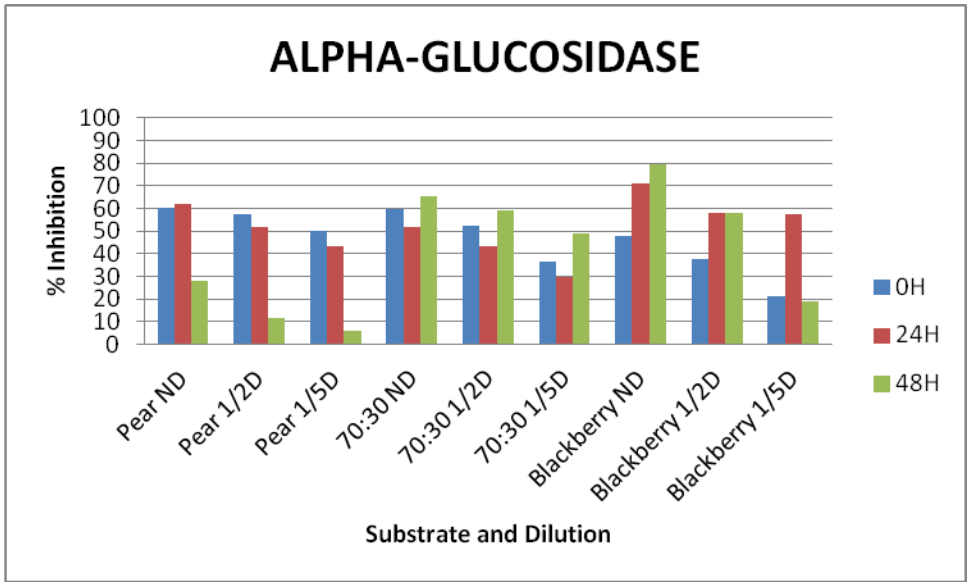

Figure 3: Alpha-glucosidase inhibition for the substrates with fermentation. Samples were undiluted (ND), 50% diluted (1/2D) and diluted to 1/5 concentration (1/5D). The inhibition usually peaked at 48 hours, with exception of the pear samples and the highest diluted blackberry sample.

4.4.4: Alpha-Amylase

Pear exhibited complete inhibition of alpha-amylase when fermented, while blackberry exhibited relatively little in any circumstance (Figure 4). A 70:30 combination was balanced between the two extremes, with good results under

fermentation and poor effects when pH adjusted. When blackberry was fermented, it seems to lose inhibitory effects in some cases. Alpha-amylase inhibition was lowered in almost all results when the pH is adjusted to 6.0.

Inhibition of alpha-amylase peaks at the 24 hour timeframe of fermentation for 100% pear juice, and at 48 hour timeframe for 100% blackberry juice. The 70:30 pear:blackberry juice has a more interesting profile, as it peaks at 48 hours but only when left at a lower dilution. At a highly diluted state, the 48 hour fermentation shows little to no activity, unless pH adjusted. Similar results were found in previous results for pear having a prolific ability to inhibit alpha-amylase under all circumstances, while blackberry had relatively very little ability.

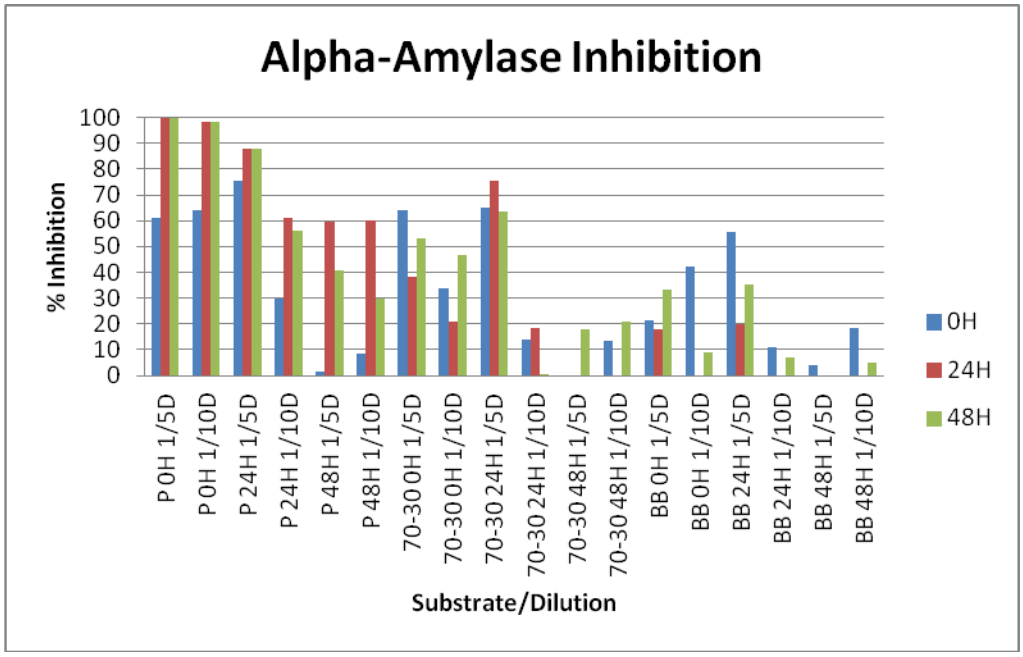

Figure 4: Alpha-amylase inhibition by fermented and non-fermented fruit juices. Substrate was diluted to 1/5 (1/5D) and 1/10 (1/10D) concentrations. Pear showed the highest inhibition of alpha-amylase, followed by the 70:30 pear: blackberry combination. Blackberry showed poor inhibition overall.

4.4.5: Angiotensin Converting Enzyme

ACE inhibition was best in pears, and almost negligible in blackberry: hence the reason blackberries were omitted entirely from Figure 5. Fermentation increased ACE inhibition in all cases except for diluted 70:30. A pH adjustment lowered ACE inhibition in all cases and diluted pH adjusted and fermented 70:30 showed no inhibitory effects.

