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Lactic Acid Bacteria Mediated Phenolic Bioactive Modulation From Fruit Systems For Health Benefits

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LACTIC ACID BACTERIA MEDIATED PHENOLIC BIOACTIVE MODULATION FROM FRUIT SYSTEMS FOR HEALTH BENEFITS

A Dissertation Presented

By

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DEDICATION

To my parents, my brother and Gitanjeli who have made this possible

ACKNOWLEDGMENTS

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ABSTRACT

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FEBRUARY 2013

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Chronic oxidation linked diseases are on a rise and are one of the leading causes of death globally. Epidemiological evidence increasingly points towards consumption of fruits and vegetables as a preventive way to manage early stages of chronic oxidation linked diseases. Oxidation linked diseases are caused by excessive reactive oxygen species (ROS) generated by a disruption in cellular antioxidant homeostasis due to an overload of calories combined with stress, no excerise and a diet low in antioxidants. Phenolic compounds can not only act as antioxidants but also stimulate the activities of antioxidants enzyme through protective pathways which can help modulate cellular protection.

The aim of this dissertation was to use probiotic fermentation to enhance the phenolic and antioxidant compounds in fruit systems which can form the basis of functional food design. The potential of these food systems for disease prevention was investigated in eukaryotic systems through understanding the role of critical metabolic pathways involed in prevention of oxidation linked chronic diseases. Based on

structure-function rationale, antioxidant, anti-hyperglycemia and anti-hypertensive potential of phenolic compounds in tea and the effect of extraction time and different degrees of fermentation were investigated in *in vitro* models. Results indicated that the most fermented teas and a longer extraction time had the highest potential. Further these extracts also had higher *H. pylori* inhibition potential. Probiotic fermentation of fruit juices with *L. helveticus* was used to mobilize phenolics and improve biological functionality by maintaining a consistent phytochemical profile. Results indicated that total phenolic and antioxidant potential decreased with feremnetation. However α-glucosidase inhibitory activity and *H. pylori* inhibitory potential increased with fermentation. Investigation into the mechanism of *H. pylori* inhibition with fermented cherry extracts revealed inhibition of proline dehydrogenase as the likely mode of action. The potential of fermented apple extracts was further investigated as a phytochemical elicitor in eliciting phenolic and antioxidant response in germinating fava bean. The results indicated a stimulation of phenolic and antioxidant response likely through the stimulation of carbon flux through glycolytic pathways. In yeast, fermented apple extracts accelerated cell death in the presence of peroxide stress in pretreatment model whereas it provided protection against oxidative stress and prevented cell death in concurrent model. Chitosan oligosachharide treatment was investigated as a potential replacement of cancer causing diphenylamine treatment for scald reduction in Cortland apples. Although the treatment did not have any effect on scald reduction, it provides better protection in storage by stimulating phenolic and antioxidant response which related to better health relevant functionality.

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CHAPTER 1

INTRODUCTION

1.1 Biological Role of Lactic Acid Bacteria

Lactic acid bacteria have the ability to produce lactic acid from sugars and are classified as a group of organisms that are non-sporeforming, aerotolerant anaerobic cocci or rods with a molar G + C content of less than 55% in their DNA (Stiles and Holzapfel, 1997). Yogurt has been used as a functional food for treating diarrhea since antiquity and recent studies suggest that yogurt, individual LAB species or both can beneficially affect host physiology for gastrointestinal conditions, including lactose intolerance, constipation, diarrheal diseases, colon cancer, inflammatory bowel disease, *Helicobacter pylori* infection, and allergies (Adolfsson *et al.,* 2004). Lactic acid bacteria can produce only end product; lactate or more than one end product; lactate, acetate, CO_2 or ethanol via homofermentation or heterofermentation pathways respectively (Kandler, 1983). The term "Probiotcs" can be broadly defined as viable microorganisms which provide beneficial health effects, beyond basic nutrition, when ingested in certain numbers (Ljungh and Wadström, 2006). LAB constitute the biggest group of probiotic bacteria used (Perdigon *et al.,* 2001) and although they may be naturally present in some foods, they may be externally added for a more consistent product. Many of the LAB metabolites such as low molecular weight acids, alcohols, carbon dioxide, diacetyl and hydrogen peroxides have broad spectrum antimicrobial activity against other species of bacteria (Helander *et al.,* 1997).

1.2 Fermented Foods for Human Health

Refrigeration has long replaced fermentation and drying as the most popular technique for preservation however, the ever expanding scientific evidence of its health benefits continue to surge interest in research in fermented foods while trying to extend this technique to novel foods for enhanced health benefits. Earliest evidence of fermentation originates from China which has been dated back to 7000 B.C. (McGovern *et al.,* 2004). Biochemical changes caused during fermentation include formation of acid/alcohol, macro and micro nutrient formation, flavor, aroma and texture development and detoxification of substrates (Kazanas and Fields, 1981; Kandler, 1983; Steele and Unlu, 1992; Hinrichsen and Pedersen, 1995; Mierau *et al.,* 1997; McSweeney, 2000; Olasupo, 2006). Lactic acid bacteria as individual species alone can have antimicrobial, physiological, immunomodulatory and anticarcinogenic effects (Naidu *et al.,* 1999; Perdigon *et al.,* 2001; Rafter, 2002). Antidiabetic, antiatherogenic and lipid lowering effects of lactic acid bacteria fermented Kimchi have been reported (Choi *et al.*, 1997; Islam and Choi, 2009). Fermented sea food rich in bioactive peptides have shown antihypertensive ability (Ichimura *et al.*, 2003; Je *et al.*, 2005a; Je *et al.*, 2005b). In rat model systems, *in vitro,* and *in vivo* studies the antidiabetic effect of fermented milk and milk product have been reported (Teruya *et al.,* 2002; Apostolidis *et al.*, 2006a; Yadav *et al.*, 2006; Apostolidis *et al.*, 2007).

1.3 Biological Role of Phenolic Bioactives

Phenolics in the diet mainly come from plant foods that contain a variety of these compounds including phenolic acids, phenylpropanoids, benzoic acid derivatives, flavonoids and tannins. Phenolics compounds originate from one of the major secondary metabolite biosynthetic pathways such as shikimate, phenylpropanoid and flavonoid pathway (Schijlen *et al.*, 2004). Plant phenolics can beneficially affect host physiology by acting as antioxidants primarily by accepting free radicals, by metal chelation or by quenching singlet oxygen (He and Shahidi, 1997). These secondary metabolites are induced by biotic and abiotic stresses as a part of the plant defense system response (Hahlbrock and Scheel, 1989; Graham, 1991; Conceicao *et al.*, 2006). Fruits such as apples, cranberries, grapes, peaches, raspberries, and strawberries and beverages like tea, red wine, apple and orange juices; vegetables such as cabbage and onion; food grains such as sorghum, millet, barley, peas, and legumes such as soybean, black bean are the richest sources of phenolic phytochemicals in the diet. Oxidative stress and ROS have been involved in the pathogenesis of a number of diseases and diet rich in phenolic phytochemicals have been shown to reduce the risk of a number of these diseases including cardiovascular disease (CVD), diabetes and some forms of cancer (Cook and Samman, 1996; Yao *et al.*, 2004).

Phenolic phytochemicals can act as antimicrobials by damaging bacterial membranes and inhibiting important cellular enzymes required for metabolism (Fogg and Lodge, 1945; Juven *et al.*, 1972). Cranberry proanthocyanidins have shown positive effects against urinary tract infection which is linked to inhibition of *E. coli* adherence

to the epithelial cells of the urinary tract (Howell *et al.,* 1998; Foo *et al.,* 2000). Polyphenolic antioxidants can quench electrons from electron transport chain (ETC) along the bacterial membrane and act as antimicrobials by disrupting oxidative phosphorylation or inhibit dehydrogenases linked proton efflux by interfering with the flow of electrons at the level of cytochromes (Vattem *et al.,* 2005a). It is also thought that soluble phenolics can disrupt the H^+-ATPase required for ATP synthesis by causing hyper-acidification via proton donation at the plasma membrane (Shetty and Labbe, 1998; Shetty and Wahlqvist, 2004).

1.4 Innovative Strategies for Improving Food Functionality by Understanding the Role of Lactic Acid Bacteria and Phenolics

Probiotic lactic acid bacteria individually, fermented foods and phenolic phytochemicals from fruits have shown numerous health benefits from simple prevention of diarrhea to protection against complex oxidative stress linked diseases such as diabetes and cardiovascular disease (CVD) (Cook and Samman, 1996; Yao *et al.,* 2004). Currently only milk based fermented products such as yogurt are the popular probiotic based fermented food in the market. The overall goal of this dissertation was to extend this lactic acid bacteria based fermentation concept to fruit juices to explore their anti-hyperglycemia and anti-hypertensive potentials. Further these fermented juices were evaluated for their antimicrobial effects against *H. pylori* and probiotic *Bifidobacterium longum*. This design of functional foods and ingredients was rationalized on the basis of linking antidiabetic, antihypertensive and antimicrobial effect to the biochemical changes in the fruit phenolic bioactives mediated by probiotic

bacteria. Probiotic fermented juices can potentially exhibit a dual functionality by potential management of metabolic syndrome stages by bioactive ingredients coupled with stimulation of gut health and innate immunity by probiotic lactic acid bacteria. Further these fermented bioactive extracts will be used to induce phenolic associated antioxidant enzyme response in fava bean, yeast and *Caenhorbditis elegans* as model systems which will be investigated using mechanistic rationale of the role of proline associated pentose phosphate pathway

CHAPTER 2

LITERATURE REVIEW

2.1 Lactic Acid Bacteria

Pioneering scientific and technical developments in the latter part of 19th century led to the concept of lactic acid bacteria (LAB) as a group of organisms with potential in food fermentation and human health (Stiles and Holzapfel, 1997). Certain species of *Lactobacillus, Lactococcus (Streptococcus), Enterococcus, Leuconostoc, Bifidobacterium* and *Pediococcus* are generally referred to as LAB (Klein *et al.,* 1998). Although recent chemotaxonomic and phylogenetic studies have resulted in a change in their nomenclature they can be generally classified as phylogenetically belonging to the clostridial branch of gram positive bacteria that are non-sporeforming, aerotolerant anaerobic cocci or rods with a molar G + C content of less than 55% in their DNA (Stiles and Holzapfel, 1997).

Glucose utilization takes place via two major pathways in lactic acid bacteria depending on whether the bacterium is homofermentative (only one end product, lactate) or heterofermentative (more than one end product, at least 50% lactate) (Kandler, 1983) (Fig 1). In homofermentative bacteria one mole of glucose is converted to 2 moles of pyruvate through glycolysis. This results into generation of 2 ATP molecules while using up one NAD^+. NAD^+ is regenerated by reduction of an internal substrate such as pyruvate which is converted to lactic acid. Heterofermentation leads to formation of gluconate-6-phosphate which on decarboxylation results into formation of xylulose-5-phosphate and carbon dioxide. Xylulose-5-phosphate on chain splitting

results into C-2 and C-3 moieties; lactate and acetate or ethanol (Kandler, 1983). As for lactose, it is taken up by a specific permease and enters the cytoplasm where it is hydrolyzed by β-galactosidase to glucose and galactose (Thompson, 1979; Kandler, 1983). Resulting glucose along with galactose which is converted to glucose-6-phosphate are fermented via glycolysis. The main function of LAB is to produce acid but some LAB with heterofermentative ability may be used to produce specific flavor components in fermented products (Johnson and Steele, 2007). Apart from being indispensible for making fermented dairy products, members of lactic acid bacteria group are used for fermentation of vegetables, meat, fish, poultry, cocoa and coffee as either a part of preserving, processing or adding flavor to these foods (Johnson and Steele, 2007). Although lactic acid bacteria is present naturally on the surface of many foods and can spontaneously ferment some of their substrates they may be added externally for a more consistent and reliable end product. Some species of lactic acid bacteria are included in the class of "probiotics" which may provide potential health benefits including a) increased resistance to intestinal infectious diseases, b) alleviating diarrheal syndromes, c) blood pressure and serum cholesterol reduction for CVD management, d) regression of tumors and reduction in carcinogen and co-carcinogen production (Tannock, 1999).

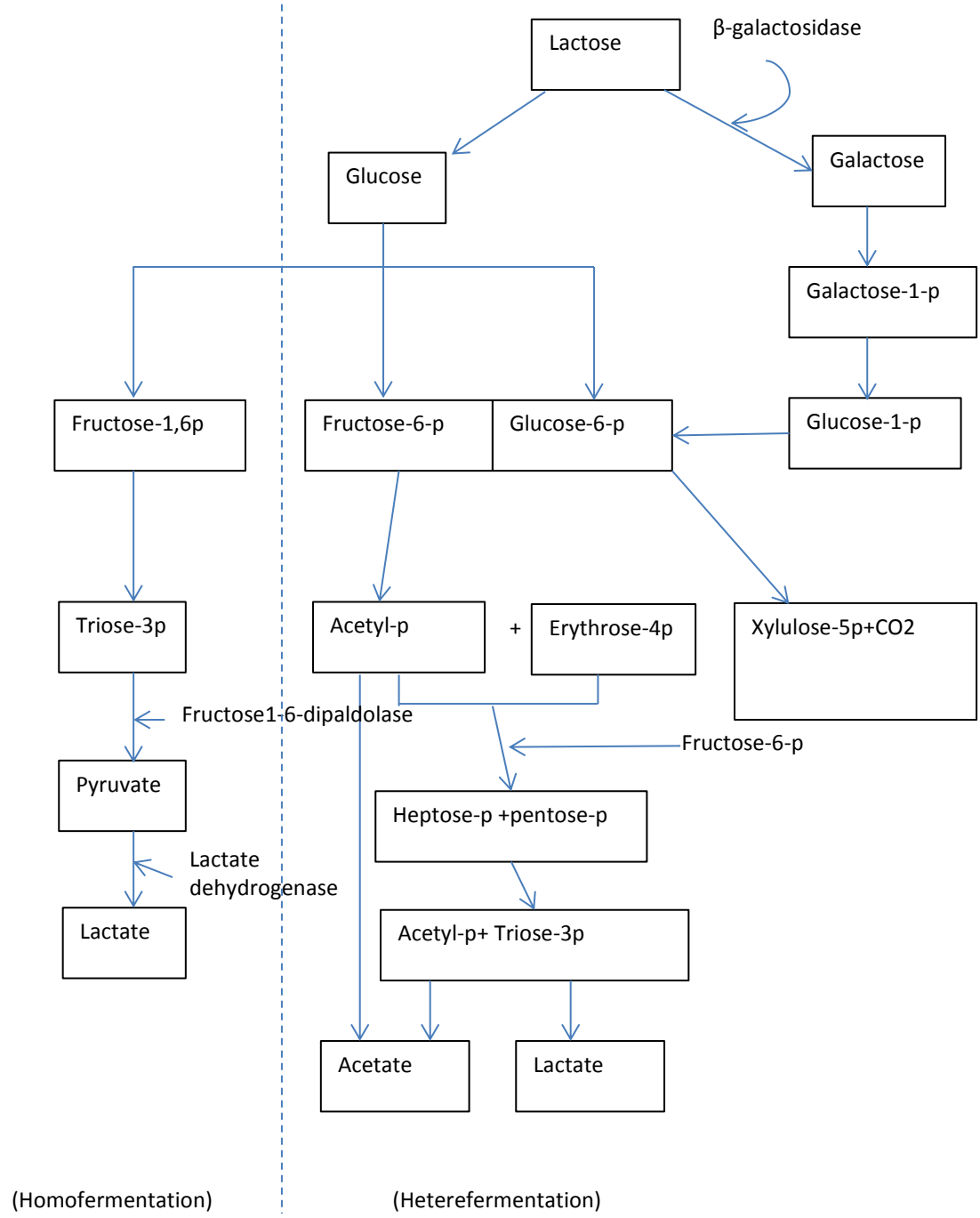

Figure 1: Hexose metabolism in lactic acid bacteria.

(Adopted from Kandler, 1983)

2.2 History of Fermentation

Fermentation as a technique has been used for thousands of years to preserve food and promote good health however, it was not until Antonie van Leeuwenhoek invented the microscope and Pasteur developed pasteurization that fermentation as a process was attributed to the activity of microorganisms growing in the food. Archaeological evidence point to the art of fermentation first originating in Henan province in China, as early as 7000 B.C. (McGovern *et al.,* 2004) and the earliest large scale wine production dated to 5400 B.C. in modern day Iran (McGovern *et al.,* 1996). From Mesopotamia fermentation technology spread to the rest of the world. Therapeutic properties of Dahi (Indian analogue of yogurt) and Chhash, (stirred diluted yogurt) and their role in preventing stomach and intestinal disorders are mentioned in Ayurveda which is said to be compiled around 1500 B.C (Prajapati and Nair, 2003). Yogurt and other fermented milks were prescribed by ancient physicians in the Middle East for intestinal and stomach disorders and stimulation of appetite (Rasic and Kurmann, 1978). King Francis I of France was cured of a debilitating illness after eating yogurt made from goat's milk and it is said that longevity and fecundity of Abraham was attributed to the regular consumption of fermented milk product such as yogurt (Deeth and Tamime, 1981). It is reported that cheese was first developed near the Tigris and the Euphrates rivers near Iraq with the idea that cheese presents an advantage over milk for having a longer shelf life, being more nutritious and high energy food (Ross and Kasum, 2002; Heller *et al.,* 2008). Sauerkraut which developed using lactic acid bacterial (LAB) fermentation has a long history of use as a food for therapeutic

purposes. Sauerkraut is an excellent source of Vitamin C and can prevent scurvy and has *Lactobacillus plantarum* as the dominating bacteria which can mitigate intestinal disorders. These observations were made by British explorer James Cook which led to a high survival rate for men on board his ships (Lloyd C, 1949). Metchnikoff, (1908), was the first to provide any scientific basis for the therapeutic properties of fermented food products by understanding the changes in micro ecology of the gut following consumption of fermented milk. He hypothesized that the large bowel harbored microorganisms that produced toxic compounds which when absorbed by the body, contributed to the process of ageing. A simple solution was to modify the microflora of the large intestine by consuming fermented milk containing lactic acid bacteria that inhibit putrefactive organisms and hence decrease the toxic metabolites such as amines produced by putrefactive organisms in the bowel. He further hypothesized that the unusually long and healthy lives of Bulgarian peasants was due to the large amounts of fermented milk consumed by these people (Metchnikoff, 1903; Metchnikoff, 1908; Tannock, 1997).

2.3 Role of Fermentation in Foods

Fermentation has had a deep impact on the history of food, eating habits and cultural exchange of food. Although fermentation has a number of functional aspects, it primarily evolved as a process to preserve food safely. Fermentation may increase the shelf life of food by one to two orders of magnitude while adding value by enriching it

with nutrients, flavor and in some cases detoxifying the substrate. Steinkraus (1994) listed some of the major roles of fermentation as:

2.3.1 Preservation of Food through Acid/Alcohol Formation

Lactic acid bacteria produce lactic acid, ethanol, acetic acid, diacetyl and CO_2 as one of their metabolic end products. Biochemistry of hexose metabolism is described in the earlier section 2.1. Lactic acid produced during fermentation helps preserve food by lowering the pH whereas ethanol, CO_2 acetic acid, diacetyl produced act by being toxic to pathogenic microorganisms and hence severely restricts their growth in foods keeping the food safe for long periods of time.

2.3.2 Enrichment of Food Substrates Through Formation of Micro and Macro Nutrients

Lactic acid bacteria have to rely on their complex proteolytic systems to obtain amino acid from their substrates, for synthesizing proteins and nitrogen required for growth, due to their limited amino acid synthesizing ability (Law and Kolstad, 1983; Thomas and Pritchard; 1987; Chopin, 1993). Milk serves as a good example since it has very little free amino acid (Law and Kolstad, 1983). When growing in milk, proteins are degraded to oligopeptides by an extracellular proteinase (Kunji *et al.,* 1998). Peptidases are intracellularly located which means peptides have to be internalized by peptide transport system before intracellular hydrolysis of these peptides to amino acids can take place (Juillard *et al.,* 1998). These amino acids can be used for protein synthesis and other metabolic activities (Mierau *et al.,* 1997). Both extracellular proteinases and

intracellular peptidases work in combination to produce peptides and free amino acids which contribute to texture and flavor development of fermented milk products (Steele and Unlu, 1992; Mierau *et al.*, 1997; Mcsweeney; 2000).

Changes in vitamin content depend on the substrate, microorganism involved, length of fermentation and fermentation conditions. In most cases there is an improvement in the content of B-group vitamins. During cereal and cereal-legume blend fermentation there was an increase in Thiamin, Riboflavin and Niacin content by 1-2.5 folds in all cases except in rice fermentation where Thiamin and Niacin were found to decrease (Tongnual, 1979**;** Aliya and Geervani, 1981; Kazanas and Fields; 1981)**.** During soybean fermentation (combination of aerobic growth and fermentation using mixed cultures) to tempeh riboflavin and nicotinic acid content were seen to increase whereas thiamine content decreased to an undetectable level (Van der riet *et al.*, 1987).

2.3.3 Flavor, Aroma and Texture Development

Microorganisms growing in food cause a number of changes resulting in formation of new compounds which are perceived as flavor components. In cured hams, secondary metabolism of flavor improving microorganisms which is associated with amino acid catabolism produce aldehydes, methyl ketones, secondary alcohols, ethyl esters all of which contribute to the flavor (Hinrichsen and Pedersen, 1995). In sauerkraut, volatile sulfur compounds such as hydrogen sulfide, dimethyl sulfide along with carbonyls contribute to the flavor (Lee *et al.,* 1974). In sourdough fermentation

homofermentative bacteria mainly produce diacetyl and other carbonyls, yeast mainly produce iso-alcohols, and heterofermentative bacteria produce ethylacetate with some alcohol and aldehydes (Damiani *et al.*, 1996)**.** In milk, proteolysis of milk proteins produces taste and aroma of fermented milk (Mierau *et al.,* 1997; Juillard *et al.,* 1998; Kunji *et al.,* 1998). Some strains of lactic acid bacteria can metabolize citrate producing various aroma compounds such as diacetyl, acetaldehyde, acetoin which can have significant effect on the flavor of fermented food (De Figueroa *et al.,* 1998). Changes in texture due to fermentation are mostly when proteins undergo changes due to fermentation. In milk, lactic acid produced denatures the protein to form a three dimensional network forming a gel. In breads, lactic acid produced can delay retrogradation which depends on the level of acidification and the lactic acid bacteria strain (Corsetti *et al.,* 2000).