The distinction between fermented non-pH adjusted and fermented pH adjusted was smaller than the difference between fermented and non-fermented fruit juices. Even with modification to reduce acidity, the fermentation still showed benefits for ACE inhibition when compared to the control samples.

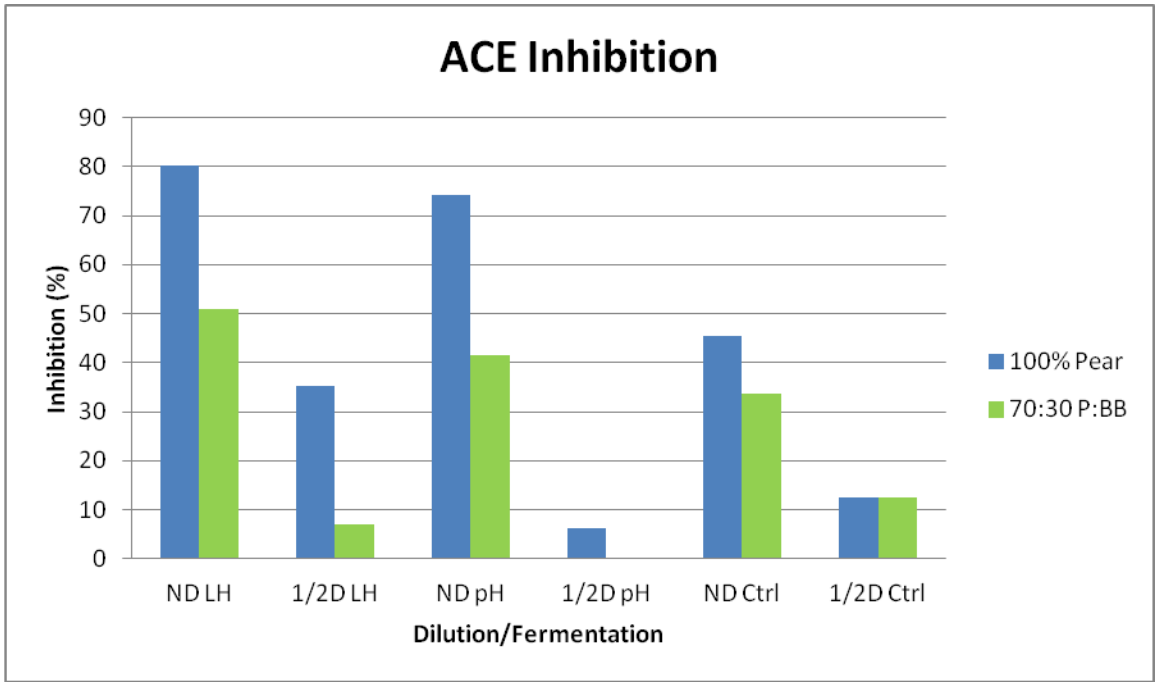

Figure 5: ACE inhibition using 100% pear and 70:30 pear:blackberry substrates. 100% blackberry was omitted due to negligible inhibition levels. Samples were undiluted (ND), or diluted to 50% (1/2D). 100% pear shows high inhibition, especially after fermentation.

4.4.6: *H. pylori*

Helicobacter pylori was only inhibited by fermented 100% blackberry (Figure 6). No other sample inhibited the growth of *H. pylori* whatsoever. Longer fermentation caused greater results by zone of inhibition testing.

The reason behind this inhibition is still not clear. pH was not the suspected cause, as all samples were pH adjusted prior to plating. Most phenolic compounds prevent bacteria from utilizing proline dehydrogenase to obtain proline for energy needs; to understand this likely metabolism strategy, *H. pylori* was grown on plates containing proline to supplement the bacteria. Even on these plates, the bacteria remained inhibited solely by fermented blackberry juice. The 70:30 combination juices also had very little effect on *H. pylori*, even though it contained 30% blackberry juice. A compound in the Bartlett pear juice may be preventing the Kiowa blackberry juice from functioning as an inhibitor.

Figure 6: A lawn plate of H, pylori with four sterile filter papers placed on top. These papers were infused with 48 hour fermented blackberry juice. The area

of inhibition around the three infused papers shows clear inhibition of growth, while the control (bottom left) shows none.

4.5: Conclusion

Fermentation increases the stability of phenolic compounds and their functionality in pear and blackberry juices, as well as the 70:30 P:BB combination. This was most likely caused by the production of compounds due to bacterial growth and pH changes due to the formation of lactic acid.

The 70:30 P:BB combination showed promising results in improving glucose metabolism and stability, especially after fermentation. Stability was increased in every measured assay that was tested in this study. Alpha-amylase, alpha-glucosidase, ACE, soluble phenolics, and antioxidant capacity were all stable with the ratio of 70 % Bartlett pear and 30% Kiowa blackberry juices after fermentation for 48 hours.

A 100% blackberry juice inhibited the proliferation of *H. pylori* on an agar plate when fermented by *L. helveticus.* This is postulated to be due to some form of protonation effect that is caused by *L. helveticus* when grown in blackberries. Pear juice does not exhibit the same effects, and the 70:30 pear:blackberry mixture likewise does not have any ability of halt the growth of *H. pylori*; even though there is blackberry juice in the combination. Varying levels of proline was used in additional plates to test if the amino acid would affect the ability of *H. pylori* to be inhibited by phenolics, but it did not alter the results whatsoever. Proline dehydrogenase inhibition is therefore ruled out as the reason for the inability of *H. pylori* to grow around 100% blackberry juice fermented with *L. helveticus.*

CHAPTER 5

IMPROVMENT OF FUNCTIONAL BIOACTIVITY IN PEAR:BLACKBERRY SYNERGIES FOR TYPE 2 DIABETES AND HYPERTENSION UTILIZING *BIFIDOBACTERIUM LONGUM* FERMENTATION

5.1: Abstract

In this study, *Bifidobacterium longum* was utilized to ferment juices sourced from Bartlett pear and Kiowa blackberries. *Bifidobacterium longum* is a known probiotic, and should also potentially increase the phenolic content and related bioactivities of the fruit juices being evaluated for health benefits. This study is a continuation of the previous investigation which utilized *Lactobacillus helveticus*, another lactic acid producing bacterium. Evaluating the same fruit juices will allow for the comparison of benefits between similar organisms but with overall differnet metabolic capabilities as *Bifidobacterium* is more oxygen sensitive. The secondary goal of the experiment is to validate if any of these fruit juices, when fermented with *B. longum,* can inhibit the growth of *Helicobacter pylori*; which is a common bacterium found in the gut that can cause ulceration of the stomach and duodenum and can subsequently lead to potential cancer development in the aforementioned organs.