2.3.4 Detoxification of Substrates During Fermentation

Cassava is a starchy tuberous root which may contain cyanogenic gluCOSides, consumption of which may cause severe intoxication. It is reported that cassava can be completely detoxified by the fermentation process (Olasupo, 2006). Microorganisms reportedly cause cellular breakdown and activate endogenous enzymes which cause detoxification (Olasupo, 2006)**.** Microorganisms can produce phytases, which can hydrolyze phytates that cause reduction in the bioavailability of iron and other minerals (Khetarpaul and Chauhan, 1991).

2.4 Fermented Foods for Human Health

With the increasing Cost of health care in an era where the human population is plagued by an epidemic of chronic diseases has led the consumer to adopt a "preventive" approach with a more careful and conscious selection of food for leading a healthy lifestyle. Probiotic fermented foods with their low-Cost and multi-faceted therapeutic potential has been one of the top choices in this first step towards adopting a healthier lifestyle. Fermented foods may have health beneficial properties from fermenting microorganism and/or from the end products they form.

2.4.1 General Benefits from Lactic Acid Bacteria

Probiotic which means life in Greek was first used to describe the stimulation of one microorganism by compounds secreted by another (Lilly and Stillwell, 1965). Fuller (1989), redefined the term as "a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance." As new health promoting properties were being discovered the definitions were annexed. Now the term "Probiotcs" can be broadly defined as viable microorganisms which beneficially affect host physiology, beyond basic nutrition, when ingested in certain numbers (Ljungh and Wadström, 2006). Lactic acid bacteria are by far the most commonly used probiotic cultures in food products (Perdigon *et al.,* 2001). Lactic acid bacteria as Probiotics may initiate/stimulate a number of different beneficial host

physiological responses such as a) antimicrobial effects: by synthesis of a number of compounds which are toxic to other bacteria and *in vivo* antimicrobial action by inhibiting attachment of pathogens to the intestinal wall and microbial antagonism b) physiological effects: reduction in serum cholesterol, alleviating lactose intolerance, increasing nutrient absorption, anti-hyperglycemic effects c) immunomodulatory effects by stimulating innate immunity d) preventing colon cancer: by pro-carcinogen binding and inhibition, changes in physiochemical condition of the colon e) management of diarrhea: infantile, travellers and antibiotic induced diarrhea (Fig 2).

Organic acids such as lactic and acetic acid, hydrogen peroxide, carbon dioxide and flavor compounds like diacetyl and acetaldehyde produced by heterofermentative LAB have shown antimicrobial activity against gram positive and/or gram negative bacteria (Piard and Desmazeaud, 1991). Many fermented food products have higher keeping qualities because of broad spectrum antimicrobial activity resulting from a general reduction in pH caused by the acids produced during fermentation (Naidu *et al.,* 1999). The antimicrobial activity of acids is higher in their undissociated forms (Presser *et al.,* 1998) and lactic acid has a higher dissociation constant than acetic acid which is why acetic acid is a more potent antimicrobial than lactic acid (Rasic and Kurmann, 1983; Piard and Desmazeaud, 1991). Oxidation of membrane lipids and sulfhydryl groups of cell proteins has been attributed to the detrimental effects hydrogen peroxide can cause to a bacterial cell (Ouwehand and Vesterlund, 2004). Certain metabolic end products, antibiotic substances and bactericidal proteins synthesized by bacteria which have a broad inhibitory spectrum are referred to as bacteriocins.

Nisin and reuterin are two well-known bacteriocins which are produced by LAB. Bacteriocins reportedly act on the cell membrane causing pore formation leading to exposure of the intracellular components to the environment which causes cell death (Montville and Chiknidas, 2007). Carbon dioxide can create an unfavorable environment for strictly aerobic microorganisms, can cause a decrease in pH and can destroy cell membranes which makes many organisms susceptible to carbon dioxide (Clark and Takacs, 1980; Eklund, 1984). LAB may inhibit pathogens in the colon by number of different ways that include production of antimicrobial compounds mentioned before, competitive growth, not allowing pathogens to attach to the colonic muCOSa and thereby preventing their establishment and multiplication and by changing the redox potential of the muCOSa by fermentative action (Naidu *et al.*, 1999).

Lactic acid bacteria may enable nutrient absorption by further breaking down undigested food passed down from the small intestine. About 60-85% of the monosaccharides and disaccharides released in the colon can be converted into short chain fatty acid which can be easily absorbed (Naidu *et al.*, 1999). It has been reported that lactose intolerant individuals can manage lactose from yogurt better because of the β-galactosidase activity of the yogurt culture (Martini *et al.*, 1991). Serum cholesterol reduction by probiotic bacteria has been attributed to a number of mechanisms including inhibition of a rate limiting enzyme involved in endogenous cholesterol biosynthesis, cholesterol assimilation by bacteria, physiological effect of short chain fatty acids produced by the bacteria, enzymatic deconjugation of bile acids and stimulating cholesterol excretion (Naidu *et al.*, 1999; Pereira and Gibson, 2002).

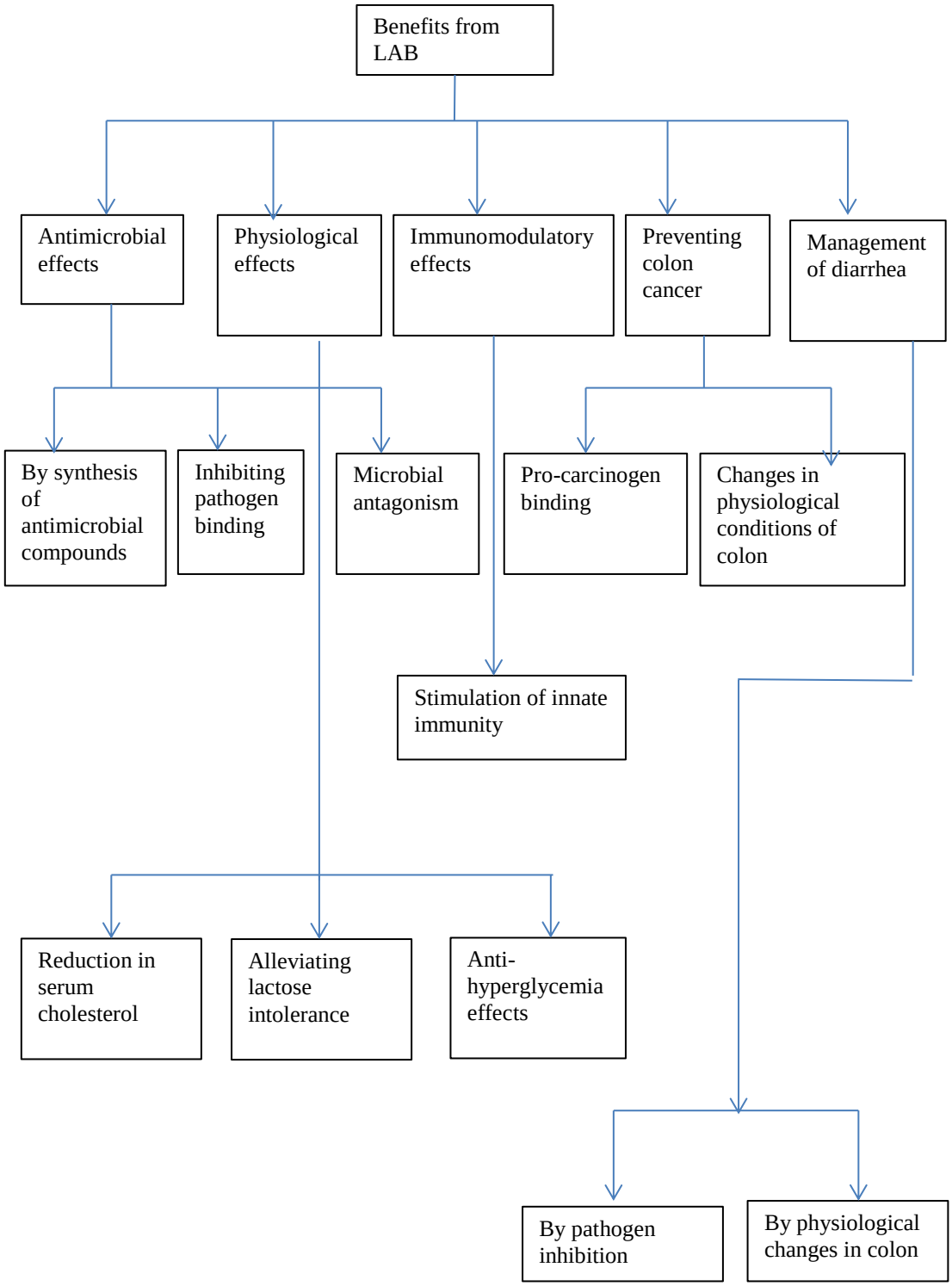

Figure 2: Health benefits from lactic acid bacteria

Some strains of *L. helveticus*, *L. bulgaricus* and *L. casei* have been shown to increase non-specific immune response which was determined by the increase in phagocytic activity of macrophages and increase in activity of lysosomal enzymes β-glucuronidase and β-galactosidase (Perdigon *et al.*, 2001).

Although there is no direct evidence for tumor suppression in the colon there are a number of ways lactic acid bacteria could affect the host beneficially to counter the onset by a) binding and degrading potential carcinogens b) changing the balance of microflora in the intestine c) changes in physio-chemical conditions of the intestine d) production of anticancer compounds e) improving host broad specific immunity (Rafter, 2002).

Lactic acid bacteria and fermented foods with live cultures have been used to alleviate diarrhea since antiquity. Lactic acid bacteria can help in management of diarrhea in several different ways a) they can inhibit pathogenic bacteria and restore balance in the colon b) they can shorten the duration and ameliorate the symptoms c) reverse intestinal permeability and d) promote recovery.

2.4.2 Lactic Acid Bacteria Based Fermented Foods for Health Benefits

Kimchi is a traditional Korean dish made by fermenting vegetables using LAB. Chinese cabbage Kimchi was reported to have some anti-diabetic effect in type 2 diabetes model rats when fed on a high fructose diet (Islam and Choi, 2009). Antiatherogenic and lipid lowering effects of fermented Chinese cabbage Kimchi has been widely reported (Kwon *et al.*, 1999; Kwon *et al.*, 2004; Sheo and Seo, 2004; Choi

et al., 1997). Cytotoxic effects of hydrogen peroxide on human skin cells were drastically reduced when treated with extracts of 2 week fermented Kimchi which indicates that fermentation can improve antioxidant ability of the fermenting substrate (Ryu *et al.,* 1997). A three week fermented Kimchi was shown to have 99% inhibitory activity towards 3-methylcholanthrene (MCA)-induced cytotoxicity in C3H/10T1/2 cells which suggests fermented Kimchi may have anti-carcinogenic potential (Choi *et al.,* 1997).

Fermented fish paste is rich in bioactive peptides which have shown potential in reducing blood pressure in hypertensive rats. Fermented fish paste also stimulated insulin secretion by cultured insulinoma cells which indicates that fermentation can enrich fish paste with bioactive compounds which have potential to lower blood pressure and diabetes (Ichimura *et al.,* 2003). Peptides isolated from fermented blue mussel sauce and oyster sauce have shown beneficial effects against blood pressure in hypertensive rats (Je *et al.,* 2005a; Je *et al.,* 2005b). Similar results were shown in fish sauce by Okamoto *et al.,* (1995).

Milk fermented by LAB has been suggested to be antihypertensive. Ile-Pro-Pro and Val-Pro-Pro are the most studied ACE inhibitors in fermented milk (Pihlanto *et al.,* 2010). ACE inhbitors produced during milk fermentation by proteolysis has been reviewed (Fitzgerald and Murray, 2006; Korhonen and Pihlanto, 2006). Antihypertensive effects of a salt free soy sauce have been demonstrated by Matsui *et al.,* (2010), in spontaneously hypertensive rats.

Fermentation of milk for diabetes management has been reported by various authors (Teruya *et al.,* 2002; Apostolidis *et al.,* 2006a; Yadav *et al.,* 2006; Apostolidis *et al.,* 2007). Anti-diabetes functionality of soymilk fermented with Kefir culture has been recently documented by Kwon *et al.,* (2006a) in a mixed substrate that also included botanical extracts of *Rhodiola crenulata*. A heat stable, pH stable small molecule was revealed to be the active agent in water soluble fraction of Kefir that increased glucose uptake in L6 myotubes both with and without insulin stimulation (Teruya *et al.,* 2002). Similarly, supplementing a high fructose diet of rats with dahi (Indian analogue of yogurt) resulted in delaying of glucose intolerance until fourth and fifth week as compared to control group where it began in the third week (Yadav *et al.,* 2006).

2.4.3 Health Benefits of Foods Fermented by Other Class of Bacteria

A solid material where rice bran and soybean were the main ingredient was fermented using bacteria of the *Bacillus* spp. This fermented product was shown to reduce serum glucose and serum triglyceride in a diabetic animal model. Further the mechanism of action was shown to be by stimulation of a glucose transporter and by inhibition of a glucose synthase kinase-3 beta, a negative modulator of insulin signal transmission (Lim, 2010). Martin and Matar, (2006), reported a significant increase in total phenolic and antioxidant capacity of lowbush blueberry juice after fermentation with *S. vaccinii*. Increase in phenolic compounds was thought to be due to deglycosylation of phenolic compounds and by biosynthesis of new phenolics by the bacterium. Vuong *et al.,* (2007), reported an increase in glucose uptake, with or without

insulin, in myotubes and adipocytes by a lowbush blueberry juice fermented using *S. vaccinii* bacteria whereas nonfermented juice had no effect on transport. This biotransformed juice further showed antiobesity and antidiabetic effect in mice. This effect was hypothesized to be due to a significant increase in antioxidant and phenolic compounds during fermentation which reduces oxidative stress in diabetic mice (Vuong *et al.,* 2009). Further this juice protected neurons against hydrogen peroxide induced cell death thereby being a potent therapeutic agent against neurodegenerative diseases (Vuong *et al.,* 2010). Berry juices when fermented with *S. vaccinii* showed increased antioxidant activity and inhibited LPS/IFN-gamma-activated macrophage $NO^{\cdot}$ production however with an increase in TNF-α production (Vuong *et al.,* 2006).

2.5 Phenolic Bioactives: Nature, Function and Biosynthesis

Phenolic phytochemicals can be defined as compounds having an aromatic ring structure with one or more hydroxyl groups derived from plant secondary metabolism that possess supplemental function (Shahidi, and Naczk, 2004; Jaganath and Crozier, 2010) (Fig 3). Phenolic compounds serve a number of purposes in plants including contributing to plant structure and stability, attracting pollinators, acting as natural pesticides, defense roles against pathogen attack and playing a key role in the plant signaling system (Shahidi, and Naczk, 2004; Jaganath and Crozier, 2010). Phenolic compounds can be classified into at least ten different classes however the ones that are obtained significantly from our diet are simple phenols, phenolic acids, hydroxycinnamic acids, coumarins and flavonoids (Bravo, 1998). Flavonoids form the

most abundant class of phenolic phytochemicals and to date almost 6000 flavonoids have been identified (Jaganath and Crozier, 2010).

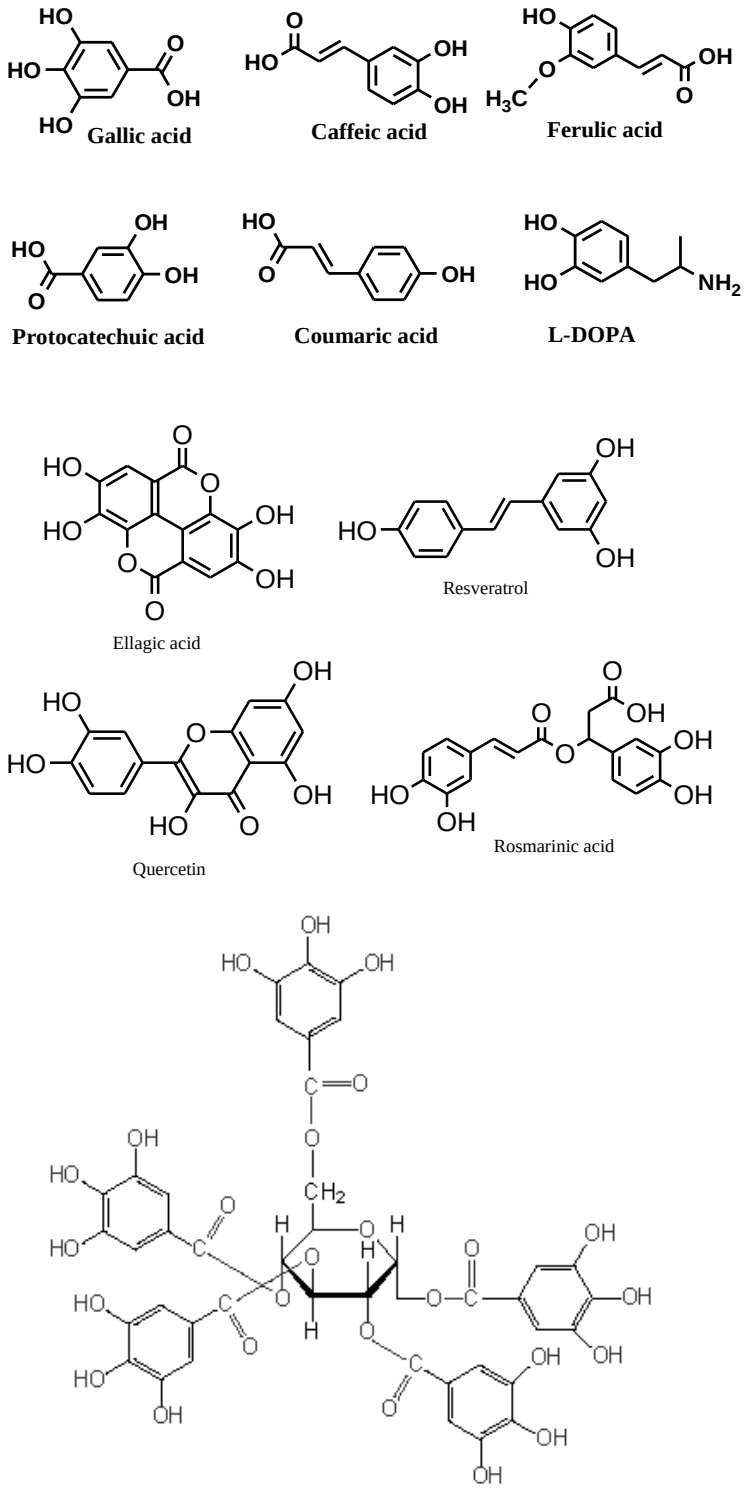

Figure 3: Common simple phenol, biphenyls, flavanoids and tannins in plants.

Plants are excellent sources of phenolic phytochemicals and recent research has highlighted the potential of phenolic phytochemical components including flavonoids, phenylpropanoids, and phenolic acids as important contributors of antioxidants in the diet. Polyphenols can act as antioxidants by stabilizing and delocalization of an unpaired electron accepted from a free radical, by donating hydrogen or electrons and by chelating transition metal ions (Rice-Evans *et al.,* 1997). Phenolic phytochemicals can provide health benefits in various other ways such as 1) acting as substrates or cofactors for biochemical reactions 2) inhibitors of enzymatic reactions 3) scavengers of reactive or toxic compounds 4) enhancing the stability and absorption of nutrients 5) as substrates for bacterial fermentation in the intestine 6) inhibitors of pathogens in the colon (Dillard and German, 2000). Phenolic phytochemicals can alleviate oxidative stress not only by acting as an antioxidant but also in conjunction with the products of the pentose phosphate pathway to stimulate antioxidant enzyme response (Shetty and Wahlqvist, 2004). Phenolic compounds in plants are mainly synthesized by the skimimate, phenylpropanoid and the flavonoid pathway (Fig 4). Phenolic acids and phenylpropanoids are synthesized from precursors which are derived from carbohydrate metabolism. Carbohydrate metabolism and phenylpropanoid pathway provide the necessary precursors malonyl-CoA and *p*-Coumaroyl CoA respectively, for flavonoid biosynthesis, which is initiated by enzymatic action of chalcone synthase. The pathway then proceeds to other class of flavonoids through many complex and biologically regulated enzymatic steps and finally to the major water soluble compounds in fruits and flowers, anthocyanins (Schijlen *et al.,* 2004).

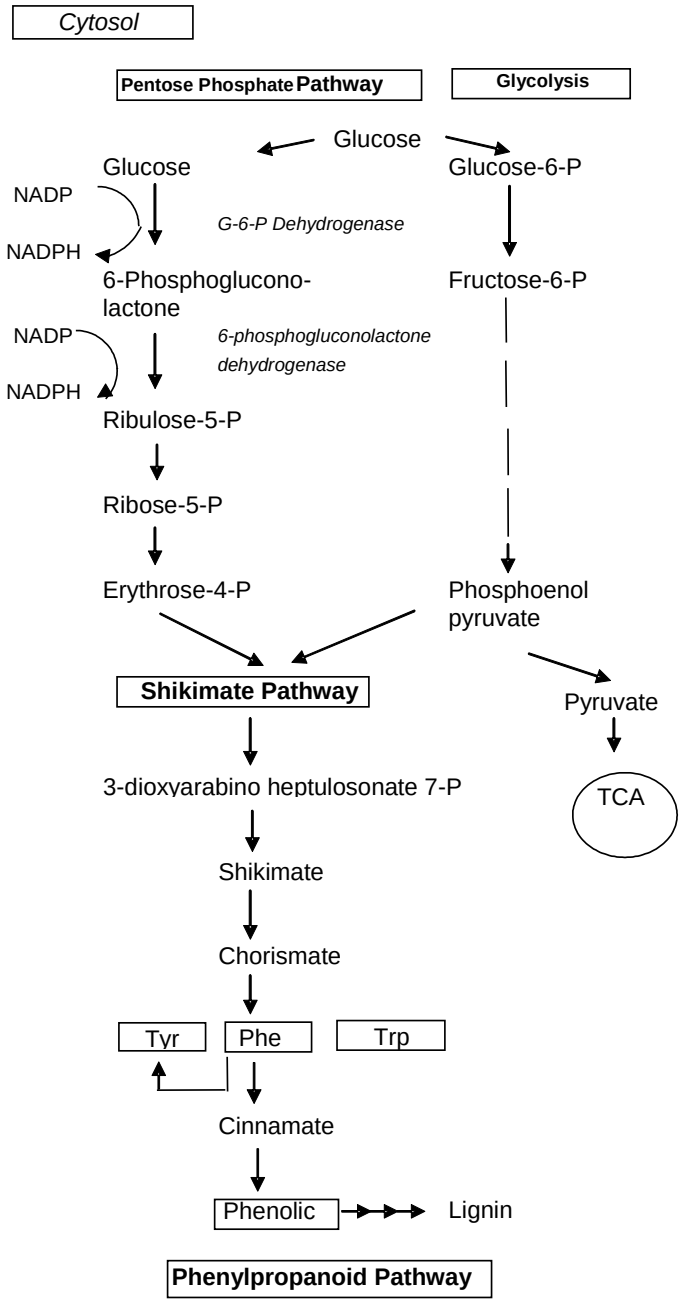

Figure 4: Biosynthesis of phenolic phytochemicals.

2.6 Phenolic Bioactives for Human Health

Oxidative stress can be defined as the change in the oxidant/antioxidant balance in favor of the oxidants which leads to biochemical and cellular damage. Chronic oxidative stress linked diseases are reaching pandemic status and continue to increase worldwide at alarming rates. Mitochondria are the biggest source of oxidants such as superoxide, hydrogen peroxide, and hydroxyl radicals which are continuously produced as a result of aerobic respiration (Shigenaga *et al.,* 1994). Every cell has numerous antioxidant defenses such as catalase, peroxidase and superoxide dismutase which can protect the cell against oxidants however oxidants that escape can cause damage to macromolecules and especially nuclei acids leading to mutation and cancer (Ames *et al.*, 1993a).

Obesity associated with current life style changes increase the oxidative stress and has been stated to be an important causative mechanism in obesity related metabolic syndromes (Urakawa *et al.,* 2003; Furukawa *et al.,* 2004). Free radicals produced from mitochondrial oxidative metabolism, nitric oxide, phospholipid metabolism and proteolytic pathways may shift the oxidant/antioxidant balance which may lead to neurodegenerative diseases such as Alzheimer's, Parkinsons and amylotropic lateral sclerosis (Simonian and Coyle, 1996). ROS may cause acute and chronic cell injury resulting in oxidative malfunction based diseases. Recently it has been reported that reactive oxygen species have a causal role in multiple forms of insulin resistance and antioxidant therapy may be a useful strategy to control type 2 diabetes (Houstis *et al.,* 2006). Excess calorie intake produces an increased amount of

ROS from various mechanisms and cellular response to protect themselves from the increased ROS is by inhibiting insulin dependent nutrient uptake (Ceriello and Motz, 2004). ROS may act by intracellular signaling pathway to induce insulin resistance (Evans *et al.,* 2003). Further β –cells are sensitive to ROS since they are low in antioxidant enzymes (Tiedge *et al.*, 1997) Pathogenesis of diabetes by reactive oxygen species may be by acting on the membranes of β-cells which are an important site for the glucose transporter GLUT2 (West, 2000). Many animal models suggest that ROS may play a causal role in CVD by mediating various cellular signaling pathway from initiation of fatty streak development to ultimate plaque rupture (Madamanchi *et al.,* 2005). ROS can induce cancer by nuclear and mitochondrial DNA damage, lipid peoxdation, forming DNA adducts and by altering cellular signaling pathways (Valko *et al.,* 2006). Persistent oxidative stress has been suggested to play an important part in tumor biology of cancer including activated transcription factors, proto-oncogenes, genomic instability, chemotherapy resistance, invasion and metastasis (Toyokuni *et al.,* 1995). ROS may interact with and modify DNA, cellular lipids and proteins and may result into altered cell function (Klaunig *et al.,* 1998).

Fruits, vegetables, herbs and other foods rich in phenolic phytochemicals with potential antioxidant activity can counteract and alleviate damage resulting from oxidative stress (Dembinska-Kiec *et al.,* 2008; Bisbal *et al.,* 2010). Phenolic structures found in foods can stabilize reactive oxygen species by donating electrons and by delocalization of unpaired electron on the resulting phenolic-radical derivative (Rice-Evans *et al.,* 1997).

2.6.1 Role in Type 2 Diabetes Management

Excess calorie intake and reduced physical activity induces insulin resistance. Impaired glucose tolerance results from the loss of ability of the β-cells to compensate for the insulin resistance which evolves into diabetes (Ceriello and Motz, 2004). Phenolic phytochemicals from fruits and vegetables is a low Cost, widely accepted and accessible, no side effect approach to diabetes control and management. Bisbal *et al.,* (2010), have reviewed a list of antioxidant compounds from common food sources that exhibit beneficial activities on glucose metabolism and type 2 diabetes. Early onset type 2 diabetes is associated with a postprandial increase in blood glucose level a condition known as hyperglycemia when intestinal, pancreatic and salivary enzymes (α-glucosidase and α-amylase) rapidly break down soluble carbohydrates. Inhibition of intestinal α-glucosidase is a good strategy to control the postprandial rise in blood glucose since it delays the breakdown of starch and sucrose (Bischoff, 1994). There are several commercial drugs that have a similar mode of action; however these drugs may lead to side effects including abdominal distention, flatulence, meteorism and possibility diarrhea (Puls *et al.*, 1977; Bischoff, 1994). Many phenolic compounds such as catechin, caffeic acid, resveratrol, quercetin, protocatecheuic, anthocyanins and rosmarinic acid found in common foods have been reported to have moderate to high α-glucosidase and α-amylase activity *in vitro* (Matsui *et al.*, 2001; McCue and Shetty, 2004; Kwon *et al.,* 2006a). Hanhineva *et al.,* (2010), in their review provide an excellent compilation of effect of pure phenolics and dietary plants or extracts on carbohydrate homeostasis measured *in vitro*. Phenolic phytochemicals can bring about inhibition of enzyme by binding to the active site or by blockage at several sub site of

the enzyme (Randhir *et al.*, 2007). Polyphenols can bring about enzyme inhibition by formation of a polyphenol-enzyme complex at the active center through the interactions of hydroxyl groups in phenols with polar groups (amide, amino and carboxyl group) in enzyme (He *et al.,* 2007). Dietary phenols can inhibit glucose metabolism enzymes and thereby reduce glucose absorption rates and shift glucose absorption to more distal parts of the small intestine (Johnston *et al.,* 2003).

Intestinal cells express glucose transporters such as sodium dependent SLGT1, GLUT2 or GLUT5 that mainly perform glucose uptake (Shimizu *et al.,* 2000). Many phenolic compounds including quercetin (Welsch *et al.,* 1989; Ader *et al.,* 2001; Cermak *et al.,* 2004), green tea catechins (Kobayashi *et al.,* 2000; Shimizu *et al.,* 2000), tannic acid, cholorogenic acid, caffeic acid, ferulic acid (Welsch *et al.,* 1989) inhibited SLGT1 in the brush border membrane vesicles. Inhibition of GLUT2 by myricetin, quercetin, and its glycoside, isoquercetin has been reported (Kwon *et al.,* 2007a). It was also reported that quercetin aglycone and quercetin-3-rhamnogluCOSide (rutin) were unable to inhibit glucose transporter SLGT1 (Ader *et al.,* 2001).

Flavanols from dark chocolate increased insulin sensitivity and β-cell function in insulin sensitive patients (Grassi *et al.,* 2008). Water soluble polyphenolic fraction from cinnamon was found to potentiate insulin action (Anderson *et al.,* 2004) and could be beneficial to diabetic individuals by delaying the gastric emptying rate and reduction in postprandial blood glucose level (Hlebowicz *et al.,* 2007). Green tea polyphenols can regulate genes involved in insulin signaling pathways in rats fed on high fructose diet (Cao *et al.,* 2007). Green tea has been shown to enhance insulin activity and sensitivity

and green tea polyphenols are the key compounds involved in this process (Anderson and Polansky, 2002; Wu *et al.,* 2004a).

2.6.2 Role in Cardiovascular Diseases

Insulin resistance, impaired glucose tolerance and diabetes all resulting from reactive oxygen species are associated with an increased risk of cardiovascular diseases (CVD) (Ceriello and Motz, 2004). Polyphenols play a key role in the many physiological events involved in cardiovascular diseases such as hypertension, arthrosclerosis and stroke. Phenolic bioactives may prevent hypertension by ACE inhibition which converts Angiotensin I to Angiotensin II a potent vasoconstrictor (Kwon *et al.,* 2006b). Platelet aggregation is a key step in acute coronary heart disease and flavonoids are suggested to prevent CVD by improving endothelial function and inhibiting platelet aggregation (Vita, 2005). Green tea polyphenols can inhibit oxidation of LDL, decrease lipid peroxidation, inhibit NO production by suppression of the iNOS pathway and ameliorate pro-inflammatory cytokine production. Function of Green tea polyphenols extend beyond its simple antioxidant activity in regulating factors that lead to oxidative stress which in turn can lead to CVD (Tipoe *et al.,* 2007). Perez-Vizcaino *et al.,* (2006), reviewed the potential of protective effects of quercetin and wine polyphenols by improving endothelial function in preventing CVD.

2.6.3 Chemoprevention of Cancer Using Phenolic Phytochemicals

It is estimated that approximately two thirds of the cancers can be prevented by appropriate changes in diet and lifestyle and people who eat more than five servings of fruits and vegetables reduce their chances of developing cancer by 50% as compared to people who eat less than 2 servings (Surh, 2003). Chemoprevention refers to the use of chemical agents to retard, inhibit, or reverse specific stages of carcinogenic process (Surh, 2003). Some of the chemopreventive phenolic compounds with their common foods sources are resveratrol from grapes (Jang *et al.,* 1997), genistein from soybean (Peterson and Barnes, 1991), catechins from tea (Naasani *et al.,* 1998), curcumin from turmeric (Kawamori *et al.,* 1999), and gingerol from ginger (Bode *et al.,* 2001). Carcinogen activation/detoxification by xenobiotic metabolizing enzymes; DNA repair; cell-cycle progression; cell proliferation, differentiation and apoptosis; expression and functional activation of oncogenes or tumour-suppressor genes are some of the mechanisms by which phenolic phytochemicals show their chemopreventive effects (Surh, 2003). Green tea catechins have shown reduced levels of markers of oxidative DNA damage in oral cells, urine and leukocytes of smokers (Thomasset *et al.,* 2007). Although individual phenolic compounds have shown health beneficial properties including anticancer effects, the natural combination of phenolic phytochemicals present in whole fruits and vegetables exhibit additive and synergistic effects that no single phenolic compound can replace (Liu, 2004). Future of phytochemical chemoprevention would focus on using synergistic and additive effects of phenolics to design specific foods with different mechanisms to interfere with a specific stage of metabolic syndrome.

2.7 Phenolic Phytochemicals as Antimicrobials

Phenolic compounds can act as antimicrobials by a number of different mechanisms. Phenolic compounds can quench electrons from electron transport chain (ETC) along the bacterial membrane and act as antimicrobials by disrupting oxidative phosphorylation or inhibit dehydrogenases linked proton efflux by interfering with the flow of electrons at the level of cytochromes (Vattem *et al.,* 2005a). It is also possible that simple phenolics can disrupt the H^+-ATPase required for ATP synthesis by causing hyper-acidification via proton donation at the plasma membrane (Shetty and Labbe 1998; Shetty and Wahlqvist 2004). Another explanation is that partially hydrophobic polyphenols can attach to the cell wall and stack themselves causing destabilization of the membrane leading to membrane disruption or transport inhibition (Shetty and Wahlqvist, 2004). Phenolic compounds from berries were shown to inhibit an *E. coli* mutant which lacked DNA repairing ability indicating these compounds act by damaging the DNA (Puupponen-Pimia *et al.,* 2001). The hydroxyl group and a system of delocalized electrons of some phenolic compounds can destabilize the cytoplasmic membrane and cause cell death by a change in proton motive force across the membrane (Ultee *et al.,* 2002).

Berry extracts generally inhibit gram-negative bacteria but not gram-poisitve bacteria which reflects the differences in the cell surfaces of these bacteria. Gram-negative bacteria use their lipid bilayer as a barrier against hydrophobic compounds. Antimicrobial activity against LAB was correlated to the hydroxyl groups present on the B ring of these compounds (Puupponen-Pimia *et al.,* 2001).

Moreno *et al.,* (2006), suggested that carnosic acid and rosmarinic acid are the major antimicrobial compounds present in rosemary extracts and that inhibition is associated with specific phenolic composition. Protocatechuic acid, caffeic acid, coumaric acid, rosmarinic acid and quercetin were all detected in ethanolic extract of clonal rosemary that inhibited *Helicobacter pylori*. Further antioxidant activity was correlated to pathogen inhibition in these studies (Chun *et al.,* 2005). Gallic acid and *p*-hydroxybenzoic were effective in inhibiting the gastrointestinal pathogen C*ampylobacter jejuni* even at concentration of 1mg/L whereas quercetin and catechin were not effective even at 1g/L. This suggests that the type of phenolics is more important than its concentration (Ganan *et al.,* 2009). Cranberry concentrate was shown to inhibit four major food borne pathogen including *E. coli, Salmonella, Listeria* and *Staphylococcus aureus* and transmission electron microscopy revealed damage to bacterial walls and membranes (Wu *et al.,* 2008). Vattem *et al.,* (2004), showed that antimicrobial activity using fungal bioprocessing of cranberry pomace was enhanced and inhibition of *vibrio, E. coli* and *listeria* correlated to the enrichment of ellagic acid content. Rauha *et al.,* (2000), reported inhibition of nine microbial species with Finnish plant extracts containg phenolic compounds using agar diffusion assay. Antimicrobial activity of wine phenolics against *S. aureus*, *E. coli* and, *C. albicans* was shown using agar diffusion assay and the zone of inhibition related to the total phenolic content (Papadopoulou *et al.,* 2005). Several phenolic acids were tested for against Listeria and results showed that the antibacterial activity was related to pH. Caffeic, coumaric and ferulic acid showed bactericidal effect at a pH of 4.5 and bacteriostatic at higher pH whereas chlorogenic acid was effective at inhibition only at pH 6.5 (Wen *et al.,* 2003).

Polyphenols on entering the colon are degraded into low molecular weight phenolic acids (Scalbert *et al.,* 2002; Aura, 2008). Flavonoids undergo ring fission in which hydroxylated aromatic compounds are formed from the A-ring, phenolic acids from the B- ring and the C ring is degraded (Aura, 2008). Tannins of gallic acid did not show inhibitory properties against Bifidobacteria or Lactobacillus however methyl gallate tannins showed inhibitory activity against intestinal LAB (Ahn *et al.,* 1998). Gallic acid and anthocyanins were reported to stimulate growth of *Leuconostoc oenos* whereas vannilic acid showed inhibitory effects and protocatechuic acid showed no effect (Vivas *et al.,* 1997). Tannins were reported to strongly affect cell viability however catechin and epicatechin, quercetin, kaempeferol did not affect growth significantly (Figueiredo *et al.,* 2008). Single phenolic fraction of hydroxytyrosol from olive strongly inhibited *L. plantarum* and combined phenolic fractions containing gluCOSides, oleuropein and verbasCOSide showed moderate inhibition (Ruiz-Barba *et al.,* 1993). By contrast, Alberto *et al.,* (2001), reported stimulation and higher cell densities of *Lactobacillus hilgardii* by catechin and gallic acid in two different media tested. It has been reported that the number of hydroxyl groups on the B- ring of flavonols influence inhibition of LAB (Puupponen-Pimia *et al.,* 2001). Flavonol myricetin has been shown to inhibit all lactic acid bacteria of the intestine whereas quercetin and kaempeferol with two less hydroxyl groups less on the B-ring has no effect (Puupponen-Pimia *et al.,* 2001). They further suggested that berry phenolics did not inhibit gram-positive bacteria (Puupponen-Pimia *et al.,* 2001).

Helicobacter pylori is a gram-negative, flagellate, microaerophilic pathogen that colonizes in the hostile environment of the human stomach (Warren and Marshall,

1983; Mitchell, 1999; Montecucco and Rapuoli, 2001) and has been implicated in the etiology of several diseases including gastritis, gastric cancer, peptic ulcer, muCOSa associated lymphoid tissue lymphoma and recently, cardiovascular diseases (Warren and Marshall, 1983; You *et al.,* 2000; Uemura *et al.,* 2001). Phenolic compounds showing inhibitory activity against *H. pylori* include tannins, catechins, quercetins, naringin, individually or in combination with other compounds (Yanagawa *et al.,* 2003; Funatogawa *et al.,* 2004; Shin *et al.,* 2005). Bioactives from Tea (Yee and Koo, 2000; Matsubara *et al.,* 2003; Ruggiero *et al.,* 2007), Wine (Daroch *et al.,* 2001; Ruggiero *et al.,* 2007), Herbs (Tabak *et al.,* 1996; Chun *et al.,* 2005; Li *et al.,* 2005; Mahony *et al.,* 2005), and fruits including berries (Kubo *et al.,* 1999; Malekzadeh *et al.,* 2001; Chatterjee *et al.,* 2004) have shown inhibition against stomach ulcer causing bacterium *H.* pylori.

2.8 Tea Polyphenols: Chemistry and Health benefits

Tea is the most consumed beverage in the world and although it originated in Southeast Asia it is now cultivated in more than 30 countries. Green tea is made by steaming fresh leaves and preventing oxidation whereas black and oolong tea are made by allowing withered tea leaves to undergo fermentation by crushing and rolling the tea leaves (Mukhtar and Ahmad, 2000). Flavanols are the most abundant polyphenolics found in tea and dry leaves may contain up to 30-40% catechins by weight (Lambert and Yang, 2003). Epicatechin, epicatechin-3-gallate, epigallocatechin and epigallocatechin-3-gallate are the major flavanols found in unfermented green tea whereas in black tea the major polyphenolics are polymers of catechins; theaflavins and

thearubigens due to enzymatic catalysis by polyphenol oxidase (Ahmad and Mukhtar, 1999). The antioxidant potential of tea catechins is in the following order of decreasing effectiveness : epicatechin gallate- epigallo catechin gallate > epigallo catechin > gallic acid > epicatechin – catechin (Rice-evans *et al.*, 1996). While the health beneficial effects of tea may be attributed to its antioxidative abilities, other mechanisms such as enzyme inhibition, anti-proliferative effects may be important. Tea and its constituents have not only shown inhibitory activity against initiation, promotion and progression of carcinogenesis and but also exhibited inhibition of carcinogenesis in the skin, lung, liver, intestine, pancreas, colon and mamamary gland (Lambert and Yang, 2003). Tea catechins have shown protective effects in *in vivo* studies on markers of cancer, CVD, and other degenerative diseases (Crespy and Williamson, 2004; Cabrera *et al.*, 2006)

2.9 Phenolic Bioactive Compounds from Apple, Pear and Cherry

Apples are one of the most consumed fruit in the world and in the United States of America. However, in the U.S, only ten cultivars account for more than 90% of the Apple production out of almost 100 cultivars cultivated. Health beneficial properties of apple such as protection against cancer, CVD, asthma, weight loss, type 2 diabetes and cholesterol lowering effects have been demonstrated in many epidemiological, animal and *in vitro* studies (Boyer and Liu, 2004). Lee *et al.*, (2003), determined the major phenolics present in apple and reported that quercetin glycosides were present in the highest amount followed by procyanidin, cholorogenic acid and epicatechin. In their study, antioxidant activity was correlated to the amount of phenolics present. Tsao *et al.*, (2003), did a similar study and reported that procyanidin was the dominant

polyphenol found in both flesh and peel whereas quercetin glycoside was dominant almost in peel and hydroxycinnamic acid in the flesh. Eight different varieties of apple were analyzed and the mean polyphenol content was reported to be between 66-211 mg/100g fresh weight (Vrhovsek *et al.,* 2004). Further up to 20 phenolic compounds were identified and the synergestic/additive effects of these compounds might explain the large number of health beneficial properties exhibited by this fruit.

Pear in the USA is consumed fresh, canned, juiced and dried. Pear production in the USA is mostly concentrated in the northwest around the region of Washington and Oregon and per capita consumption of all pear products was reported to be around 5.4 pounds (Agricultural marketing resource centre, www. agmrc.org). In a study on pear phenolics, Schieber *et al.,* (2001), reported cholorogenic acid to be the major phenolic present followed by isorhamnetin glycoside, quercetin glycoside and epicatechin. At harvest browning in pear is related to the hydroxycinnamic acid esters and flavanols present. However, after 4 days browning depended on cultivar rather than phenolic content or composition (Amiot *et al.,* 1995). Sun-drying has caused 64% decrease in the amount of phenolic compounds however not all compounds were equally affected. Hydroxycinnamic acids and procyanidin were most affected whereas catechins and arbutin were less affected (Ferreira *et al.,* 2002).