Over the course of the study, results showed that the 70:30 combinations had good synergy, the highest content of soluble phenolics, higher antioxidant activity, and the fermentation with *B. longum* leading to higher phenolic activity retention over time. Fermentation increased the potency of phenolic compounds in regards to their ability to inhibit certain key digestive enzymes associates with type 2 diabetes

and demonstrated an increased stability of the phenolic bioactives. A 100% Kiowa blackberry juice was shown to inhibit the growth of *H. pylori,* but only after fermentation. The control showed no inhibition whatsoever, nor did any 70:30 combinations or 100% Bartlett pear show any inhibition of the bacterium either. The 70:30 combinations are the most cost-effective way to reap the greatest benefits of the fruits studied. Fermentation with *B. longum* increases this cost-effectiveness by adding shelf life and additional health benefits.

5.2: Introduction

Bifidobacterium longum is a bacterium commonly used as probiotics in cultured yogurts along with other lactic acid bacterial fermentation such as *Lactobacillus bulgaricus*. In this study *Bifidobacterium longum* was utilized in order to compare phenolic content and associate bioactive capabilities of a fermented juice from Bartlett pear and Kiowa blackberry to their non-fermented counterparts. The aim is to increase the ability of the juice to inhibit the alpha-amylase, alpha-glucosidase and angiotensin converting enzymes while also evaluating if the antioxidant functions are affected by the fermentation. This experiment was conducted in order to examine the bioavailability and stability of phenolic compounds that are naturally found in fruits when fermentation is involved. These phenolic compounds are important in the inhibition of key metabolic enzymes that break down carbohydrates within the digestive system. The reasoning behind inhibiting carbohydrate metabolizing enzymes is to slow the absorption of sugar into the body from digestion to reduce insulin shock and other metabolic damage to counter the development of Type 2 diabetes mellitus in humans. Refined sugars are quickly absorbed into the blood, causing a spike in blood glucose. These spikes in glucose can cause insulin resistance in cells over time, which is a factor in

developing Type 2 diabetes mellitus. Altering and slowing glucose metabolism is a key way to avoid these sugar spikes in the blood by slowing their uptake rate in the intestines.

When glucose is absorbed into the bloodstream, insulin is produced by the pancreatic beta-cells to keep blood sugar regulated. When too much glucose is absorbed, insulin production must also increase. If the pancreas is overworked for too long of a period, cellular damage can occur in the organ. The body can also become resistant to insulin in the same way that it acclimates to any change in atmosphere. This acclimation to the sustained production of insulin can lead to insulin resistance, and then ultimately beta-cell dysfunction as the beta-cells of the pancreas are overworked even further (Ceriello, 2000).

Phenolic compounds within fruits can also inhibit the angiotensin converting enzyme, which can cause oxidative stress and hypertension. Hypertension is a co-factor for the development of Type 2 diabetes mellitus, and is seen as a co-morbid disease due to the increased chance of developing one of the conditions when the person already has the other. One way of developing hypertension is through the renin-angiotensin-aldosterone pathway which occurs naturally to regulate blood pressure. The angiotensin converting enzyme in the body converts angiotensin I, an inert compound, to angiotensin II which increases blood pressure and can lead to hypertension. Managing ACE in the blood can help alleviate hypertension by stalling the production of angiotensin II (DeFronzo, 1999) .

Bartlett pear and Kiowa blackberries have been studied previously in this laboratory, and have been shown to effectively reduce glucose metabolism enzymes like alpha-amylase and alpha glucosidase using *in vitro* models (Warner, 2012). As a supporting evidence to the historical preservation and stability aspect of lactic acid

fermentation, the change in total soluble phenolic content over time will be monitored to investigate if the biological function of the phenolics present in the juice is preserved along with the juice itself. Phenolic compounds are what is inhibiting the enzymes and are also partly responsible for the antioxidant properties of the fruit juices. The preservation of phenolic activity over time would increase the shelf life of a product with regard to the nutritional properties it exhibits. This preservation technique could also be further utilized to repurpose fruits that would not be consumed before their expiration, which would increase economic profits and decrease losses due to spoilage.

5.3: Materials and Methods

The enzymes, α-glucosidase from yeast *S. cerevisiae*(EC 3.2.1.20), porcine pancreatic α-amylase (EC 3.2.1.1) and angiotensin-1-converting enzyme from rabbit lung (EC 3.4.15.1) were purchased from Sigma Chemical Co. (St. Louis, MO). Unless noted, all chemicals were also purchased from Sigma Chemical Co. (St. Louis, MO).

The Bartlett pear was sourced from a local Big Y supermarket, while the Kiowa Blackberries were from the Arkansas Agricultural Experiment Station breeding program via Auburn University in Alabama.

5.3.1: Sample Preparation

Fresh Bartlett pears were homogenized using a Waring blender for 3 min and subsequently the supernatant of the pear was collected following centrifugation at 15,000g for 15 min. Thawed blackberry fruit was homogenized for 3 min using a Waring blender and then centrifuged two times at 15,000g for 15 min each to produce a clear juice. Supernatants were collected and stored at -20 °C during the period of study. The pear and blackberry supernatants were either used as 100%

pure samples, or combined at a ratio of 70/30. Pear and blackberry juice alone were used as controls. Some combinations and controls were prepared in the adjusted pH 6 (with several drops of 0.1N NaOH) while some were used with natural acidic pH conditions. The juices were stored at 4 °C during their analysis.