In the United States only two types of cherries are commercially produced: sweet and tart cherries. Neochlorogenic acid, coumaroylquinic acid, chlorogenic acid, epicatechin and rutin were the major phenolics detected in sweet cherries (Usenik *et al.,* 2008). Antioxidant capacity and total phenolics decreased during early developmental

stages of sweet cherry however exponentially increased at a stage which coincided with anthocyanin accumulation and fruit darkening (Serrano *et al.,* 2005).

2.10 Proline-Linked Pentose Phosphate Pathway

Cellular antioxidant homeostasis is governed by a fine balance of antioxidant defense systems that can absorb and buffer a large pool of reductants and oxidants (Foyer and Noctor, 2005). Low molecular weight cellular antioxidants such as glutathione, ascorbate, tocopherol can provide information about the cellular redox state and influence gene expression associated with biotic and abiotic stress by interacting with cellular components (Foyer and Noctor, 2005). Reactive oxygen species (ROS) is continuously produced as a result of aerobic respiration and it is suggested that ROS in low amounts may be useful in intracellular messaging and defense against pathogens however at high concentration they are harmful to cellular components (Schulze-Osthoff *et al.,* 1997; Lee *et al.,* 1998). Antioxidant enzymes such as superoxide dismutase (SOD), Catalse (CAT), Glutathione S-transferase (GST), Glutathione peroxidase (GPX) and molecular antioxidants such as ascorbate, tocopherol, β-carotene, gluthione (GSH) are efficient in removing ROS and maintaining cellular antioxidant homeostasis by a series of redox reactions but there are many sites of ROS production and few mechanisms for removal (Mates *et al.,* 2008). To maintain the efficiency of this defense system cellular antioxidants which have been used up have to be regenerated. Regeneration is an energy intensive process and requires a group of oxido-reductases using cellular reducing equivalents (RE) NADPH and $FADH_2$ (Mates and Sanchez-Jimenez, 1999; Mates *et al.,* 1999; Nordberg and Arner, 2001). In order to meet the

cellular demand for these RE a model has been proposed by Shetty, (2004), where phenolic antioxidants may mediate the antioxidant response of the cell, not only by acting as direct scavengers of ROS but also by stimulating certain enzymatic pathways in the cell that can replenish the need for reducing equivalents to aid the antioxidant response system. One such pathway which can provide NADPH and can be up-regulated is the pentose phosphate pathway (PPP). While primarily being anabolic, PPP does involve oxidation of glucose and is a major source of reducing power in the form of NADPH, and supplies metabolic intermediates for biosynthetic processes by committing glucose to the production of 5-carbon sugars (Kruger and von Schaewen, 2003).

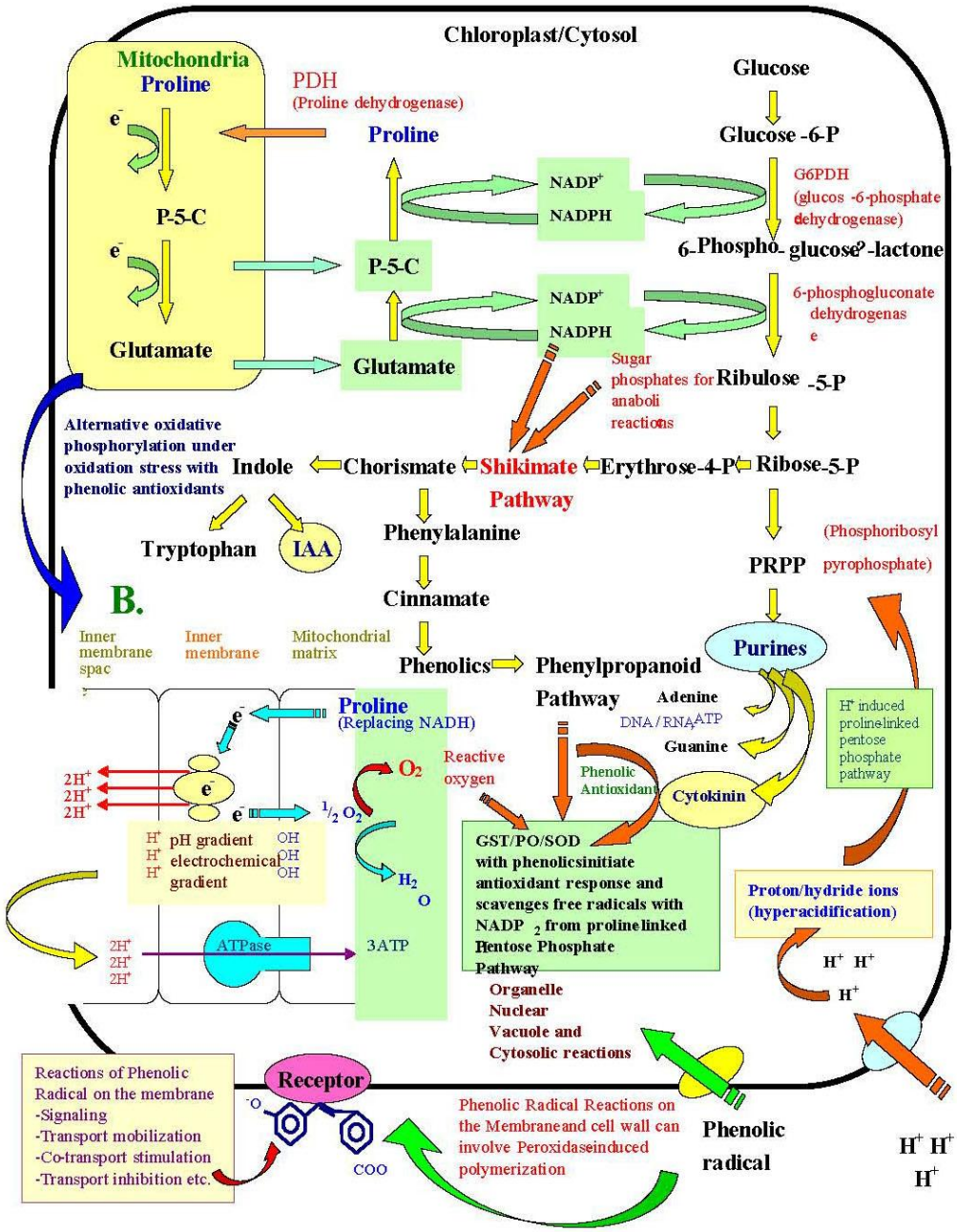

Figure 5: Proline linked pentose phosphate pathway

(Adapted from Shetty, 2004; Shetty and Wahlqvist, 2004)

Proline biosynthesis requires NADPH and initiates with glutamate being converted to pyroline-5-carboxylate (P5C) by a series of reactions and finally P5C is reduced to proline by P5C reductase (Kishor *et al.,* 2005). Proline and P5C are important metabolic regulators and can act as a redox couple (Hagedorn and Phang, 1983; Phang, 1985). Proline synthesis in the cytosol also generates NADP+ which is a co-factor for G6PDH, the first rate limiting enzyme of PPP. PPP has been shown to be up-regulated by a decrease in NADPH/$NADP^+$ ratio in rat liver (Fabregat *et al.,* 1985). In this proposed model, proline biosynthesis is coupled to stimulation of PPP through NADPH/$NADP^+$ redox balance (Shetty, 2004) (Fig 5). Phenolic antioxidants can channel the carbon flux from the TCA cycle for proline biosynthesis which in turn can stimulate PPP (Shetty and Wahlqvist, 2004). The coupling of proline biosynthesis and PPP should produce increased amounts of NADPH which can feed into anabolic reactions such as phenolic synthesis through the shikimate and the phenylpropanoid pathway and antioxidant response pathway involving SOD, GST and Peroxidases (Shetty, 2004; Shetty and Wahlqvist, 2004). During stress, proline linked pentose phosphate pathway may be especially active to a) drive the PPP to provide intermediates for anabolic reactions b) drive the PPP to provide NADPH for anabolic synthesis and antioxidant response pathways c) activate biosynthesis of proline which can enter the mitochondria and provide the reducing equivalents for oxidative phosphorylation and hence provide rapid, non-NADH route to ATP synthesis (Shetty, 2004).

2.11 Application of Proline Linked Pentose Phosphate Pathway for Disease Management, Antimicrobial Applications and Phenolic Phytochemical Elicitation

Understanding the role of proline associated pentose phosphate pathway in different systems can be used for metabolic regulations by inducing antioxidant response, maintain cellular function and biosynthesis of secondary metabolites. While some are promising and well-studied, others are in their initial stages and system specific.

2.11.1 PLPPP for Oxidative Stress Linked Disease Management

Reactive oxygen species (ROS) are continuously produced as a result of cellular metabolism either through mitochondrial electron transport chain (Shigenaga *et al.*, 1994) or through their involvement in normal physiological processes such as innate immunity, signal transduction or other biochemical reactions (Lambeth, 2007). Degenerative diseases of ageing such as cancer, CVD, immune system breakdown and brain dysfunction are associated with ROS involved in oxidation of lipids, DNA, proteins and other biological entities (Ames *et al.*, 1993b). Cells have an efficient antioxidant defense system against ROS, involving antioxidant enzymes and cellular antioxidants however, they have to be regenerated and this requires NADPH (Mates and Sanchez-Jimenez, 1999; Mates *et al.*, 1999; Nordberg and Arner, 2001).

Phenolic antioxidant induced stimulation of the proposed proline-associated PPP can positively modulate and potentially protect against oxidation-linked diseases by replenishing the cellular need for NADPH required for the antioxidant enzyme

response. In eukaryotic systems phenolic phytochemicals can modulate the carbon flux through the TCA cycle through α-ketoglutarate towards glutamate and finally to proline in the cytosol (Shetty, 2004). This proline biosynthesis through glutamate requires NADPH which is produced in the oxidative steps of the PPP (Kruger and von Schaewen, 2003). This increased demand for NADPH up-regulates the PPP (Shetty and Wahlqvist, 2004). NADPH generated through the stimulated PPP can be used for maintaining the efficiency of the antioxidant response system by regenerating the cellular pool of antioxidants (Shetty, 2004).

Proton gradients across the cell membrane can be altered through the proton donating ability of phenolic acids and chelating ability of flavonoids (Shetty, 2004). Alterations in the proton gradient across the membrane can initiate a number of signaling cascades and can result into changes in certain pathways, one of which being stimulation of PPP (Pugin *et al.*, 1997; Stout and Charles, 2003; Cohen and Fields, 2004). Partially hydrophobic phenolics by interacting with membrane micro domains, channels and proton pumps can have a similar effect eventually leading to stimulation of PPP (Tsuchiya, 2001; Tachibana *et al.*, 2004; Hendrich, 2006). Stimulation of PPP will generate NADPH required for maintaining cellular redox balance by cellular antioxidant regeneration and antioxidant enzyme functioning.

Oregano phenolics in stressed porcine muscle have shown increased G6PDH activity, an enzyme that controls the rate limiting step of PPP, which can provide NADPH required for antioxidant enzymes. Reduced MDA levels were found in the muscle which indicated an increase in antioxidant protective response along with high concentration of proline possibly indicating proline biosynthesis being associated with

PPP (Randhir *et al.,* 2005). In a similar study, cranberry phenolics showed up regulation of PPP along with accumulation of free proline. Further the increase in antioxidant enzyme response correlated with decreased MDA (Vattem *et al.,* 2005b). Higher G6PDH and antioxidant content was shown in dark germinated corn, mung bean sprouts and velvet bean which were shown by soaking the seeds with oregano extract (Randhir and Shetty, 2005; Randhir *et al.,* 2009).

Phenolic antioxidants can exhibit their biological functionality in eukaryotes by modulating cellular redox response by acting as direct scavengers of antioxidants by accepting and delocalization of free radicals from substrates or by mediating of antioxidant response through proline associated pentose phosphate pathway (Shetty, 2004; Shetty and Wahlqvist, 2004). By understanding the role of phenolic antioxidants in mediating antioxidant response through PPP functional foods and ingredients can be designed to counter oxidative malfunction based diseases including CVD, diabetes, and neurodegenerative diseases.

2.11.2 PLPPP for Antimicrobial Applications

Proline can serve as an important energy source since it can generate ATP by oxidative phosphorylation in the mitochondria (Shetty and Wahlqvist, 2004) or by gluconeogenesis (Kowaloff *et al.,* 1977; Adams and Frank, 1980). Regulation of proline metabolism by PDH, localized at the mitochondria inner membrane of eukaryotes and at the cytoplasmic surface of the bacterial plasma membrane, catalyzes the conversion of proline to pyrroline-5-carboxylate (P5C), and may serve as an important mechanism

for allocation of fuel sources (Kowaloff *et al.,* 1977). Shuttling of redox equivalents between cytosol and mitochondria in the proline cycle is mediated by PDH (Hagedorn and Phang, 1983; Phang, 1985). Inhibition of PDH when proline associated PPP is induced may be an important mechanism involved in inhibition of pathogenic bacteria. Oxidative phosphorylation on the outer plasma membrane could be disrupted in prokaryotic cells by proline mimicking small phenolics which can inhibit proline oxidation via PDH. If this is assumed to be true then addition of proline to the growth media should overcome pathogen inhibition. Kwon *et al.,* (2007b), reported that the inhibitory activity of caffeic acid against *S. aureus* was reversed with the addition of 0.5 mM proline to the media. Listeria inhibition by oregano, cranberry phenolics was reversed by addition of proline (Apostolidis *et al.,* 2008). Further *H. pylori* inhibition by cranberry and oregano synergies was suggested to be by disruption of energy production by inhibition of PDH (Lin *et al.,* 2005). Inhibition of *H. pylori* by fermented cherry extracts was reversed by addition of 0.5mM proline (Unpublished results).

Induction of proline associated PPP may increase PDH activity to provide reducing equivalents through a non-NADH route for energy synthesis (Shetty, 2004). PDH activity may generate ROS, especially superoxide radicals, which can cause apoptosis via mitochondrial pathway (Liu *et al.,* 2006; Phang *et al.,* 2008).

2.11.3 PLPPP for Phytochemical Elicitation

Phenolic phytochemicals are compounds that present numerous health beneficial properties and can reduce the risk of various oxidation linked diseases including type 2 diabetes, cancer, CVD, neurodegenerative diseases (Szajdek and Borowska, 2008). Phenolic biosynthesis involves linking of the enzymatic steps of the shikimate, phenylpropanoid and the flavonoid pathway (Jaganath and Crozier, 2010). Shikimate pathway which is the starting point of phenolic biosynthesis, is initiated by phosphenolpyruvate from the TCA cycle and erythrose-4-phosphate from the PPP (Tzin and Galili, 2010). Stimulation of proline associated PPP can provide excess erythrose-4-phosphate and hence drive the shikimate and the phenylpropanoid pathway towards phenolic synthesis (Shetty, 2004). Proline biosynthesis can be stimulated by the use of proline analogue, azetidine-2-carboxylate (PDH inhibitor) and hydroxyproline (competitive inhibitor) which in turn will up-regulate PPP providing excess E4P for phenolic biosynthesis (Shetty, 2004). Proline analogues (A2C and hydroxyproline) and proline precursors (ornithine and arginine) have been used to stimulate rosmarinic acid in clonal oregano (Yang and Shetty, 1998) and thyme (Kwok and Shetty, 1998) and total phenolics, antioxidant activity and L-DOPA in Fava bean (Shetty *et al.,* 2003). Natural elicitors like fish protein hydrolysates, lactoferrin and oregano extracts have shown an increase in total phenolics with stimulation of rate limiting enzyme G6PDH of the PPP in Fava bean (Randhir et al., 2002; Randhir and Shetty, 2003), mung bean (Randhir *et al.,* 2004) and pea (Andarwulan and Shetty, 1999).

Induction of protective secondary metabolite has been shown in response to biotic and abiotic stresses (Dixon *et al.,* 1994; Dixon and Paiva, 1995). Plants produce

phenolic compounds as a part of their stress adaptation response and biosynthesis may be through stimulation of proline associated pentose phosphate pathway (Shetty, 2004).

In response to wounding (Hahlbrock and Scheel, 1989), nutritional stress (Graham, 1991), and temperature stress (Christie *et al.*, 1994; Zimmerman and Cohill, 1991) induction of many phenolic compounds synthesized in the phenylpropanoid pathway has been shown. Pentose phosphate pathway has been suggested to play an important role in protecting yeast against oxidative stress (Juhnke *et al.,* 1996; Slekar *et al.*, 1996). In response to polymeric dye tolerance in clonal oregano, increased proline and phenolic accumulation was observed indicating the aromatic pollutant stress tolerance may be regulated through proline associated pentose phosphate pathway (Zheng *et al.*, 2001). In creeping bentgrass, proline associated pentose phosphate pathway regulated antioxidant enzyme response for enhanced stress tolerance in response to cold stress (Sarkar *et al.,* 2009a) and application of marine peptide and chitosan oligosaccharide (Sarkar *et al.,* 2009b). Stimulation of proline associated pentose phosphate pathway in response to microwave (Randhir and Shetty, 2004) and UV (Shetty *et al.,* 2002) stress has been reported in germinating fava bean.

Cell suspensions of *Hypericum perforatum* L. have been shown to accumulate xanthones when inoculated with *Colletotrichum gloeosporioides* (Conceicao *et al.,* 2006). Phenolic accumulation was shown in red pines after inoculation with bark bettle-vectored fungus (Klepzig *et al.,* 1995). Insect and fungal attacks have shown to increase phenolic content in sorghum depending on the stage of growth of the plant and cultivar (Woodhead, 1981). Organically grown plants are exposed to greater environmental stresses which are countered by higher accumulation of phytochemical suggesting

organic farming may be a good strategy to improve phenolic phytochemical content of plants (Young *et al.,* 2005). In clonal lines of thyme *Pseudomonas spp* inoculation has shown high phenolics (Shetty *et al.,* 1996) indicating a possible link of the proline associated pentose phosphate pathway in countering environmental stresses by phenolic biosynthesis and antioxidant enzyme response.

Based on the rationale of health benefits of dietary phenolics as outlined in this literature review the major goals of this dissertation proposal are to understand the benefits of phenolic bioactives in the context of management of type 2 diabetes and ulcer causing bacteria using specific whole food extracts and their fermented derivatives. To this end we initially we explored tea polyphenols as models to optimize my experimental system and further explored similar benefits in fruits apple, pear and cherry of the rosaceae family. Further at the metabolic response level this dissertation explored how host eukaryotic/prokaryotic systems responded to these phenolic bioactives from rosaceae fruit family.

CHAPTER 3

OBJECTIVES

3.1 Major Objectives

The major objectives of this dissertation research are:

a) Evaluation of antihyperglycemia, antihypertension, *Helicobacter pylori* inhibition properties of tea phenolic bioactives and influence of extraction time as an initial model for further studies using *Rosaceae* family.

b) Dietary management of hyperglycemia, hypertension, and modulation of beneficial bacterial responses using probiotic bacteria mediated fermentation of apple, pear and cherry juice from the *Rosaceae* family.

c) Induction of phenolic biosynthesis in fava bean and antioxidant enzyme response using mechanistic rationale of the proline associated pentose phosphate pathway in fava bean and yeast using fermented apple extracts.

d) Evaluation of superficial scald reduction using DPA and Chitosan Treatments in relation to phenolic linked metabolic regulation and its effect on in vitro anti-hyperglycemia models.

3.2 Specific Objectives

The specific aims derived from the major objectives are:

3.2.1 Evaluation of Antihyperglycemia, Antihypertension, *Helicobacter pylori* Inhibition Properties of Tea Phenolic Bioactives and Influence of Extraction Time.

a) Anti-Hyperglycemia properties of tea (*Camellia sinensis*) bioactives using *in vitro* assay models and influence of extraction time

b) Inhibitory potential of tea polyphenolics and influence of extraction time against *Helicobacter pylori* and lack of inhibition of beneficial lactic acid bacteria.

3.2.2 Dietary Management of Hyperglycemia, Hypertension, and Modulation of Beneficial Bacterial Responses using Probiotic Bacteria Mediated Fermentation of Apple, Pear and Cherry Juice.

a) Fermentation of Whole Apple Juice Using *Lactobacillus helveticus* for Potential Dietary Management of Hyperglycemia, Hypertension, and Modulation of Beneficial Bacterial Responses.

b) Phenolic Bioactive Modulation by *Lactobacillus helveticus* Mediated Fermentation of Cherry Extracts for Anti-Diabetic Functionality, *Helicobacter pylori* inhibition and Probiotic *Bifidobacter longum* Stimulation.

c) Evaluation of Antihyperglycemia and Antihypertension Potential of Pear juice Fermented with *Lactobacillus helveticus* Using *in Vitro* Assay Models

3.2.3 Induction of Phenolic Biosynthesis in Fava bean and Antioxidant Enzyme Response using Mechanistic Rationale of the Proline Associated Pentose Phosphate Pathway in Fava bean and Yeast using Fermented Apple Extracts.

a) To investigate the mechanism of action of fermented apple extracts in modulating oxidative stress response and phenolic biosynthesis in light germinated fava bean through proline-associated pentose phosphate pathway.

b) To investigate the mechanism of action of fermented apple extracts in modulating oxidative stress, induced by hydrogen peroxide, in yeast eurkaryotic model through proline-associated pentose phosphate pathway.

3.2.4 Evaluation of Superficial Scald Reduction using DPA and Chitosan Treatments in Relation to Phenolic Linked Metabolic Regulation and its Effect on *in vitro* Anti-Hyperglycemia Models.

a) To investigate the mechanism of action of chitosan treatments in relation to commercially used DPA in modulating phenolic biosynthesis and oxidative stress response in preventing scald development in Cortland Apples and the possible role of proline-associated pentose phosphate pathway.

CHAPTER 4

TEA, INFLUENCE OF EXTRACTION TIME AND ITS HEALTH EFFECTS

4.1 Anti-Hyperglycemia Properties of Tea (*Camellia sinensis*) Bioactives using *in vitro* Assay Models and Influence of Extraction Time

4.1.1 Abstract

Tea (C*amellia sinensis*) has well known health benefits, which are attributed to its polyphenolic metabolites. This research explored the potential of regular tea consumption and influence of extraction time typically used in daily consumption of tea, as a therapeutic dietary support for potential management of early stage type 2 diabetes using *in vitro* assay models. Extraction times of 2 and 5 minutes were compared. Five min extraction time had significantly higher total phenolic content when compared to the 2 min extraction time. Choice Darjeeling 5 min extraction yielded highest amount of total phenolics (299.6 ± 5.9 mg/g) followed by Tazo Black 5 min (240 ± 9.7 mg/g), whereas Bigelow Green 2 min had the lowest total phenolic content (53 ± 8.2 mg/g). DPPH scavenging-linked antioxidant activity was high (81-91%) for all types evaluated and for most samples it was influenced by the extraction time. Similarly, high *in vitro* α-glucosidase inhibition was observed in almost all the samples assayed and for most samples the 5 min extraction had significantly higher inhibition when compared to the 2 min extraction time. The most fermented teas showed highest α-amylase inhibition; Choice Darjeeling 5 min had the highest inhibition (84.1%) followed by Tazo Black 5 min (71.6%). Angiotensin converting enzyme inhibition was not observed in any sample. Overall, 5 min extraction time was found to have more relevance for potential benefits for managing hyperglycemia than 2 min. This research

suggests that tea offers an attractive potential strategy to regulate postprandial hyperglycemia towards an overall dietary support for type 2 diabetes management.

4.1.2 Introduction

Polyphenols are one of the most widely distributed compounds in the plant kingdom with several thousand molecules identified in higher plants and several hundreds in edible plants (Bravo, 1998; Manach *et al.,* 2004). Polyphenols are products of secondary metabolism in plants and can range from simple molecules like phenolic acids to very large polymers like tannins. Until recently they were thought to be anti-nutritional because of their ability to inhibit digestive enzymes and reduce digestibility. However investigators have reported a potential to prevent chronic oxidation-linked diseases; most notably type 2 diabetes, cardiovascular disease and cancer which has increased the interest in food-based phenolics (Block *et al.,* 1992; Serdula *et al.,* 1996; Kim and Masuda, 1997; Shetty, 1997; Shetty and Labbe; 1998; Shetty, 1999; Shetty 2001).

Tea (*Camellia sinesis*) is one of the most consumed beverages in the world, and has enjoyed popularity for over 4000 years. Tea is usually consumed in four types Black, Oolong, Green and White. Black Tea is produced by rolling the young tea leaves to disrupt the compartmentalization and by allowing the phenolic compounds to enzymatically interact and are oxidized for about 2 hours. Oolong tea is fermented in the same way but for a shorter period. To produce Green tea, younger green leaves are rolled and steamed immediately to prevent oxidation whereas White tea is produced by harvesting very young buds during early spring (Del Rio *et al.,* 2004; Rusak *et al.,* 2008). Epicatechin, epigallocatechin, epicatechin gallate, and epigallocatechin gallate are the main phenolics in Green tea which may constitute up to 1/3rd of their dry weight (Bronner and Beecher, 1998; Rusak *et al.,* 2008). Catechins are converted to more

complex forms mainly theaflavins and their polymers thearubigins during fermentation. In recent times, studies have demonstrated the many health benefits of this beverage is generally attributed to the polyphenolic metabolites it contains. Tea has been shown to be potentially beneficial in treating many diseases such as arthrosclerosis, hypertension, infectious diseases, and cancer (Kono *et al.,* 1992; Nakayama *et al.,* 1993; Abe *et al.,* 1995; Kim and Masuda, 1997). Regular consumption of tea is also associated with stimulation of immune response system and longevity (Hu *et al.,* 1992; Sadakata *et al.,* 1992). Mice studies have shown that tea can reduce the age related oxidative damage to the brain (Kishido *et al.,* 2007). Overall; these research findings indicate that the regular consumption of tea can be potentially beneficial for prevention of oxidation-linked diseases and maintenance of overall health.

Type 2 diabetes and associated complications are increasing worldwide at alarming rates. Type 2 diabetes in part is associated with a postprandial increase in blood glucose level a condition known as hyperglycemia when intestinal pancreatic and salivary enzymes (α-glucosidase and α-amylase) rapidly break down soluble carbohydrates such as soluble starch (Bischoff, 1994). If these enzymes can be inhibited, carbohydrate absorption can be delayed which prolongs digestion and decreases the rate of glucose absorption. There are several commercial drugs that have a similar mode of action; however these drugs may lead to side effects including abdominal distention, flatulence, meteorism and possibility diarrhea (Puls *et al.,* 1977; Bischoff, 1994). Some of the early reports on anti-diabetic properties of tea are the traditional folk remedies, which a number of other authors have demonstrated the anti-diabetic potential of tea while elucidating the underlying mechanisms (Konayagi and

Minowada; 1933; Konayagi and Minowada , 1935; Isigaki, 1991; Matsumoto *et al.,* 1993; Kobayashi *et al.,* 2000; Shimizu *et al.,* 2000; Wu *et al.,* 2004a).

The basis of this investigation was to explore the relevance of daily consumption of several types of commonly consumed tea (Green, Black, Oolong, White and Earl Grey) and extraction time for determining the potential for managing hyperglycemia and related oxidative dysfunction. To this end, the optimum extraction time for everyday consumption is important and was evaluated in this study. This was undertaken by using *in vitro* biochemical and enzyme models that measured total phenolics, free radical scavenging-linked antioxidant activity by 1,1 diphenyl-2-picrylhydrazyl (DPPH) radical inhibition, and relevant α-amylase and α-glucosidase inhibitory potential.

4.1.3 Materials and Methods

4.1.3.1 Sample Extraction

A 2 g tea bag was added to 200 mL of boiling water. It was then allowed to steep for 2 or 5 min. The extract was then centrifuged at 10,000g for 10 min. Samples evaluated were Choice brand tea (Seattle, WA, USA), type included were White, Japanese Green, Oolong, and Darjeeling; Tazo brand tea (Portland, OR, USA), types included were Berry Blossom White, Green and Black as well as from Bigelow's Tea Company (Fairfield, CT, USA), types included were Green and Earl Grey. All teas were purchased from Whole Foods Market, Hadley, MA, USA.

4.1.3.2 Total Phenolics Assay

The total phenolics in all samples were determined by using a method modified by Shetty *et al.*, (1995). In brief, 0.5 mL of sample extract was added to a test tube and mixed with 0.5 mL of 95% ethanol and 5 mL of distilled water. To each sample, 0.5 mL of 50% (vol/vol) Folin-Ciocalteu reagent was added and mixed. The absorbance was read at 725 nm using a spectrophotometer (Genesys UV/Visible, Milton Roy, Inc., Rochester, NY). Different concentrations of gallic acid were used to develop a standard curve. Results were expressed as mg of gallic acid/g of sample dry weight (DW).

4.1.3.3 Antioxidant Activity by DPPH Radical Inhibition Assay

The antioxidant activity was determined by the DPPH radical scavenging method modified from Kwon *et al.*, (2006b). A 250 μL aliquot of the sample extract was mixed with 1,250 μL of DPPH (60 μM in ethanol). Absorbance was measured at 517 nm using the Genesys UV/Visible spectrophotometer. The readings were compared with the controls, containing 95% ethanol instead of sample extract. The percentage inhibition was calculated by:

$$\%\ \text{inhibition} = \frac{(Absorbance_{\text{control}} - Absorbance_{\text{extract}})}{Absorbance_{\text{control}}} \times 100$$

4.1.3.4 α-Glucosidase Inhibition Assay

The α-glucosidase inhibitory activity was determined by an assay modified from McCue *et al.,* (2005a). α-glucosidase was assayed by using 50 µL of sample extracts (diluted 1:2) and 100 µL of 0.1 M phosphate buffer (pH 6.9) containing α-glucosidase solution (1 U/mL) and was incubated in 96-well plates at 25 °C for 10 min. After preincubation, 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 incubation, absorbance readings were recorded at 405 nm by a microplate reader (Thermomax, Molecular Devices Co., Sunnyvale, CA) and compared to a control that had 50 µL of buffer solution in place of the extract. The α-glucosidase inhibitory activity was expressed as percentage inhibition and was calculated with the same equation as for percentage inhibition in the DPPH radical inhibition assay. Dose dependency was tested using 25 µL and 10 µL of the sample, the volume was made up to 50 µL using 0.1 M phosphate buffer (pH 6.9) and same protocol was followed.

4.1.3.5 α-Amylase Inhibition Assay

The α-amylase inhibitory activity was determined by an assay modified from McCue *et al.,* (2005a). A total of 500 µL of sample extract and 500 µL of 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M NaCl) containing α-amylase solution (0.5 mg/mL) were incubated at 25 °C for 10 min. After preincubation, 500 µL of a 1% starch solution in 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M NaCl) was

added to each tube at timed intervals. The reaction was stopped with 1.0 mL of dinitrosalicylic acid color reagent. The test tubes were incubated in a boiling water bath for 5 min and cooled to room temperature. The reaction mixture was then diluted after adding 5-15 mL of distilled water, and the absorbance was measured at 540 nm using the Genesys UV/Visible spectrophotometer. The readings were compared with the controls, containing buffer instead of sample extract. The percentage α-amylase inhibitory activity was calculated with the same equation as for percentage inhibition in the DPPH radical inhibition assay. Dose dependency was tested using 250 µL and 100 µL of the sample, the volume was made up to 500 µL using distilled water and same protocol was followed.

4.1.3.6 ACE Inhibition Assay

ACE inhibition was assayed by a method modified by Kwon *et al.,* (2006b). The substrate hippuryl-histidyl-leucine (HHL) and the enzyme ACE-I from rabbit lung (EC 3.4.15.1) were used. Fifty µL of sample extracts were incubated with 100 µL of 1 M NaCl-borate buffer (pH 8.3) containing 2 mU of ACE-I solution at 37 °C for 10 min. After preincubation, 100 µL of a 5 mU substrate (HHL) solution was added to the reaction mixture. Test solutions were incubated at 37 °C for 1 hour. The reaction was stopped with 150 µL of 0.5 N HCl. Five µL of the sample was injected in a high-performance liquid chromatography (HPLC) apparatus (Agilent 1100 series equipped with autosampler and DAD 1100 diode array detector, Agilent Technologies, Palo Alto, CA). The solvents used for gradient were (1) 10 mM phosphoric acid (pH 2.5) and (2)

100% methanol. The methanol concentration was increased to 60% for the first 8 min and to 100% for 5 min and then was decreased to 0% for the next 5 min (total run time, 18 min). The analytical column used was an Agilent Nucleosil 100-5C18, 250 mm × 4.6 mm inside diameter, with packing material of 5 μm particle size at a flow rate of 1 mL/min at ambient temperature. During each run, the absorbance was recorded at 228 nm, and the chromatogram was integrated using the Agilent Chemstation (Agilent Technologies) enhanced integrator for detection of liberated hippuric acid (A). Hippuric acid standard was used to calibrate the standard curve and retention time. The percentage inhibition was calculated by:

$$\%\ \text{inhibition} = \frac{(A_{\text{control}} - A_{\text{extract}})}{(A_{\text{control}} - A_{\text{blank}})} \times 100$$

4.1.3.7 Statistical Analysis

Extractions were carried out in duplicates using two different tea bags for each kind of sample. All experiments were performed at least in duplicate or triplicate. Analysis at every time point from each experiment was carried out in duplicate or triplicate. Means, standard errors and standard deviations were calculated from replicates within the experiments and analyzed using Microsoft Excel XP. Significant difference between the two and five min extraction was determined using the student *t*-test at the 0.05 probability level.

4.1.4 Results and Discussion

4.1.4.1 Total Phenolics and Antioxidant Activity by DPPH

The amount of total soluble phenolics varied not only according to the type of tea but also according to the brands tested (Fig 6). For all brands the level of phenolics directly co-related to the degree of fermentation of the teas. However, the level varied for the same type of tea among different brands. Water extracts of Choice Darjeeling with 5 min extraction time had the highest amount of phenolics (299.6 mg/g) on DW basis followed by Tazo Black 5 min (240 mg/g) and Tazo White 5 min (202.5 mg/g). Bigelow Green 2 min had the lowest amount of total phenolics (53.0 mg/g). In all the samples tested, the 5 min extraction time yielded significantly ($P < 0.001$) higher phenolic levels than the 2 min extracts. The results for the longer extraction times leading to higher level of phenolics are in conformity with previous studies (Lachman *et al.,* 2003; Cheong *et al.,* 2005; Rusak *et al.,* 2008). In a recent study, Venditti *et al.,* (2010), reported a higher phenolic content for white tea steeped in cold water as compared to hot water suggesting breakdown or transformation of phenolic components unique to white tea when exposed to high temperatures. Contrasting nature of results found in our experiments here may be due a different brand of white tea tested or a different time of extraction used. Total phenolics relating to the degree of fermentation are somewhat contradictory as reported in previously published studies. Using a 1:10 tea leaves to distilled water ratio, Kwon *et al.,* (2008), found highest total phenolics in Green tea followed by Black, White and Oolong tea when refluxed for 1 hour, whereas Rababah *et al.,* (2004), found levels in Green and Black tea to be similar when ground tea leaves were boiled for 10 min.

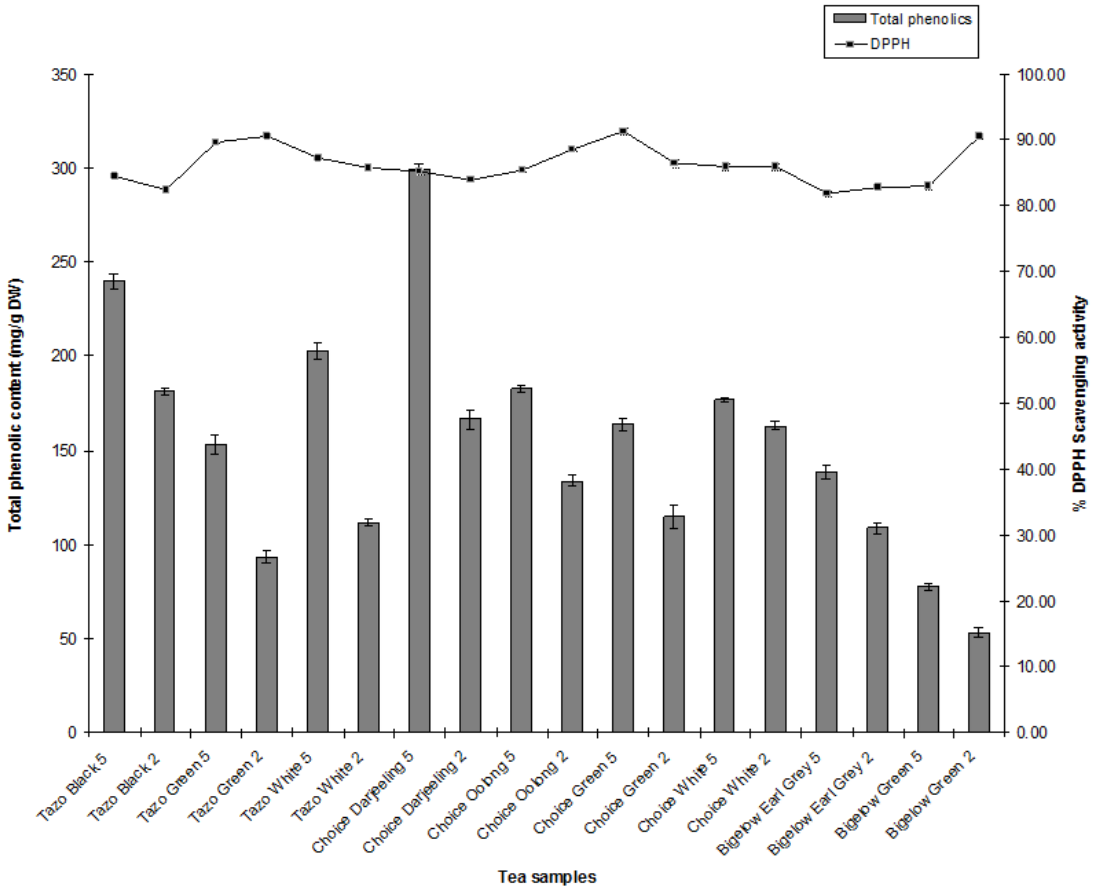

Figure 6: Total phenolic content (mg/g of sample DW) and DPPH scavenging activity (percentage inhibition) of aqueous extracts of different tea samples

Liebert *et al.*, (1999), found significantly higher total phenolics in Black tea when brewed for up to 8 min under stirring conditions than Green tea. These differences may be attributed to the different time and conditions used in the extraction methods but under typical household extraction method used in this study extent of fermentation

coupled to 5 minute extraction time was superior for extracting the highest phenolic content.

The *in vitro* antioxidant potential as measured by the free radical scavenging-linked DPPH antioxidant assay for all the teas was in a range of 80-90% irrespective of the brand and the type of tea evaluated (Fig 6). The 5 min extraction was significantly ($P<0.05$) different than the 2 min extraction for all samples except Choice White, Choice Darjeeling and Bigelow Earl Grey where no difference was found. The antioxidant capacity of Tazo Black, Tazo White and Choice Green 5 min extraction was significantly higher, whereas 2 min was higher than 5 min for all remaining samples. The antioxidant capacity did not co-relate to the level of phenolics ($r =0.05$). This may be because the process used to create these teas is highly oxidative and therefore it would not result in improvement of free radical scavenging activity. Also DPPH assay may show lower antioxidant activity potential for large phenolic molecules, because of stearic hindrance in the accessibility of N radical, (Prior *et al.,* 2005), like polymers of catechin and epicatechins which are present in large amounts in fermented teas. Also antioxidant properties are more related to the type of phenolics rather than the amount (Kwon *et al.,* 2008). Liebert *et al.,* (1999), found higher antioxidant capacity in Black tea than in Green tea by TEAC assay using slightly different extraction conditions than used in this study. By contrast, Kwon *et al.,* (2008), and Rababah *et al.,* (2004), found significantly higher antioxidant capacity in Green tea when compared to Black tea using the DPPH and the methyl linoleate assay, respectively. Rusak *et al.,* (2008), reported higher antioxidant capacity in White tea in bagged leaf form than Green tea after 5 min of extraction using the ABTS and FRAP assays. However, in some studies high

correlation between total phenolics and antioxidant capacity was reported (Kwon *et al.*, 2008; Rusak *et al.,* 2008). This could be because the extraction conditions used and the brands in the study were different which could result in a different spectrum of phenolic compounds.

4.1.4.2 α–Glucosidase Inhibition Assay

In relation to *in vitro* α–glucosidase inhibitory activity, there was a difference between tea types rather than the brands evaluated (Fig 7). The most fermented teas had the highest α–glucosidase inhibitory activity**.** Tazo Black 2 min had the highest inhibition (99.5%) whereas Tazo Green 5 min had the lowest (51.4%). Overall, 5 min extraction time showed significantly ($P<0.05$) higher α–glucosidase inhibitory activity than the 2 min extraction time in most samples. Only in case of Tazo Black, Tazo Green and Choice White, the 2 min extraction time had equal or higher inhibitory activity than the 5 min extraction. No correlation ($r = 0.22$) could be observed between α–glucosidase inhibitory activity and total soluble phenolics. Dose dependent response was observed in all samples evaluated. Numerous studies have summarized the effect of tea on anti-hyperglycemia benefits and various mechanisms have been suggested for both *in vivo* and *in vitro* responses.

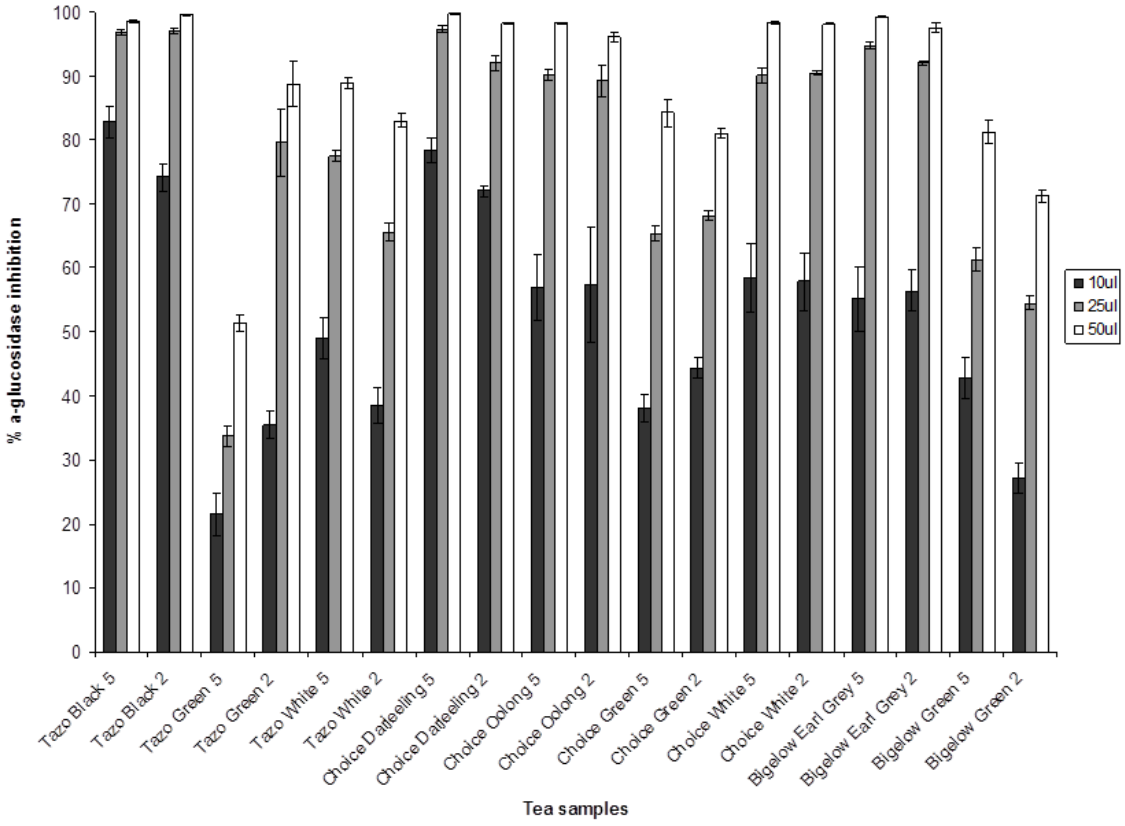

Figure 7: Dose-dependent changes in percentage α-glucosidase inhibitory activity of aqueous extracts different tea samples.