5.3.2: Bacterial Strains and Other Materials

Strains of microorganisms used in this study were *Bifidobacterium longum* isolated from a previous study (Apostolidis et al. 2007) and *H. pylori* ATCC 43579 of human gastric origin that was provided by American Type Culture Collection (Rockville, MD

5.3.3: Fermentation with *Bifidobacterium longum*

Initially, 100 µL of *B. longum* frozen stock were inoculated into 10 mL of MRS broth (Difco) and incubated at 37 ˚C for 16 h. A hundred microliter of the overnight grown strain were sub cultured to 10 mL of MRS broth, incubated at 37 ˚C for 16 h and used as an inoculum. An inoculum size of 10% (v/v) was added aseptically to the 100% Bartlett pear juice, 100% Kiowa blackberry juice, or the 70:30 P:BB combination to achieve a total volume of 90 mL in a 125-mL sterile Erlenmeyer flask. Initial pH was measured and adjusted to 6 using 1N NaOH at 0h fermentation. Fermentation was performed at 37 ˚C and 13 mL of samples were taken out at 0, 24 and 48 h. At every time point, the inoculated sample was placed into 2 different tubes. Prior to any assays, one tube was adjusted to pH 6 using 1N NaOH, while the pH of another tube was not adjusted (labeled as fermented acidic pH) and added with distilled water to keep the volume same. All samples were then centrifuged at 15,000g for 15 min and used for the assays.

5.3.4: Absorbance of Samples and Colony Counts

These measurements were done before the final pH treatment. Turbidity of the fermented samples at every time point as an indicator of bacterial growth was estimated using absorbance at 600 nm. More accurate estimation of living cells were made by plate count technique and expressed as CFU/mL. At 0, 24 and 48 h, 100 µL of the appropriate dilution were plated on MRS Agar and incubated at 37 ˚C for 24 h. Individual colonies were counted in the valid range of 25-250 to determine the CFU/mL of each sample.

5.3.5: Total Phenolics Assay

Total phenolic content of pear, blackberry, and pear/blackberry juice combinations were determined using an assay modified by Shetty et al. (1995). 500 microliters of sample and 500 microliters of distilled water were transferred into a test tube and added with 1 mL of 95% ethanol, 5 mL of distilled water and 0.5 mL of 50% (v/v) Folin-Ciocalteau reagent, respectively. The mixture was left to incubate for 5 min, followed by the addition of 1 mL of 5% Na2CO3. After thorough mixing, the reaction mixture was incubated in the dark for 60 min and the absorbance was read at 725 nm after this time had elapsed. Standard curves were generated using increasing concentrations of gallic acid in 95% ethanol. Absorbance values were converted to total phenolics and expressed as mg of gallic acid equivalent (GAE) per mL volume of the pear and blackberry combination.

5.3.6: Antioxidant Activity by 1, 1-Diphenyl-2-Picrylhydrazyl (DPPH) Radical Inhibition Assay

Antioxidant activities of pear and blackberry juices and their 70/30 combination were measured using a modified DPPH radical inhibition assay

(Cervato et al., 2000). In a microcentrifuge tube, a volume of 0.25 mL sample mixture was added to 1.25 mL of 60 µM DPPH in 95% ethanol. During 5 min of incubation, the samples were vortex mixed and then centrifuged at 15,000g for 1 min. The absorbance (*A*) was read at 517 nm. As a control, 0.25 mL of 95% ethanol was used instead of a sample mixture. The antioxidant activity was expressed as % inhibition of DPPH radical formation and calculated using the following formula:

$$\%\ Inhibition = \frac{(A_{517}\ control - A_{517}\ sample)}{A_{517}\ control}\ x\ 100$$

5.3.7: α-Glucosidase Inhibition Assay

The α-glucosidase inhibition assay was performed by mixing 50 µL of sample and 100 µL of 0.1 M phosphate buffer containing (pH 6.9) containing α-glucosidase enzyme solution (1.0 U/mL) in 96-well plates. The mixture solutions were incubated at 25 °C for 10 min. After incubation, 50 µL of 5 mM p-nitrophenyl-α-D-glucopyranoside solution in 0.1 M phosphate buffer (pH 6.9) was added to each well at timed intervals. The reaction mixtures were incubated at 25 °C for 5 min. Before and after that 5 min incubation, absorbance (*A*) reading were recorded at 405 nm by micro plate reader (Thermomax, Molecular Device Co., VA, USA) and the difference between 0 and 5 min readings were noted as Δ*A*. For the control, 50 µL of buffer solution was added instead of sample. The result was expressed as % inhibition of α-glucosidase and calculated using the following formula:

$$\%\ Inhibition = \frac{(A_{517}\ control - A_{517}\ sample)}{A_{517}\ control}\ x\ 100$$

5.3.8: α-Amylase Inhibition Assay

Two doses were tested in this assay, a total volume of 500 µL comprised of 100 µL of sample and 400 µL of distilled water (1/5 concentration) or 50 µL of sample combined with 450 µL of distilled water (1/10 concentration). The water

and sample mixtures were combined with 500 µL of 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M sodium chloride) containing α-amylase enzyme solution (0.5 mg/mL) were incubated at 25 °C for 10 min. After incubation, 500 µL of 1% (w/v) starch solution in 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M sodium chloride) was added to each tube at timed intervals and then incubated at 25 °C for 10 min. The reaction was stopped with the addition of 1.0 mL of dinitrosalicylic acid color reagent. The test tubes were incubated in a boiling water bath for 10 min and then cooled down to room temperature. The reaction mixture was then diluted with 10 mL of distilled water and the absorbance (*A*) was read at 540 nm. The result was expressed as % inhibition of α-amylase and calculated using the following formula:

$$\%\ Inhibition = \frac{(A_{517}\ control - A_{517}\ sample)}{A_{517}\ control}\ x\ 100$$

5.3.9: Angiotensin-1- Converting Enzyme (ACE) Inhibition Assay

The ACE inhibition activity was measured using a modified Cushman and Cheung (1971) method. A volume of 50 µL of sample was incubated with 200 µL of 0.1 M NaCl-borate buffer (pH 8.3) containing 2.0 mU ACE-I solution at 25 °C for 10 min. After incubation, 100 µL of 5 mM substrate solution (hippuryl-histidine-leucine, HHL) was added. The reaction mixture was incubated at 37 °C for an hour. The reaction was then stopped with the addition of 150 µL of 0.5 N HCl. The product of ACE reaction, hippuric acid, was detected and quantified using HPLC.