Kobayashi *et al.*, (2000), suggested inhibition of SGLT1, a sodium dependent glucose transporter, whereas Sabu *et al.*, (2002), indicated both; alterations in the glucose utilizing system and reduction in the oxidative stress which may cause less pancreatic injury. Other authors (Wu *et al.*, 2004b; Anderson and Polansky, 2002)

reported decrease in insulin resistance by GLUT IV expression and enhanced insulin activity to be responsible for anti-diabetic properties of tea. Matsui *et al.,* (2007), suggest direct inhibition of α–glucosidase by tea polyphenols may be one of the mechanisms by which tea regulates postprandial rise in blood glucose. Our *in vitro* results provide evidence that the selected tea samples, fermentation method and the time of extraction, typical of daily consumption, can consistently enrich bioactive compounds in water soluble form for α–glucosidase inhibition. This could potentially help delay the postprandial rise in blood glucose and provides the biochemical rationale for further animal and clinical studies.

4.1.4.3 α- Amylase Inhibition Assay

The *in vitro* α – amylase inhibition was observed for most of the samples evaluated and it was largely dependent on the type of tea rather than the brand (Fig 8). α- Amylase inhibitory activity did not correlate with total soluble phenolics (r = 0.29). Most fermented teas had the highest inhibitory activity; Choice Darjeeling 5 min had the highest inhibition (84.1%) followed by Tazo Black 5 min (71.6%) and Choice Darjeeling 2 min (68.0%).

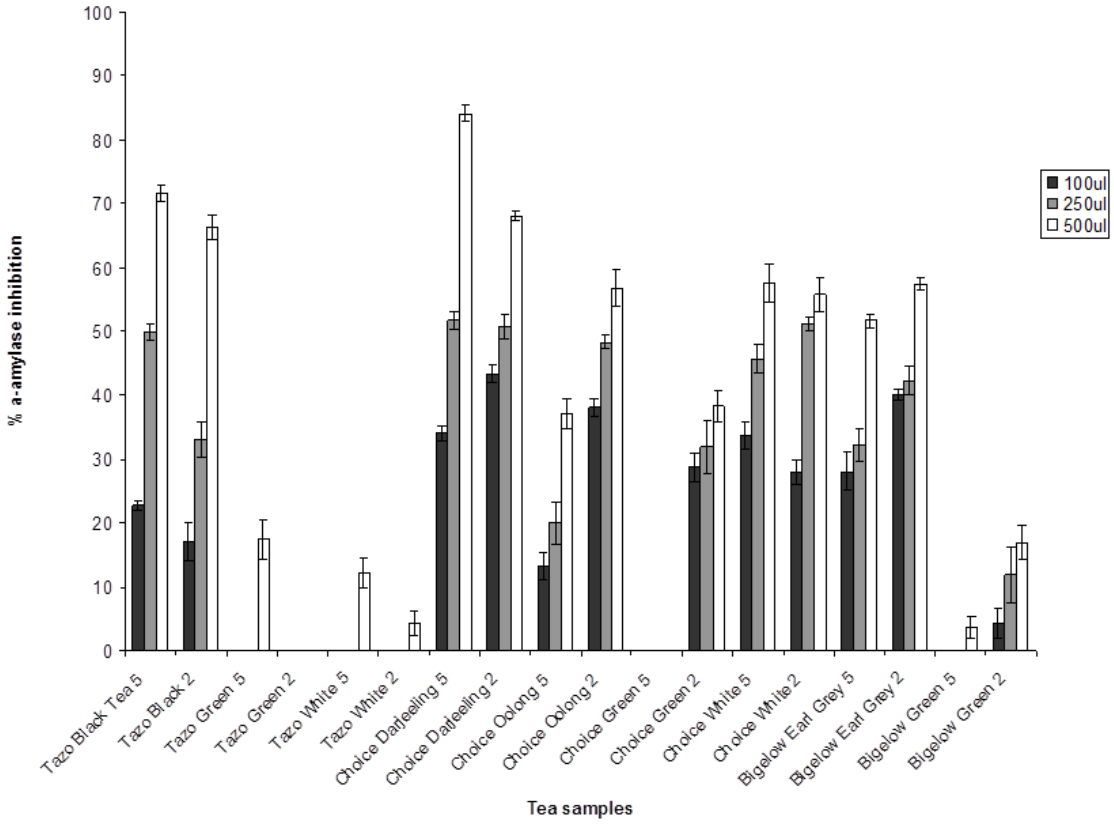

Figure 8: Percentage α-amylase inhibitory activity of aqueous extracts different tea samples

Tazo Green 2 min and Choice Green 5 min showed no inhibition. Difference between the 5 min extraction and the 2 min extraction was not found to be statistically significant ($P>0.05$). A dose dependent response was observed for all samples evaluated.

α- Amylase inhibitory activity did not correlate with total phenolic content (r = 0.29). By contrast other investigators have reported that tea polyphenols may exhibit enzyme inhibition by complexing; which may change its configuration resulting in a loss of catalytic activity (Spencer *et al.,* 1988; Shi *et al.*, 1994; He *et al.,* 2007). Black tea inhibited α- amylase at a higher level than Green Tea and contradictory to our findings here, other researchers have attributed this to the fact that the polymeric compounds in fermented teas have a stronger affinity to precipitate proteins (Hara and Honda, 1990; Zhang and Kashket, 2000).

4.1.4.4 Angiotensin Converting Enzyme Inhibition Assay

None of the samples showed Angiotensin Converting Enzyme inhibitory activity which indicates tea may not possess anti-hypertensive properties i*n vitro* (data not shown). In the literature, contrasting results have been reported. Stensvold *et al.,* (1992), found lower systolic blood pressure among Norwegian men and women who consumed Black tea whereas Hodgson *et al.,* (1999), reported no significant alternation in ambulatory blood pressure of 13 Australian men drinking tea. Although the results obtained *in vitro* may not necessarily translate into similar results *in vivo,* our results provide some insight on the anti-hypertensive properties of the selected tea samples, the time and method of extraction used.

4.1.5 Conclusion

In this study we evaluated the potential of tea with a method of extraction typical of daily consumption for potential to manage hyperglycemia linked to type 2 diabetes. This study was designed to model typical daily consumption behavior as closely as possible; using hot water extraction with typical store bought tea bags. Total phenolics, antioxidant activity and inhibition of key enzymes in carbohydrate digestive process (α-glucosidase and α-amylase) were investigated. Overall, the most fermented teas were found to have the highest potential relevance to manage hyperglycemia using these *in vitro* assay models. Overall 5 min extraction time was found to be more relevant in terms of total phenolics, antioxidant capacity and α-glucosidase inhibition. The extraction time did not have any effect on α-amylase inhibitory potential. None of the extracts showed angiotensin converting enzyme inhibitory potential. This study provides a simple additional medicinal food-based strategy in a large arsenal of plant food therapies that have the potential to manage hyperglycemia in modern day life styles where there is constant demand and exposure to high soluble carbohydrate diets and beverages. Combining such after meal 5 minutes hot water-extracted tea usage offers rationale strategies to potentially delay on-set of improper glucose homeostasis in pre-diabetic stages, when diet and exercise can help combat early stages of type 2 diabetes.

4.2 Inhibitory Potential of Tea Polyphenolics and Influence of Extraction Time Against *Helicobacter pylori* and Lack of Inhibition of Beneficial Lactic Acid Bacteria

4.2.1 Abstract

Tea polyphenolics such as catechins are known to have the potential to inhibit many bacterial pathogens. *Helicobacter pylori* has been identified as an etiological agent in the development of gastric ulcer, peptic ulcer, gastritis and many other stomach related diseases. In this study, we investigated the effect of nine tea extracts, of three different brands representing four different processed types (White, Green, Oolong and Black), on the inhibition of *H. pylori*. Extraction times of 2 and 5 min were compared. Most 5 min extracts showed *H. pylori* inhibition whereas 2 min extracts of only Choice Darjeeling Black and Tazo White showed inhibition. No recovery was observed after the addition of 0.5 and 5 mM proline indicating tea polyphenols do not inhibit *H. pylori* by inhibition of proline oxidation via proline dehydrogenase. Extracts that showed inhibition were further evaluated for their effect on beneficial lactic acid bacteria. None of the samples showed inhibition indicating that tea might be able to inhibit *H. pylori* without affecting the beneficial lactic acid bacteria. HPLC analysis indicated the presence of gallic acid, quercetin, caffeine and tea catechins including catechin, epicatechin, and epigallocatechin in all the tea samples. Our study here indicates that tea can be potentially used as a low cost dietary support to combat *H. pylori* linked gastric diseases without affecting the beneficial intestinal bacteria.

4.2.2 Introduction

Helicobacter pylori is a gram-negative, flagellate, microaerophilic pathogen that is prevalent in more than half of the human population; colonizing in the hostile acidic environment of the human stomach (Warren and Marshall, 1983; Mitchell, 1993; Montecucco and Rappuoli, 2001; Vattem *et al.,* 2005a). *H. pylori* infection is regarded as an important factor in the etiology of several diseases including gastritis, gastric cancer, peptic ulcer, mucosa associated lymphoid tissue lymphoma and recently it has been shown to be related to cardiovascular diseases (Warren and Marshall, 1983; You *et al.,* 2000; Uemura *et al.,* 2001). Although more than 50% of the populations harbor *H. pylori* only 15-20% of the infected populations develop clinically significant diseases which are generally influenced by complex interactions between the host, bacterial and environmental factors (Go, 1997; Parsonnet, 1998; Vattem *et al.,* 2005a). *H. pylori* survives in the acidic environment by biosynthesis of urease, an enzyme that can catalyze the hydrolysis of urea into ammonia and carbon-dioxide. With the help of ammonia and a proton gated urea channel which prevents over alkalization, it buffers the cytosol to create a neutral environment around the bacterial surface (Mobley *et al.,* 1991; Weeks *et al.,* 2000). There are a number of reasons for the failure of *H. pylori* eradication therapy; these include antibiotic resistance by chromosomal mutation, limited efficacy of antibiotics because of low gastric pH, and high bacterial load (Megraud, 1997; Megraud and Lamouliatte, 2003). Investigations are underway to find whole food and healthy dietary support agents and/or alternatives in lieu of Costly and rigorous antibiotic treatment regimens which are associated with a number of side effects.

Phenolic compounds which have been shown to be inhibitory to *H. pylori* include tannins, tea catechins, quercetins (Yanagawa *et al.,* 2003; Funatogawa *et al.,* 2004; Shin *et al.,* 2005). Tea, which is a commonly consumed household beverage, has also been shown to be inhibitory to *H. pylori* (Yee and Koo, 2000; Matsubara *et al.,* 2003; Ruggiero *et al.,* 2007).

Lactic acid bacteria such as *Lactobacillus sp, Bifidobacterium sp* are a class of probiotics, which are defined as living microorganisms which exert health benefits beyond basic nutrition, when ingested in certain numbers (Ljungh and Wadstrom, 2006). Lactic acid bacteria have been shown to confer several benefits to the host including better absorption of nutrients from food, decrease in lactose intolerance in some individuals, control of diseases originating from intestinal infections, potential to control of some types of cancer, and stimulation of host immune response (Gilliland, 1990; Perdigón *et al.,* 2001; Rafter, 2002). Oregano and rosemary have been reported to delay the growth and acid production of lactic acid bacteria (Zaika and Kissinger, 1981; Zaika *et al.,* 1983). Other authors have reported tannins, tannic acid and related compounds did not affect the growth of lactic acid bacteria or effected very slightly at high concentrations (Ahn *et al.,* 1998; Chung *et al.,* 1998). Contradictory reports exist in literature on the effect of tea on some species of lactic acid bacteria. Horiba *et al.,* (1991), reported that green tea inhibited the growth of *Bifidobacterium bifidum* whereas Ahn *et al.,* (1990a), reported Green tea did not affect *Bifidobacterium*. Also stimulatory effect of water extract of *Panax ginseng* has been reported on the growth of *Bifidobacteria* (Ahn *et al.,* 1990b).

Antibacterial effects and bactericidal effect of tea has been shown against many bacteria (Ahn *et al.,* 1990a; Horiba *et al.,* 1991; Friedman, 2007). Therefore, the basis of this investigation was to explore the relevance of daily consumption of several types of commonly consumed tea (Green, Black, Oolong, and White) and extraction time for *H. pylori* inhibition and its effects on different strains of lactic acid bacteria with probiotic potential such as *Lactobacillus helveticus, Bifidobacterium longum* and *Lactobacillus plantarum*. Furthermore, investigations on the mode of action of *H. pylori* inhibition by tea were carried out on the basis of the rationale that simple phenolics may mimic proline analogs and may exhibit antimicrobial activity by inhibition of proline oxidation via proline dehydrogenase (Shetty and Wahlqvist, 2004; Lin *et al.,* 2005; Kwon *et al.,* 2007b; Apostolidis *et al.,* 2008).

4.2.3 Materials and Methods

4.2.3.1 Sample Extraction

One tea bag was added to 200 mL of boiling water. It was then allowed to steep for 2 or 5 min. The extract was then allowed to cool at room temperature and centrifuged at 10,000g for 10 min. Samples evaluated were Choice Brand tea (Seattle, WA, USA), type included were White, Japanese Green, Oolong, and Darjeeling; Tazo Brand tea (Portland, OR, USA), types included were Berry Blossom White, Green and Black as well as from Bigelow's tea Company (Fairfield, CT, USA), type included were Green and Earl Grey. All teas were purchased from Whole Foods Market, Hadley, MA, USA. Absorbance of the extracts were measured at 400 and 600 nm to determine whether the extraction were uniform

4.2.3.2 Bacterial Strains

Helicobacter pylori (strain ATCC 43579, which originated from human gastric samples) was obtained from the American Type Culture Collection (Rockville, MD). The lactic acid bacteria strains used in this study were the following: *Lactobacillus bulgaricus* and *Bifidobacterium longum* were isolated from yogurt in a previous study.[35] *Lactobacillus helveticus* was supplied by Rosell Institute Inc. Montreal, Canada (Lot#: XA 0145, Seq#:00014160) and *Lactobacillus plantarum* (1) (ATCC 9019).

4.2.3.3 High performance Liquid Chromatography (HPLC) Analysis of Phenolic Profiles

Hot water extracts (2 mL) were filtered through a 0.2 μm filter. A volume of 5 μL of extract was injected using an Agilent ALS 1100 auto sampler into an Agilent 1100 series HPLC (Agilent Technologies, Palo Alto, CA) equipped with a 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, 25 min). The analytical column used was Agilent Zorbax SB-C_{18}, 250 x 4.6 mm i.d., with packing material of 5 μm particle size at a flow rate of 1 mL/min at room temperature. During each run the absorbance was recorded at 226 nm and 305 nm and the chromatogram was integrated using Agilent Chemstation enhanced integrator. Pure standards of gallic acid, catechin, epicatechin, quercetin, caffeine, and epigallocatechin in 100% methanol

were used to calibrate the standard curves and retention times. The peaks were identified taking into account the retention time and the UV absorption spectra of the corresponding standards.

4.2.3.4 Preparation of Starter Culture of *Helicobacter pylori*

Helicobacter pylori were cultured according to Stevenson *et al.*, (2000). Standard plating medium (*H. pylori* agar plates) were prepared by using 10 g of special peptone (Oxoid Ltd., Basingstoke, England) per liter, 15 g of granulated agar (Difco Laboratories, Becton, Dickinson and Co., Sparks, MD) per liter, 5 g of sodium chloride (EM Science, Gibbstown, NJ) per liter, 5 g of yeast extract (Difco) per liter, 5 g of beef extract (Difco) per liter of water.

Broth media were prepared by 10 g of special peptone (Oxoid Ltd., Basingstoke, England) per liter, 5 g of sodium chloride (EM Science, Gibbstown, NJ) per liter, 5 g of yeast extract (Difco) per liter, 5 g of beef extract (Difco) per liter of water. A volume of 1 mL of frozen stock of *H. pylori* was added into test tubes containing 10 mL of sterile broth media. This was incubated at 37 ^{o}C for 48 h. Ten mL of sterile broth media was inoculated with 1 mL of the 48 h culture and incubated at 37 °C for 24 h. The active culture was then spread on *H. pylori* agar plates to make bacterial lawn for the agar-diffusion assay.

4.2.3.5 Agar-Diffusion Assay

The antimicrobial activity of sample extracts was analyzed by the agar-diffusion method. The assay was done aseptically using sterile 12.7 mm diameter paper disks (Schleicher & Schuell, Inc., Keene, NH) to which 100 µL of test extracts were added. Saturated disks were placed onto the surface of seeded agar plates. Controls consisted of disks with distilled water only. 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). The diameter of clear zone (no growth) surrounding each disk under static incubation with plates in GasPak jars was measured and the zone of inhibition was determined and expressed in mm. For the dose dependency studies, 50 µl and 75 µl of the sample were used.

4.2.3.6 Proline Growth Response Assay

A model for mode of action of phenolic phytochemicals was developed based on the rationale that small phenolics could behave as proline analogs or proline analog mimics and likely inhibit proline oxidation via proline dehydrogenase (Shetty and Wahlqvist, 2004). Further, the likely inhibitory effects of phenolic phytochemicals should be overcome by proline if the site of action is proline dehydrogenase.

Bacterial lawns of *H. pylori* were prepared as described previously. Plating media were prepared by using standard plating medium as described in agar-diffusion assay with some modifications. Proline (Sigma, Louis, Mo) was added into the medium to a final concentration of 0.5 and 5 mM. Then a similar protocol as mentioned in the agar diffusion assay was followed.

4.2.3.7 Lactic Acid Bacterial Proliferation Assay

Initially 100 µL of frozen stock from the lactic acid bacterial strains were inoculated into 10 mL MRS (de Man, Rogosa, Sharpe) broth (Difco) and incubated for 24 h at 37 °C. Then, 100 µL of the 24 h grown strain was re-inoculated into 10 mL MRS broth for 24 h at 37 ^{o}C. Sample volumes of 1 mL were filter sterilized using sterile filters Millex GP 0.22 µm (Millipore Corporation, Bedford, MA). Filter sterilized sample extracts (1 mL) and 100 µL of the 24 h grown strain with approximately 10^{8} CFU/mL (diluted 100 times with sterile distilled water) were added into 9 mL of MRS broth tubes and incubated at 37 °C for 24 h. A control with 1 mL of sterile distilled water instead of sample extract was also included. One hundred µL of the serially diluted samples were plated in triplicates every 0, 6, 9, 12, and 24 h on MRS agar (Difco) plates and incubated in anaerobic BBL GasPak jars (Becton, Dickinson and Co., Sparks, MD) with BD GasPak EZ anaerobe container system sachets (Becton, Dickinson and Co., Sparks, MD) at 37 °C for 48 h to determine the CFU/mL.

4.2.3.8 Statistical Analysis

All experiments were performed by duplicate and the analysis at every time point from each experiment was carried out in triplicate (n=6). In case of HPLC analysis, the experiments were performed at least in triplicates. Results were expressed as means ± standard deviation. Data were subjected to Student's *t* test.

4.2.4 Results and Discussion

4.2.4.1 Extract Absorbance Analysis

The absorbance of the 2 and 5 min extracts were measured at 400 and 600 nm. Results of the analysis are presented in Table 1. In general black teas as expected had highest absorbance followed by partially fermented Oolong tea then green tea and white tea with the lowest absorbance. The differences in absorbance did not indicate a correlation to phenolic profiles based on HPLC analysis below and total phenolics or antioxidant activity reported previously (Ankolekar *et al.*, 2011). In general it reflects the state of oxidation as black tea are considered the most oxidized teas from mature leaves and white tea least oxidized from leaf buds.

Table 1: Weight, origin of the teas with absorbance of the 2 and 5 min extracts at 400 and 600 nm

Tea sample	Weight in g per tea bag	Origin	Absorbance at 400 nm		Absorbance at 600 nm	
			2 min	5 min	2 min	5 min
Tazo Black	2.55	Blend of teas from	3.24	3.45	0.209	0.209
Tazo Green	2	China	0.655	0.991	0.056	0.15
Tazo White	1.5	Blend of Chinese	0.358	0.517	0.033	0.065
Choice Darjeeling	2	India	1.562	1.880	0.120	0.139
Choice Oolong	2	China	1.094	1.295	0.136	0.183
Choice Green	2	Japanese	1.189	1.248	0.158	0.215
Choice White	2	China	0.697	0.927	0.054	0.076
Bigelow Earl Grey	1.65	Not mentioned	2.30	2.49	0.168	0.230
Bigelow Green	1.4	Not mentioned	0.879	1.119	0.096	0.187

4.2.4.2 High Performance Liquid Chromatography (HPLC) Analysis of Phenolic Profiles

HPLC analysis of the tea samples was carried out to determine specific phenolic compounds present in the samples. The amount of each compound detected by HPLC analysis in each sample with a 2 and 5 min extraction is shown in Table 2. Gallic acid, quercetin, caffeine and tea catechins including catechin, epicatechin, and epigallocatechin were found in all the tea samples. No epigallocatechin gallate or epicatechin gallate were found in any of the samples evaluated. A strong correlation (Pearson's correlation coefficient) was found between catechin (R=0.91), epicatechin (R=0.88), epigallocatechin (R=0.85) and the zone of inhibition in the samples that showed inhibition. However, even some samples that did not show inhibition showed the presence of these phenolic compounds. This suggests that these compounds may work in combination with other compounds that were not detected in the HPLC analysis in inhibiting *H. pylori*. Zhu *et al.*, (1997), have reported that the structures of tea catechins were not affected for 18 h at pH<4 which is similar to the pH phenolic structures would encounter in the stomach. This shows that phenolic structures involved in *H. pylori* inhibition will not be affected by stomach pH.