Five microliters of sample was injected using Agilent ALS 1100 autosampler into an Agilent 1100 series HPLC (Agilent Technologies, Palo Alto, CA) equipped with DAD 1100 diode array detector. The solvents used for gradient elution were (A) 10 mM phosphoric acid (pH 2.5) and (B) 100% methanol. The methanol

concentration was increased to 60% for the first 8 min and to 100% for the next 5 min, then decreased to 0% for the last 5 min (total run time is 18 min). The analytical column used was Nucleosil 100-5C18, 250x4.6 mm i.d., with packing material of 5 µm particle size at a flow rate 1 mL/min at ambient temperature. During each run, the chromatogram was recorded at 228 nm and integrated using Agilent Chemstation enhanced integrator for detection of liberated hippuric acid. The peak area of hippuric acid (*E*) chromatogram was noted. Pure hippuric acid was used to calibrate the standard curve and retention time. The result was expressed as % inhibition of ACE and calculated using the following formula:

$$\%\ Inhibition = \frac{(A_{517}\ control - A_{517}\ sample)}{A_{517}\ control}\ x\ 100$$

5.3.10: HPLC Analysis of Phenolic Profiles

Two ml of pear, blackberry or the 70/30 juice mixture was filtered through a 0.2 µm filter. A volume of 5 µL sample was injected using Agilent ALS 1100 autosampler into an Agilent 1100 series HPLC (Agilent Technologies, Palo Alto, CA) equipped with DAD 1100 diode array detector. The solvents used for gradient elution were (A) 10 mM phosphoric acid (pH 2.5) and (B) 100% methanol. The methanol concentration was increased to 60% for the first 8 min and to 100% over the next 7 min, then decreased to 0% for the next 3 min and was maintained for the next 7 min (total run time is 25 min). The analytical column used was Agilent Zorbax SB-C18, 250x4.6 mm i.d., with packing material of 5 µm particle size at a flow rate of 1 mL/min at ambient temperature. During each run, the chromatogram was recorded at 225 nm and 306 nm and integrated using Agilent Chemstation enhanced integrator. Pure standards of gallic acid, protocatechuic acid, chlorogenic acid, caffeic acid, resveratrol, rutin, p-coumaric acid, m-coumaric acid and rosmarinic acid in 100% methanol were used to calibrate the standard curves and retention times.

5.3.11 Preparation of *H. pylori* Culture

Culture of *H. pylori* was grown according to Stevenson et al. (2000). Standard plating medium was composed of 10 g of special peptone (Oxoid Ltd, Basing-Stoke, England), 15 g of granulated agar (Difco Laboratories, Becton, Dickinson and Co., Sparks, MD, USA), 5 g of sodium chloride (EM Science, Gibbstown, NJ, USA), 5 g of yeast extract (Difco) and 5 g of beef extract (Difco) per liter of water. Broth media were consisted of 10 g of special peptone (Oxoid Ltd) per liter, 5 g of sodium chloride (EM Science) per liter, 5 g of yeast extract (Difco) per liter and 5 g of beef extract (Difco) per liter of water. One milliliter of *H. pylori* stock culture was inoculated to 10 mL of sterile broth medium and incubated at 37 ˚C for 24 h. The active culture was then spread on *H. pylori* standard plating agar plates to make bacterial lawn for the agar-diffusion assay.

5.3.12: Agar-Diffusion Assay

The antimicrobial activity of the fermented sample extracts on *H. pylori* was analyzed by agar-diffusion method. Sterile 12.7 mm diameter paper disks (Schleicher & Schuell, Inc., Keene, NH) were placed on the surface of seeded agar plates. The test extracts were sterilized using 0.22 µm Milipore filter membrane (Fisher Scientific, Pittsburgh, PA). One hundred microliters of test extracts were aseptically added onto the paper disks. Distilled water was used as control. Treated plates were incubated at 37 ˚C for 48 h in BBL GasPak jars (Becton, Dickinson and Co., Sparks, MD) with BD GasPak Campy container system sachets (Becton, Dickinson and Co., Sparks, MD). Each experiment was repeated twice and consisted of triplicates (3 disks per sample or treatment in 1 plate). Diameter (D) of clear zone surrounding each disk was measured (in millimeter). The result was expressed as an index of inhibition using the following formula.

5.3.13: Proline Growth Response Assay

The inhibition mechanism of *H. pylori* mediated by phenolic phytochemicals was proposed by Shetty and Wahlqvist (2004). Bacterial lawns of *H. pylori* were prepared as described previously. The standard plating medium was modified by the addition of Proline to a final concentration of 5 mM. A similar protocol as mentioned in the agar-diffusion assay was followed.

5.3.14: Statistical Analysis

All experiments were performed in triplicate and repeated three times each. Means and standard errors were calculated from the replicates within the experiments and analyzed using Microsoft Excel XP. Significant differences were determined using one way ANOVA, then least significant difference test at $p < 0.05$.

For all assays except the phenolic profiles, the results were compared using a basic mathematical calculation. The results generated by various formulas were dependent on the combination ratios of 100% Kiowa blackberry juice and 100% Bartlett pear juice.

5.4 Results and Discussion

5.4.1: Total Soluble Phenolics

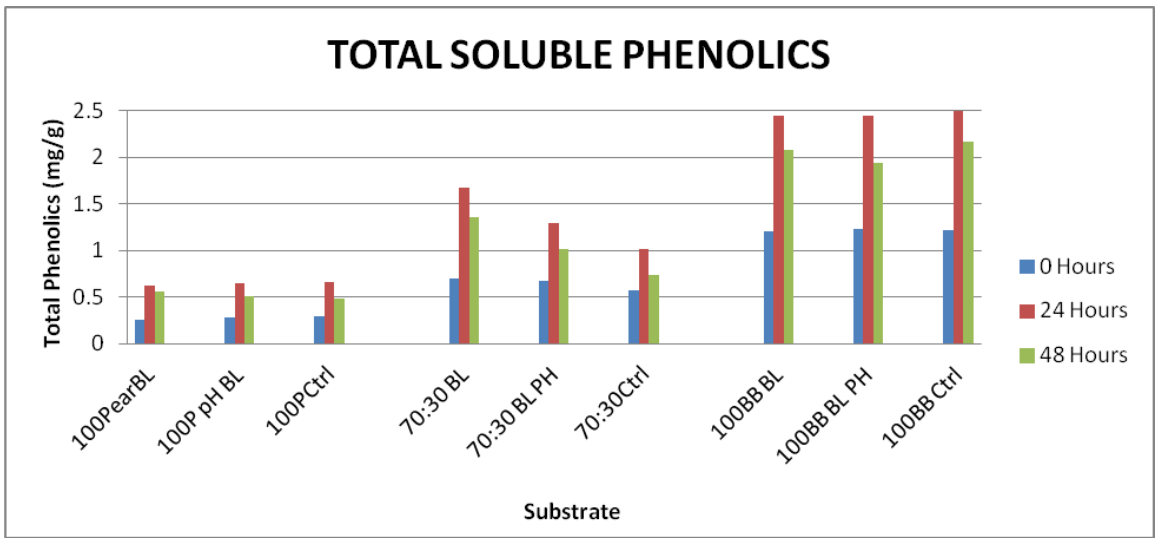

Figure 7: Total soluble phenolics for B. longum fermented substrate and control substrate. 100% pear shows lower levels than blackberry overall. 24 hours after blending shows the best results.

Phenolic contents of some fruit juices increased substantially with fermentation by *B. longum.* In this case, the 24 hour samples appear to exhibit the highest amount of soluble phenolic compounds in both fermented and non-fermented juices. It could be assumed that after 24 hours the bacteria begin to degrade the phenols with lactic acid or other metabolic byproducts along with light and heat degradation. Pear juice naturally contained the least phenolic compounds, while blackberry had the most. A 70:30 pear:blackberry combination was naturally between the two, but had a very good bioactive potential when fermented with *B. longum*. A 70:30 combination had the highest increase of total soluble phenolics after fermentation for 24 hours, without pH adjustment the amount of soluble phenolics more than doubled. The ability of *B. longum* to affect total soluble phenolics in fruit juices should be investigated further. Evaluating after holding the

juices for 72 hours at 37 ° C may yield interesting results on phenolic longevity and associated shelf life of overall bioactive potential.

5.4.2: DPPH

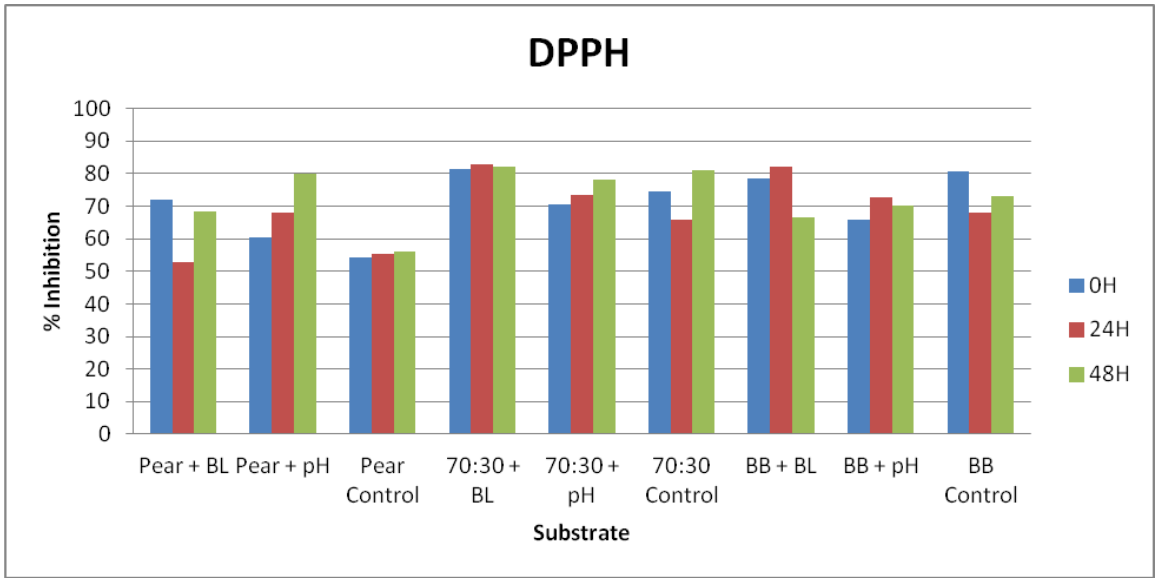

Figure 8: DPPH inhibition assay for the substrates. Samples were fermented with B. longum (BL), fermented and also modified to a steady pH of 6.2 (pH) and left unfermented (Control). Inhibition was measured at 0, 24 and 48 hours. Strong inhibition overall, with 70:30 pear: blackberry showing the highest overall results.

Juices evaluated by the DPPH radical scavenging oxidative stress test showed good results in all assays at all times. The lowest amount of inhibition was around 55% by the pear control juices; while the highest results were from 70:30 fermented with *B. longum* which were measured upwards of 80% inhibition. A 70:30 pear:blackberry juice had the best indicators in almost all aspects of free radical quenching. Pear juice seemed to be affected the most by fermentation, with good increases in radical quenching ability when compared to the control.