Table 2: Phenolic compounds and Caffeine (mg/g dw) content in 2 and 5 min extracts of tea

Sample	Gallic acid	Quercetin	Catechin	Epicatechin	Epigallocatechin	Caffeine
Tazo Black 5	0.066 ± 0.0015	0.031 ± 0.0006	0.194 ± 0.0007	6.092 ± 0.381	0.715 ± 0.260	58.147 ± 1.38
Tazo Black 2	0.052 ± 0.00003	0.022 ± 0.0002	0.099 ± 0.013	3.115 ± 0.35	0.482 ± 0.15	44.534 ± 0.91
Tazo Green 5	0.014 ± 0.0004	0.017 ± 0.00012	0.319 ± 0.054	6.639 ± 0.257	1.139 ± 0.082	33.832 ± 0.54
Tazo Green 2	0.012 ± 0.0004	0.013 ± 0.00092	0.243 ± 0.04	5.748 ± 0.03	0.950 ± 0.04	28.888 ± 1.04
Tazo White 5	0.030 ± 0.007	0.021 ± 0.0004	0.408 ± 0.036	8.391 ± 0.12	1.262 ± 0.14	47.530 ± 5.7
Tazo White 2	0.024 ± 0.007	0.013 ± 0.0007	0.280 ± 0.01	7.008 ± 1.42	0.945 ± 0.036	18.402 ± 2.46
Choice Darjeeling	0.096 ± 0.00034	0.032 ± 0.002	0.547 ± 0.083	9.467 ± 0.76	1.193 ± 0.247	70.213 ± 4.58
Choice Darjeeling	0.065 ± 0.007	0.022 ± 0.001	0.365 ± 0.086	7.197 ± 0.63	1.143 ± 0.038	63.163 ± 0.71
Choice Oolong 5	0.055 ± 0.005	0.026 ± 0.003	0.402 ± 0.064	7.142 ± 0.08	1.098 ± 0.133	48.292 ± 1.87
Choice Oolong 2	0.037 ± 0.0022	0.016 ± 0.0004	0.427 ± 0.001	8.193 ± 1.2	1.544 ± 0.23	41.121 ± 2.47

Choice Green 5	0.011 ± 0.0009	0.029 ± 0.001	0.421 ± 0.091	11.664 ± 0.054	2.391 ± 0.216	37.972 ± 4.51
Choice Green 2	0.007 ± 0.00026	0.015 ± 0.0012	0.311 ± 0.003	10.550 ± 2.34	1.538 ± 0.059	28.243 ± 1.60
Choice White 5	0.037 ± 0.00068	0.018 ± 0.0014	0.309 ± 0.059	5.756 ± 0.476	0.709 ± 0.108	46.353 ± 1.97
Choice White 2	0.029 ± 0.00079	0.013 ± 0.00079	0.202 ± 0.059	6.684 ± 1.71	0.521 ± 0.052	33.964 ± 1.71
Bigelow Earl Grey	0.033 ± 0.001	0.021 ± 0.00063	0.096 ± 0.0288	2.196 ± 0.37	0.543 ± 0.016	38.361 ± 2.12
Bigelow Earl Grey	0.024 ± 0.00066	0.013 ± 0.00012	0.101 ± 0.0036	1.484 ± 0.258	0.417 ± 0.025	27.046 ± 1.78
Bigelow Green 5	0.011 ± 0.00035	0.011 ± 0.0017	0.142 ± 0.026	3.948 ± 0.273	0.585 ± 0.0491	21.646 ± 0.253
Bigelow Green 2	0.011 ± 0.001	0.014 ± 0.0004	0.141 ± 0.021	3.673 ± 0.247	0.561 ± 0.0344	22.273 ± 0.692

Values are means ± Standard error;

2 and 5 in the first column indicate the time of extraction

4.2.4.3 *Helicobacter pylori* Inhibition and Proline Growth Response Assay

Potential of several commercial tea varieties and the influence of extraction time on *H. pylori* inhibition were evaluated. Of all the tea varieties tested with 5 min extraction time Choice Darjeeling, Choice Oolong, Choice White, Tazo White and Tazo Black showed inhibition whereas with the 2 min extraction time only Choice Darjeeling and Tazo White showed inhibition (Fig 9). Bigelow brand did not show any inhibition. Highest inhibition zone was observed for Choice Darjeeling 5 min (16.95 mm) whereas lowest was observed for Tazo Black 5 min (14.68 mm). Significant difference ($P<0.05$) was observed in *H. pylori* inhibition of the 2 and 5 min sample for both Choice Darjeeling and Tazo White with 5 min sample being superior.

The proline growth response assay is based on the rationale that small phenolics in tea could behave as proline mimics and can inhibit proline oxidation via proline dehydrogenase (PDH) at the plasma membrane level in prokaryotic cell disrupting the oxidative phosphorylation linked proton motive force resulting in inhibition of the bacterium (Vattem *et al.,* 2005a). If so, then addition of proline could overcome inhibition of the bacterium by proline mimicking phenolics present in the tea extract. The results obtained in this study (Fig 10) indicate that microaerophillic *H. pylori* inhibition by water extracts of tea is not associated to the proline dehydrogenase based oxidative phosphorylation and there could be other mechanisms involved associated with membrane disruption or acidification.

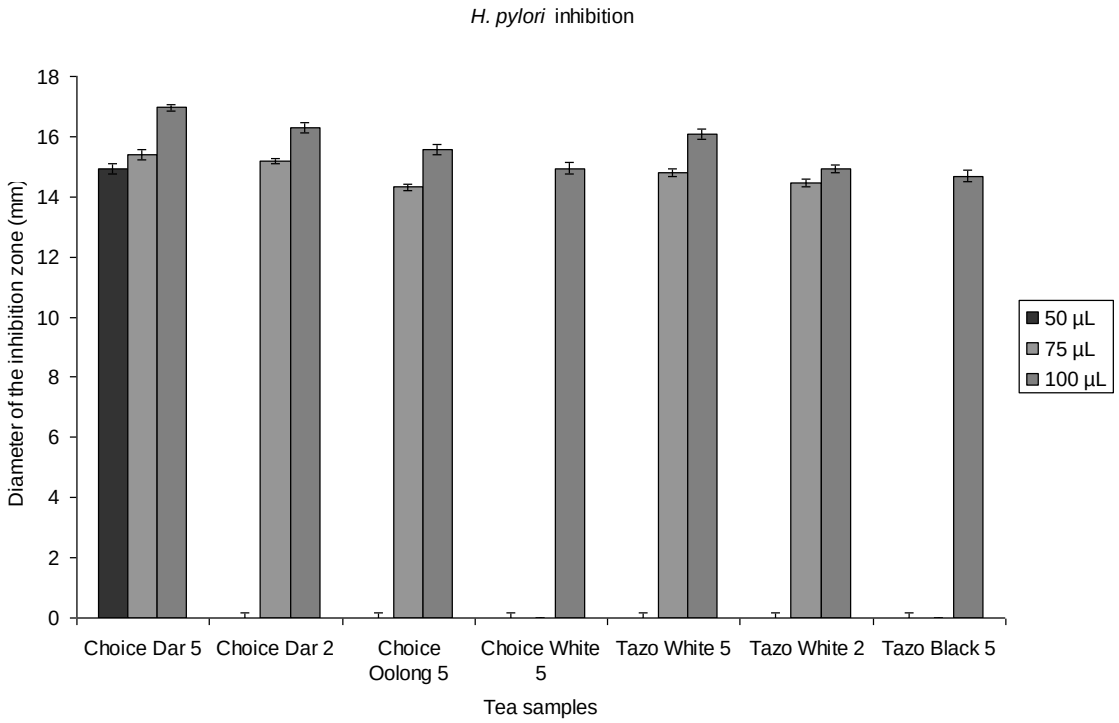

Figure 9: Anti-*Helicobacter pylori* activity of tea extracts at three different doses of sample concentration (50 µL, 75 µL, 100 µL) as determined by agar spread plate inhibition zones in replicates of 3.

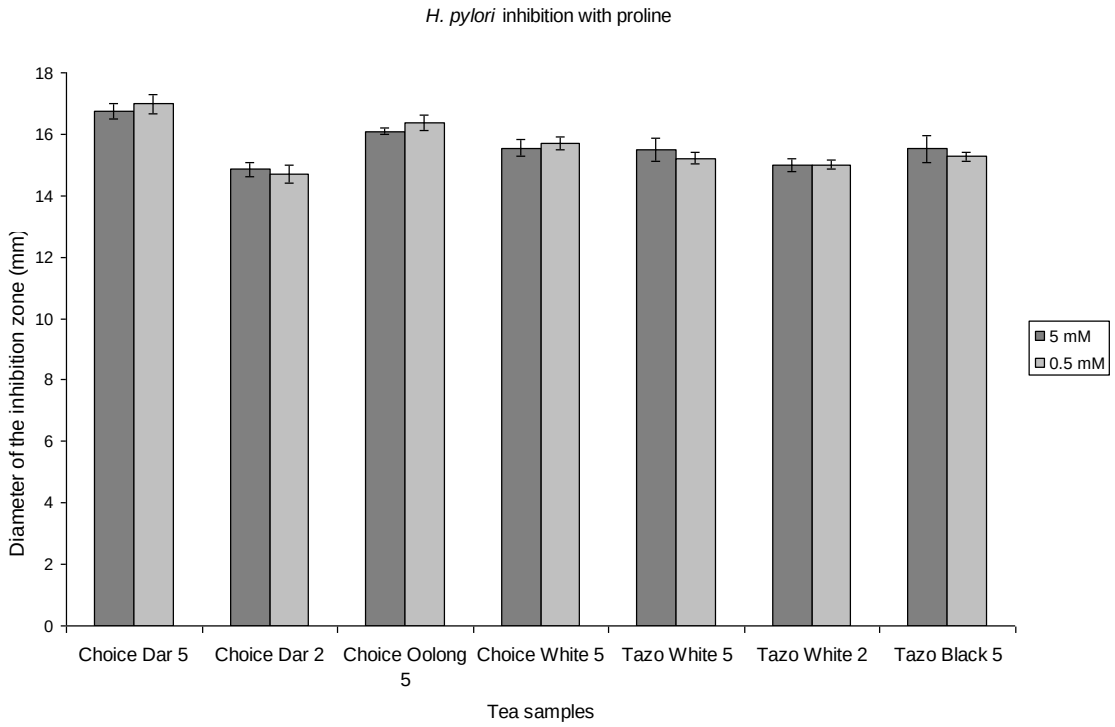

Figure 10: Effect of tea extracts on anti-*Helicobacter pylori* activity using proline (0.5 and 5 mM) as determined by agar spread plate inhibition zones in replicates of 3. Control, without proline

It is possible that tea polyphenolic antioxidants can quench electrons from electron transport chain (ETC) along the bacterial membrane and act as antimicrobials by disrupting oxidative phosphorylation or inhibit dehydrogenases linked proton efflux by interfering with the flow of electrons at the level of cytochromes (Shetty and

Wahlqvist, 2004; Vattem *et al.*, 2005a). However, simple soluble phenolics which are not very hydrophobic may not be effective in disrupting oxidative phosphorylation because of the external lipopolysachharide layer of the gram-negative *H. pylori* which creates a hydrophobic microenvironment along the bacterial surface. It is also thought that soluble phenolics can disrupt the H^+-ATPase required for ATP synthesis by causing hyper-acidification via proton donation at the plasma membrane and by intracellular cytosolic acidification (Shetty and Wahlqvist, 2004; Kwon *et al.*, 2007b). Another explanation is that partial hydrophobicity of polyphenols in more fermented teas can attach to the cell wall and allowing them to possibly stack themselves causing destabilization of the membrane leading to membrane disruption or transport inhibition. All possibilities exist that these mechanisms may work synergistically i.e. disrupting and destabilizing effect of the polyphenols may make it easier for the simple soluble phenolics to exert their hyper-acidification.

Ikigai *et al.*, (1993), have reported that the bactericidal effect of catechins is by damage to bacterial membranes and Funatogawa *et al.*, (2004), have demonstrated this effect of tea catechins in *H. pylori*. Damage to DNA of gram-negative *H. pylori* could be one of the underlying mechanisms involved in inhibition. Puupponen-Pimia *et al.*, (2001), with their studies on gram-negative *E. coli* reported that the reaction of phenolic compounds with DNA was the main cause of inhibition.

Polyphenols may show anti-*H. pylori* properties by inhibiting ureases. Matsubara *et al.*, (2003), reported inhibition of *H. pylori* ureases by Green tea, Oolong tea, Jasmine tea and Black tea. In that study, both tea catechins and Green tea extracts suppressed *H. pylori* induced gastric lesions in Mongolian gerbils. Lin *et al.*, (2005),

reported urease inhibition by phenolic phytochemicals *in vitro.* By contrast, Shin *et al.*,(2005), examined the effect of some flavonoids including, tea catechins, quercetin, naringin and reported no inhibitory effect on urease. Also catechins may restrict the supply of ions Fe^{3+} and Ni^{2+} essential for bacterial growth by forming complexes with these metal ions (Kumamoto *et al.*, 2001; Montecucco and Rappuoli, 2001). Overall it is not clear from our studies whether the effects of tea extracts are bacteriostatic or bacteriocidal and what the precise mode of action is. Further studies based on these initial studies will clarify these mechanisms proposed on disruption of membrane functions and will form the basis and foundation of further studies.

4.2.4.4 Probiotic Lactic Acid Bacteria Proliferation Studies

Tea samples which showed inhibition against *H. pylori* were further investigated for their effects on lactic acid bacteria to determine relevance for positive health benefits. The concentrations of extracts used in this study were similar to those used in *H. pylori* inhibition studies. No inhibitory or stimulatory effects of the extracts were observed on probiotic strains of *Bifidobacterium longum* (Fig 11 and Fig 14), *Lactobacillus plantaraum* (Fig 12 and Fig 15) *and Lactobacillus helveticus* (Fig 13 and Fig 16) as compared to control. This indicates that tea extracts are able to inhibit *H. pylori* without affecting the intestinal bacteria, when extracted in a way typical of daily consumption.

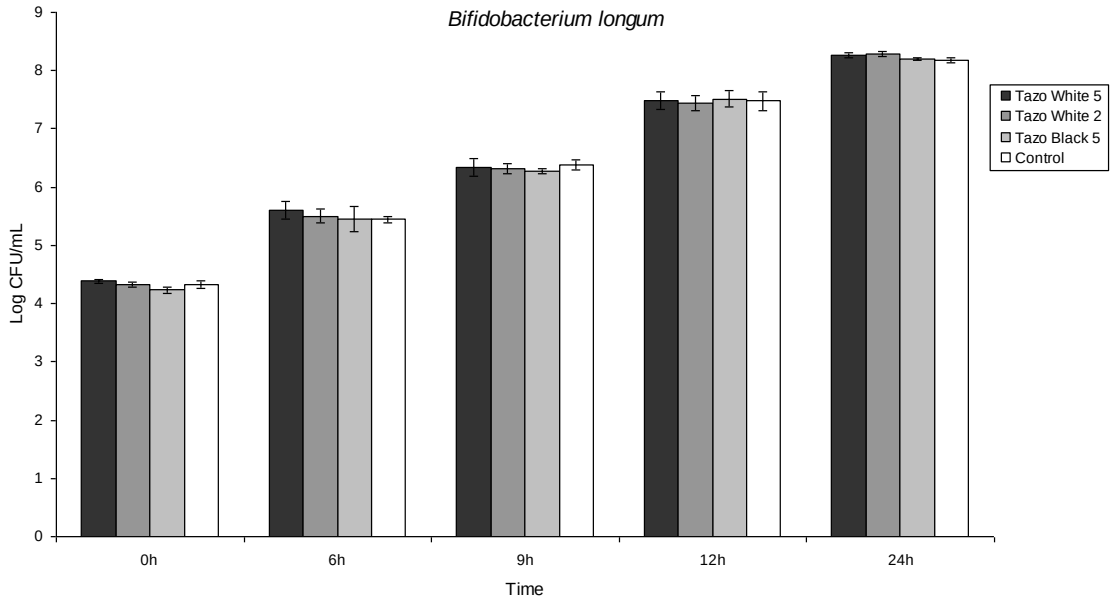

Figure 11: Effect of Tazo brand tea extracts on *Bifidobacterium longum* proliferation (cfu/mL) as determined by colony count method in replicates of 3.

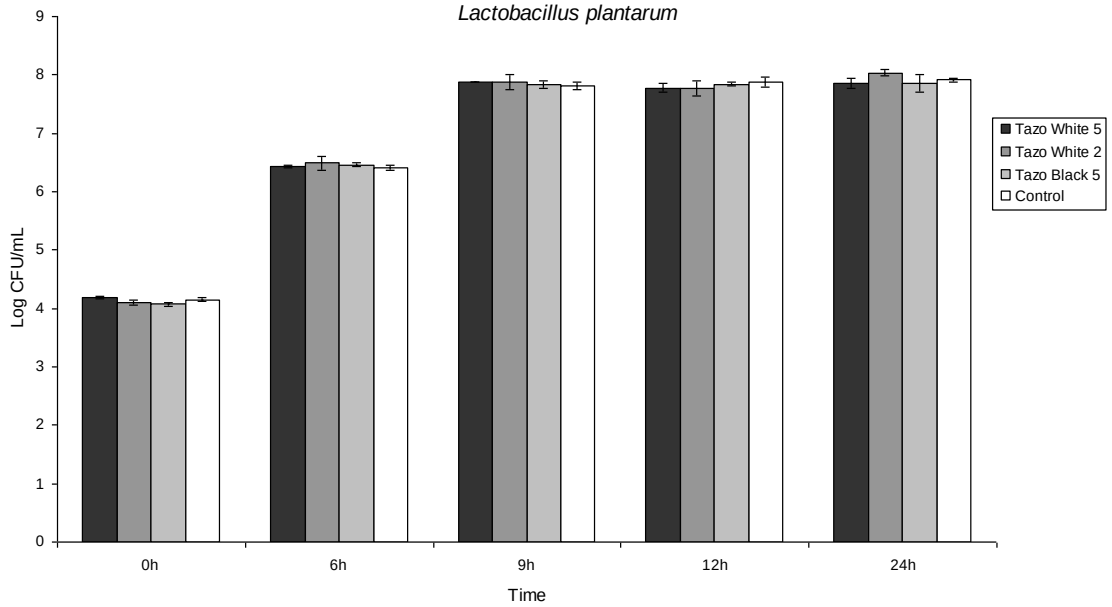

Figure 12: Effect of Tazo brand tea extracts on *Lactobacillus plantarum* proliferation (cfu/mL) as determined by colony count method in replicates of 3.

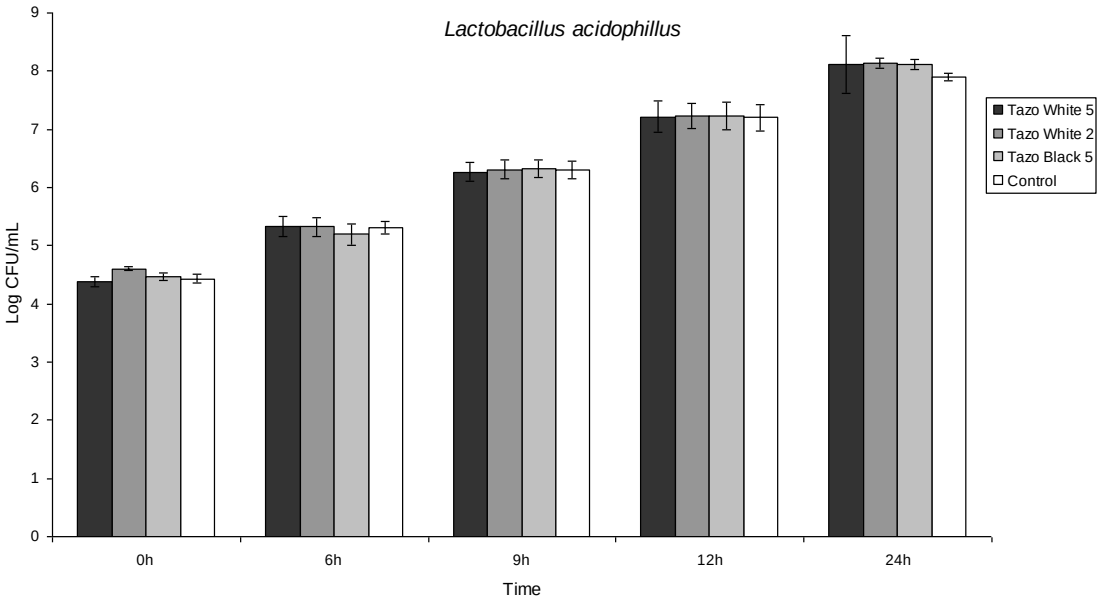

Figure 13: Effect of Tazo brand tea extracts on *Lactobacillus helveticus* proliferation (cfu/mL) as determined by colony count method in replicates of 3.