Blackberry had relatively good bioactive potential with and without fermentation by *B. longum.*

5.4.3: Alpha-Glucosidase

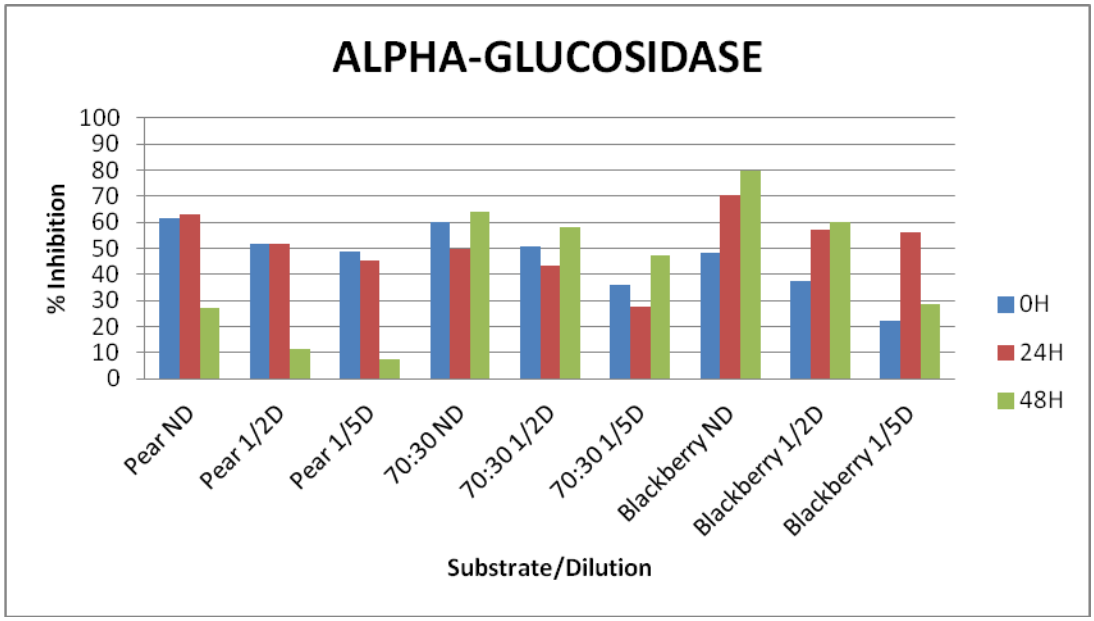

Figure 9: Alpha-glucosidase inhibition for the substrates with fermentation. Samples were undiluted (ND), 50% diluted (1/2D) and diluted to 1/5 concentration (1/5D). The inhibition usually peaked at 48 hours, with exception of the pear samples and the highest diluted blackberry sample.

Alpha-glucosidase inhibition was highest in 48 hour blackberry, while being relatively weak in fermented pear juice. Fermentation increased the alpha-glucosidase inhibitory effects of the plant phenolics tested in this experiment with the exception of pear juice. Pear juice actually seemed to decrease in inhibitory ability when left to ferment over time. At 48 hours time, the strongest inhibitor was blackberry, while the weakest happened to be pear; but interestingly enough it seemed that pear was almost as strong as blackberry when measured at 24 hours. Something occurs between 24 and 48 hours in pear juice which renders it an

ineffective alpha-glucosidase inhibitor. A 70:30 combination possesses strong inhibitory abilities which decreases at 24 hours but then surges to a peak at 48 hours. This seems to be a direct opposite of the pear juice which weakens after 48 hours elapses. A 70:30 also performed the best out of all diluted juices after 48 hours time.

5.4.4: Alpha-Amylase

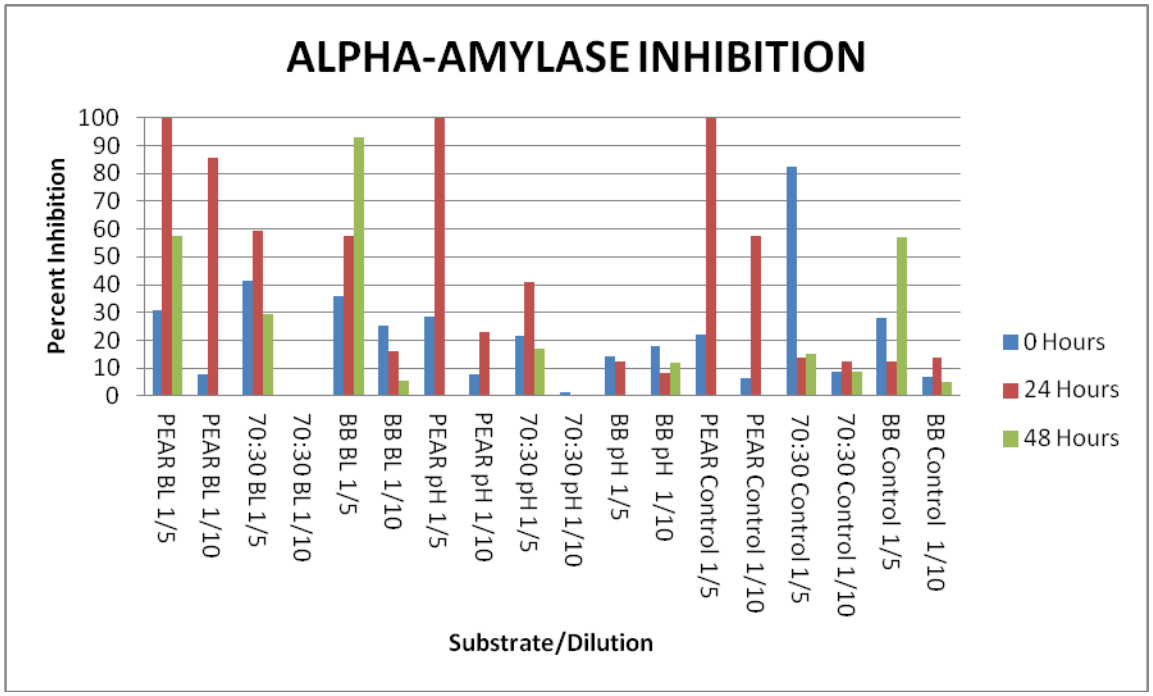

Figure 10: Alpha-amylase inhibition for samples with B. longum and control samples without added bacteria. The highest results show up at the 24 hour mark with100% pear having the highest overall results on average.

Alpha-amylase results for *B. longum* were rather erratic in nature. The most conclusive results from this experiment showed that after 24 hours, most juices showed higher inhibition of alpha-amylase, but it often dropped in inhibitory effectiveness after 48 hours with exception of in some blackberry samples. The increased effect at 24 hours was true even for the non-fermented versions of the

fruit juice. This would imply that there could be a reaction with the fruit juice and the atmosphere or raised temperature that increased its ability to counteract alpha-amylase after one day of holding. It seems that no conclusive evidence can be drawn from these experiments on the viability of *B. longum* to affect a juices ability to inhibit alpha-amylase with exception of undiluted blackberry juice.

5.4.5: ACE

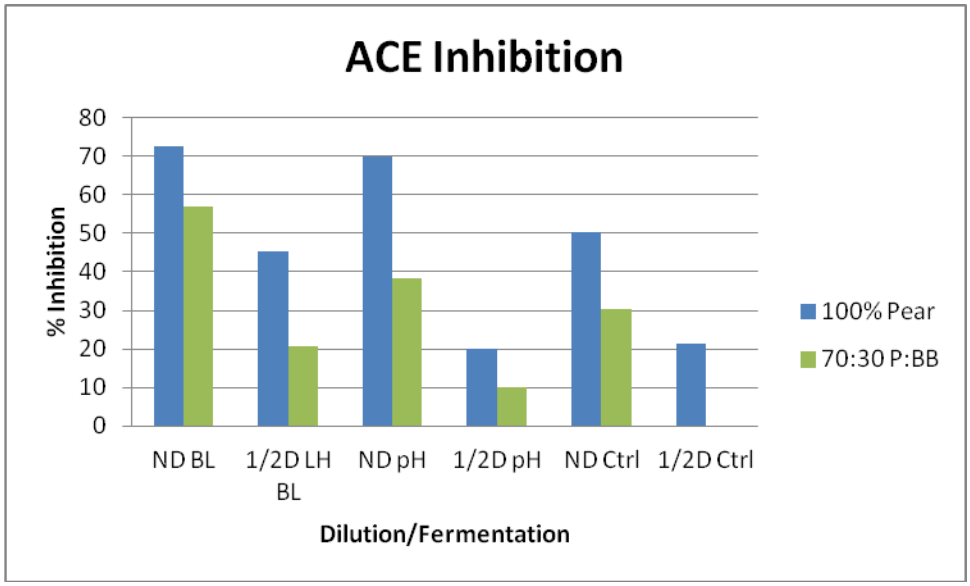

Figure 11: ACE inhibition using 100% pear and 70:30 pear:blackberry substrates. 100% blackberry was omitted due to negligible inhibition levels. Samples were undiluted (ND), or diluted to 50% (1/2D). 100% pear shows high inhibition, especially after fermentation.

Angiotensin converting enzyme inhibition highest in pear juice while being moderately lower in the 70:30 pear:blackberry juice combination. The reason for 70:30 being lower in potential ACE inhibition is the relatively non-existent ability of blackberry juice to affect ACE. The inability of blackberry juice to affect ACE is the reason behind not having it translate into measurable results. There is definite dose dependence for ACE inhibition in 70:30 combination, as these results will show.

Pear on the other hand suffers much less from dose dependence, especially for the *B. longum* fermented juice. Adjusting the pH of the pear juice seemingly augments the juice to be more dose dependent.

5.4.6: *Helicobacter pylori*

Plates of *Helicobacter pylori* were treated with *Bifidobacterium longum* fermented juices and their non-fermented control samples as well, what was recorded in this experiment closely reflected the previous *L. helveticus* findings. Kiowa blackberry that was fermented for 48 hours had relatively strong rates of inhibition on *H. pylori.* This experiment was then tested again with plates containing proline, to test the proline pathway. These plates showed the same results of Kiowa blackberry inhibition and no other juice variety having any effects. Another experiment with *H. pylori* was done to test if acidity had any bearing on the inhibition. *H. pylori* lawn plates were seeded with filter paper containing blackberry juice control modified to have the same pH as the fermented juice. The findings from the growth of those plates showed no inhibition whatsoever, further solidifying the fact that the blackberry juice requires fermentation by a lactic acid bacterium in order to inhibit *H. pylori.* The exact mechanism for why fermentation of 100% Kiowa blackberry juice inhibits the growth of *H. pylori* is enigmatic at this point, but further research could uncover interesting results and potential treatments for the pathogen.

5.5: Conclusion

Utilizing *Bifidobacterium longum* as a fermentative organism in the juice of pear, blackberry and a respective 70:30% combination of the two fruits generally increased the ability of the juices to affect the metabolic syndrome related pathways using *in vitro* models. To help prevent the metabolic syndrome from occurring in

humans, at least with dietary moderation, these fermented fruit juices could perform as a reasonably potent metabolic moderator. Fermentation with *B. longum* affects the ability of these aforementioned fruit juices to perform metabolic enzyme inhibition in a positive overall manner. Fermentation also increases the bioactive stability of phenolic compounds over time. The findings of this experiment show that a probiotic dietary supplement involving lactic acid bacteria, pear and blackberry juices is a viable suggestion for improving community health in the context of type 2 diabetes and its complications. Between the fruit juices and bacteria the supplement would be affordable for poor communities and could help provide additional dietary strategies to counter type 2 diabetes which unusually affects them in so-called food and nutritional deserts due to overabundance of inexpensive refined carbohydrates.

Nothing would be a miracle-cure, but this preventative supplement could be dispersed with little expense with careful budgeting and efficient distribution systems. This is also a "low-tech" food in the sense that it could be easily produced in less developed countries. All which is required is: fruits, bacteria and physical crushing of said fruit. The bacteria would enable the key bioactive benefits in this situation through its fermentation metabolic pathways.

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