Funatogawa *et al.,* (2004), studied the effect of hydrolysable tannins and reported that while these compounds were able to inhibit *H. pylori*, they did not affect *E. coli* a normal inhabitant of the gut microflora. Results reported elsewhere suggest tannins derived from *Galla rhois* were able to inhibit pathogens but had a very slight inhibitory effect or no effect on intestinal flora (Ahn *et al.,* 1998). Chung *et al.,* (1998), found no inhibition of lactic acid bacteria when tannic acid was used. They further indicated that this might be because of absence of heme enzymes and the crucial iron containing ribonucleotide reductase being replaced by adenosylcobalamine in some lactic acid bacteria. So while metal chelating compounds would inhibit the growth of other bacteria by sequestering essential metal ions, it would have no effect on lactic acid bacteria. The growth of lactic acid bacteria has also been shown to be stimulated by extracts from *Panax ginseng* and Green tea (Ahn *et al.,* 1990a; Ahn *et al.,* 1990b). The results might have varied because the growth was evaluated using methanol extracts as against water extracts in this study. By contrast, Horiba *et al.,* (1991), reported 10 fold decrease in number of *Lactobacillus helveticus* in broth and a significant zone of inhibition was found for *Bifidobacterium bifidum* using agar diffusion assay using 20% methanol extract of Green tea. Puupponen-Pimia *et al.,* (2001), discussed the structure-activity relationship and reported that lipophilic phenolics like quercetin and kaempferol were unable to inhibit lactic acid bacteria whereas hydrophilic phenolics like myricetin were completely inhibitory. This might partly explain our findings of why *H. pylori* were inhibited but lactic acid bacteria were not. The difference in dependency on partial oxidative phosphorylation in microaerophilic bacteria such as *H. pylori* may be another

reason for inhibition and lack of inhibition in lactic acid bacteria that use substrate level phosphorylation for energy generation.

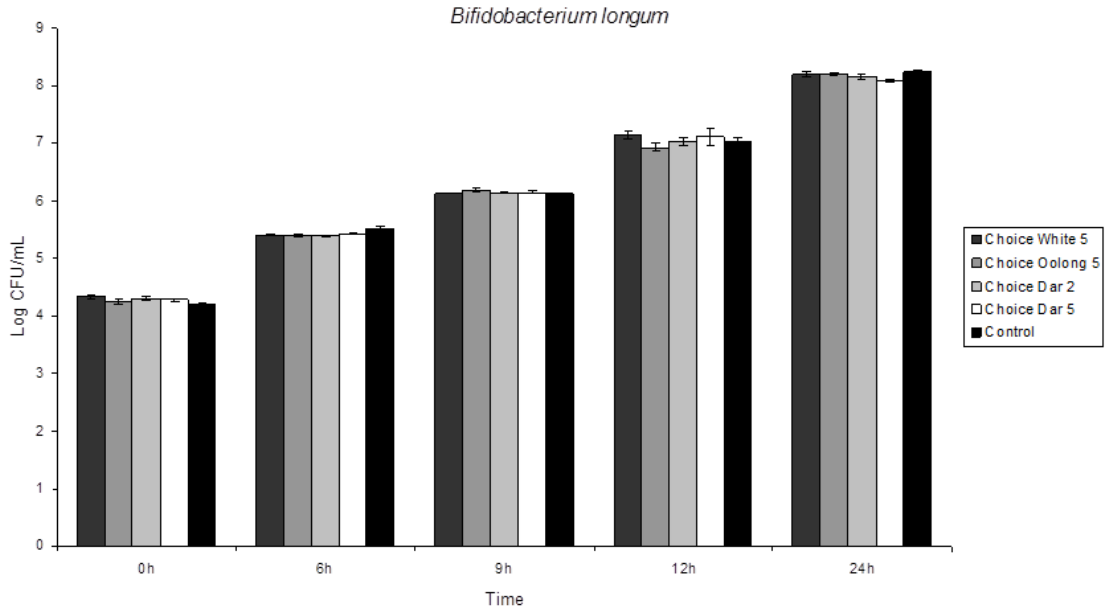

Figure 14: Effect of Choice brand tea extract on *Bifidobacterium longum* proliferation (cfu/mL) as determined by colony count method in replicates of 3

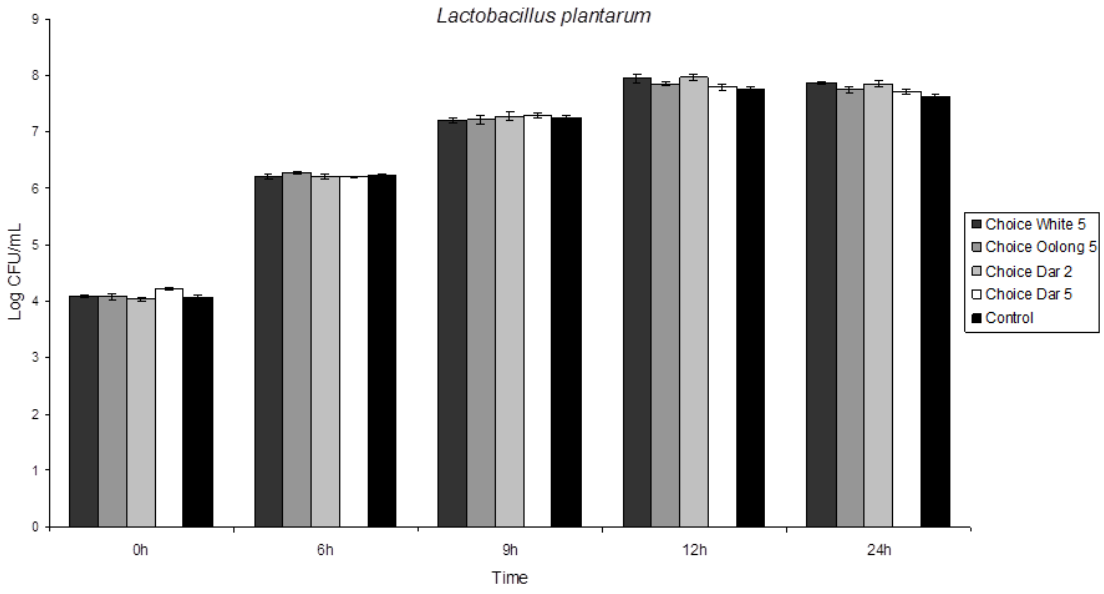

Figure 15: Effect of Choice brand tea extract on *Lactobacillus plantarum* proliferation (cfu/mL) as determined by colony count method in replicates of 3

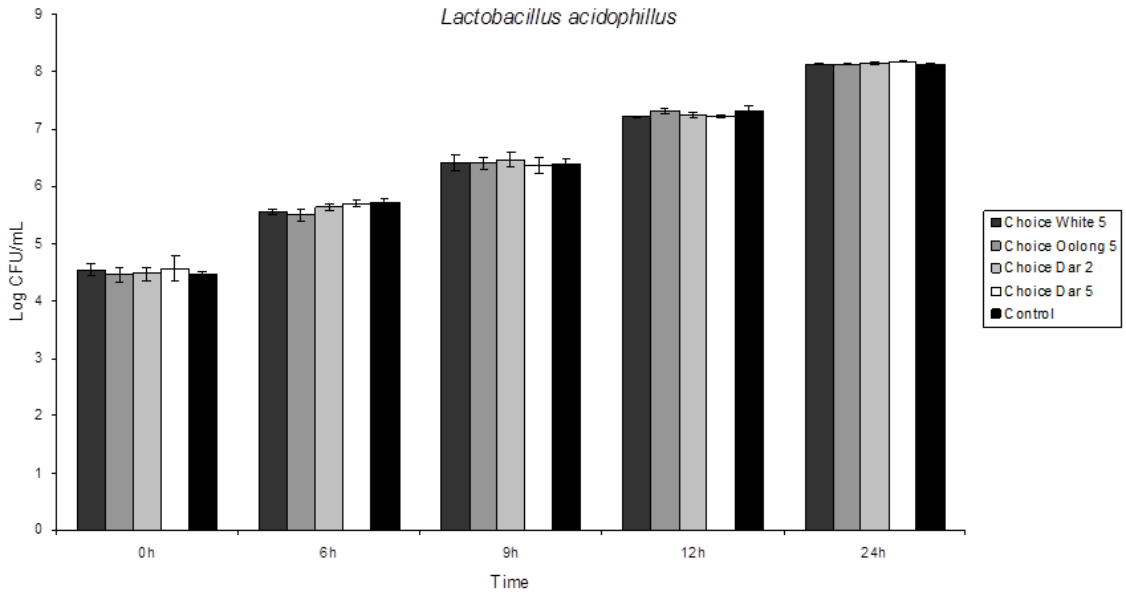

Figure 16: Effect of Choice brand tea extract on *Lactobacillus helveticus* proliferation (cfu/mL) as determined by colony count method in replicates of 3

4.2.5 Conclusion

The antimicrobial effects of tea extracts and the influence of extraction time were evaluated on the inhibition of *H. pylori*. Five min extraction was found to be superior for the *in vitro* inhibition of *H. pylori* linked to gastric diseases. Major phenolic compounds detected by HPLC analysis were gallic acid, quercetin and tea catechins including catechin, epicatechin and epigallocatechin. Preliminary insights into the mechanism of action revealed that inhibition is not linked to proline dehydrogenase based oxidative phosphorylation. Other mechanisms involving membrane disruption, DNA damage or urease inhibition could be involved and will be explored in further studies. These tea extracts did not inhibit species of the beneficial lactic acid bacteria including *Lactobacillus helveticus, Lactobacillus plantarum* and *Bifidobacterium longum*. This leads us to conclude that tea can be potentially used as a dietary support to manage *H. pylori* linked infections without affecting the intestinal probiotic bacteria.

CHAPTER 5

PROBIOTIC FERMENTED FRUIT JUICES AND THEIR HEALTH EFFECTS

5.1 Fermentation of Whole Apple Juice Using *Lactobacillus helveticus* for Potential Dietary Management of Hyperglycemia, Hypertension, and Modulation of Beneficial Bacterial Responses

5.1.1 Abstract

Apple juice from four cultivars were fermented for 0, 24, 48 and 72 h using *Lactobacillus helveticus* and their effects related to management of hyperglycemia, hypertension, inhibition of *Helicobacter pylori* and proliferation of probiotic *Bifidobacterium longum* were evaluated using *in vitro* models. All the assays were carried out at fermented acidic pH and by adjusting the pH to 6.0-7.0. Total soluble phenolics, DPPH-linked free radical scavenging activity and α-amylase inhibition decreased over 72 h. Overall α-glucosidase inhibition and hypertension-relevant ACE inhibition decreased after 72 h for pH adjusted samples whereas contrasting results were obtained for fermented acidic pH samples. Only at 48 h and 72 h fermented acidic pH samples had *H. pylori* inhibitory activity. Further investigations into mechanisms using proline revealed that *H. pylori* inhibition was not through inhibition of proline oxidation via proline dehydrogenase. No inhibition was observed when samples that had *H. pylori* inhibition were further tested for their effect on probiotic *Bifidobacterium longum* growth.

5.1.2 Introduction

Apples are one of the most consumed fruit in the world and in the United States of America. In 2006, the average per capita consumption in the USA was 17.8 lb (Agricultural Marketing resource Centre, www. agmrc.org). Apple consumption has been inversely linked to many chronic diseases including certain types of cancer, cardiovascular diseases, asthma and pulmonary function, diabetes, and inhibition of lipid oxidation (Boyer and Liu 2004). Health benefits of fruits and vegetables, including apple are mainly attributed to the phenolic phytochemicals such as flavonoids, isoflavonoids, carotenoids and phenolic acids present in them (Ross and Kasum, 2002). Consumption of apple as a fruit or its products like juices can be a significant source of phytochemicals in human diets. In the USA it is estimated that apple alone contributes to about 22% of total phenolics consumed from fruits making it the largest source of these health beneficial phenolic phytochemicals (Vinson *et al.,* 2001).

Chronic diseases such as type 2 diabetes, CVD, cancer and other oxidation related health challenges are on the rise because of chronic oxidative stress, unhealthy diets low in fruits and vegetables, excess alcohol consumption, and excess calories, which is further complicated by environmental factors such as pollution and smoking. One of the important therapeutic approaches to counter type 2 diabetes is by decreasing glucose absorption through reduction in starch breakdown by mildly inhibiting starch breakdown enzymes; mainly pancreatic α-amylase and glucose uptake relevant intestinal α-glucosidase. The drug strategies to decrease glucose uptake is associated with side effects including flatulence, abdominal distension and possible diarrhea (Bischoff 1994; Kwon *et al.,* 2006b). Natural plant-based polyphenolic α-amylase and

α-glucosidase inhibitors from fruits and vegetables offer a simple, cheap and overall a more attractive strategy in controlling post-prandial rise in blood glucose without the associated side effects (Matsui et al. 2001; McDougall and Stewart 2005; Kwon *et al.*, 2006b).

Helicobacter pylori is a gram-negative, curve shaped, microaerophilic pathogen that has been identified as an etiological agent of many stomach related diseases including gastritis, peptic ulcer, and rare cases of gastric cancer (Warren and Marshall 1983; Mitchell 1999; You *et al*. 2000; Uemura *et al*. 2001). Antibiotic resistance, low pH of the stomach and high bacterial load of the pathogen has been attributed to the failure of antibiotic based therapies (Megraud and Lamouliatte 2003; Megraud 1997). Natural dietary ingredients from fruits and vegetables offer additional options to compliment rigorous antibiotic therapies associated with side effects. Fermented milk (Felley *et al.,* 2001), wine (Daroch *et al.,* 2001; Ruggiero *et al.,* 2007), and fruits including berries (Kubo *et al.,* 1999; Malekzadeh *et al.,* 2001; Puupponen-Pimia *et al.* 2001; Chatterjee *et al.,* 2004; Lin *et al.,* 2005; Vattem *et al.,* 2005a) have been shown to inhibit *H. pylori* both *in vitro* and *in vivo.*

Probiotics are a class of living organisms which confer health benefits when ingested in certain numbers (Ljungh and Wadström 2006). Lactic acid bacteria colonize the intestine and may confer several benefits to the host including better absorption of nutrients from food, decrease in lactose intolerance in some individuals, control of diseases originating from intestinal infections, potential for control of some types of cancer and stimulation of host immune response (Gilliland 1990; Perdigón *et al.,* 2001; Rafter 2002). Alcoholic apple cider is made by fermenting apple juice using bacterial

and/or yeast strains which is mostly consumed in Europe whereas cider consumed in North America and Canada is mostly unfermented non-alcoholic raw apple juice. In this study, we fermented juice extracted from whole apples using *Lactobacillus helveticus* over a period of 72 h and investigated the changes in total phenolics, antioxidant activity, inhibition of enzymes related to type 2 diabetes, *H. pylori* inhibition, and lactic acid bacteria proliferation in acidic and pH adjusted samples. Furthermore, preliminary investigations on the mode of action of *H. pylori* inhibition by fermented apple juice were carried out on the basis of the rationale that simple phenolics may mimic proline analogs and may exhibit antimicrobial activity by inhibition of proline oxidation via proline dehydrogenase at the same time these extracts would not affect beneficial lactic acid bacteria such as *Bifidobacterium longum* (Shetty and Wahlqvist 2004; Lin *et al.*, 2005; Kwon *et al*. 2007b; Apostolidis *et al.*, 2008).

5.1.3 Materials and Methods

5.1.3.1 Sample Extraction

Four cultivars of apple: Red delicious, Fuji, Golden delicious and Granny smith were purchased from Stop and Shop, Hadley, MA, USA. Whole apples were washed with water and then cut into small pieces. These small pieces were liquefied using a Waring blender for 5 min. The thick pulp was then centrifuged for 15 min at 15,000 g; supernatants were collected and kept at -20 ^{o}C during the period of the study.

5.1.3.2 Bacterial Strains

The lactic acid bacteria strains used in this study were the following: *Lactobacillus helveticus* was provided by Rosell Institute Inc. Montreal, Canada (Lot#: XA 0145, Seq#:00014160), *Bifidobacterium longum* was isolated in a previous study (Apostolidis *et al*. 2007), *Helicobacter pylori* (strain ATCC 43579, which originated from human gastric samples) was obtained from the American Type Culture Collection (Rockville, MD).

5.1.3.3 Fermentation

Initially 100 µL of frozen *Lactobacillus helveticus* stock were inoculated into 10 mL MRS broth (Difco) and incubated for 24 h at 37 °C. Then, 100 µL of the 24 h grown strain was re-inoculated into 10 mL MRS broth for 24 h at 37 ^{o}C. Ninety mL of the juice was put into a 125 mL Erlenmeyer flask; pH adjusted to 6 using NaOH and inoculated using 10 mL of the broth culture. Fermentation was carried out at 37 ^{o}C and 12.5 mL samples were taken out at 0, 24, 48 and 72 h. At every time point except for 0 h, two 12.5 mL samples were taken out, for one sample pH was adjusted to 6 by adding drops of 0.1M NaOH. For the fermented acidic pH sample; same number of drops of distilled water was added to keep the volume same. The samples were centrifuged at 15,000 g for 15 min and then used for the assays.

5.1.3.4 Absorbance of Sample and Colony Counts

Growth of bacteria in the sample was estimated using absorbance at 600 nm. More accurate estimations of the bacterial numbers were made by plate count

technique. At 0, 24, 48 and 72 h, CFU/mL was determined by pipetting 100 µL of the sample, serially diluting and plating on MRS medium. The plates were incubated anaerobically at 37 ^{o}C for 24 h and individual colonies were counted. The pH of the samples was also measured at 24, 48 and 72 h.

5.1.3.5 Total Phenolics Assay

The total phenolics in all samples were determined by using a method modified by Shetty *et al.*, (1995). In brief, 0.5 mL of sample extract was added to a test tube and mixed with 0.5 mL of 95% ethanol and 5 mL of distilled water. To each sample, 0.5 mL of 50% (vol/vol) Folin-Ciocalteu reagent was added and mixed. The absorbance was read at 725 nm using a spectrophotometer (Genesys UV/Visible, Milton Roy, Inc., Rochester, NY). Different concentrations of gallic acid were used to develop a standard curve. Results were expressed as mg of gallic acid/mL of sample.

5.1.3.6 Antioxidant Activity by DPPH Radical Inhibition Assay

The antioxidant activity was determined by the DPPH radical scavenging method modified from Kwon *et al.*, (2006b). A 250-µL aliquot of the sample extract was mixed with 1,250 µL of DPPH (60 µM in ethanol). Absorbance was measured at 517 nm using the Genesys UV/Visible spectrophotometer. The readings were compared with the controls, containing 95% ethanol instead of sample extract. The percentage inhibition was calculated by:

$$\%\ \text{inhibition} = \frac{(Absorbance_{\text{control}} - Absorbance_{\text{extract}})}{Absorbance_{\text{control}}} \times 100$$

5.1.3.7 α-Glucosidase Inhibition Assay

The α-glucosidase inhibitory activity was determined by an assay modified from McCue *et al.,* (2005a). α-Glucosidase was assayed by using 50 µL of sample extracts and 100 µL of 0.1 M phosphate buffer (pH 6.9) containing α-glucosidase solution (1 U/mL) and was incubated in 96-well plates at 25 °C for 10 min. After preincubation, 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 incubation, absorbance readings were recorded at 405 nm by a microplate reader (Thermomax, Molecular Devices Co., Sunnyvale, CA) and compared to a control that had 50 µL of buffer solution in place of the extract. The α-glucosidase inhibitory activity was expressed as percentage inhibition and was calculated with the same equation as for percentage inhibition in the DPPH radical inhibition assay. Dose dependency was tested using 10 µL and 25 µL of the sample, the volume made up to 50 µL using 0.1 M phosphate buffer (pH 6.9) and same protocol was followed.

5.1.3.8 α-Amylase Inhibition Assay

The α-amylase inhibitory activity was determined by an assay modified from McCue *et al.,* (2005a). A total of 500 µL of 1:10 dilution (due to the high sugar content) of sample extract and 500 µL of 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M NaCl) containing α-amylase solution (0.5 mg/mL) were incubated at 25 °C for 10 min.

After preincubation, 500 μL of a 1% starch solution in 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M NaCl) was added to each tube at timed intervals. The reaction was stopped with 1.0 mL of dinitrosalicylic acid color reagent. The test tubes were incubated in a boiling water bath for 5 min and cooled to room temperature. The reaction mixture was then diluted after adding 15 mL of distilled water, and the absorbance was measured at 540 nm using the Genesys UV/Visible spectrophotometer. The readings were compared with the controls, containing buffer instead of sample extract. The percentage α-amylase inhibitory activity was calculated with the same equation as for percentage inhibition in the DPPH radical inhibition assay.

5.1.3.9 ACE Inhibition Assay

ACE inhibition was assayed by a method modified by Kwon *et al.,* (2006b). The substrate hippuryl-histidyl-leucine (HHL) and the enzyme ACE-I from rabbit lung (EC 3.4.15.1) were used. Fifty μL of sample extracts were incubated with 100 μL of 1 M NaCl-borate buffer (pH 8.3) containing 2 mU of ACE-I solution at 37 °C for 10 min. After preincubation, 100 μL of a 5 mU substrate (HHL) solution was added to the reaction mixture. Test solutions were incubated at 37 °C for 1 h. The reaction was stopped with 150 μL of 0.5 N HCl. Five μL of the sample was injected in a high-performance liquid chromatography (HPLC) apparatus (Agilent 1100 series equipped with autosampler and DAD 1100 diode array detector, Agilent Technologies, Palo Alto, CA). The solvents used for gradient were (1) 10 mM phosphoric acid (pH 2.5) and (2) 100% methanol. The methanol concentration was increased to 60% for the first 8 min and to 100% for 5 min and then was decreased to 0% for the next 5 min (total run time,

18 min). The analytical column used was an Agilent Nucleosil 100-5C18, 250 mm × 4.6 mm inside diameter, with packing material of 5 μm particle size at a flow rate of 1 mL/minute at ambient temperature. During each run, the absorbance was recorded at 228 nm, and the chromatogram was integrated using the Agilent Chemstation (Agilent Technologies) enhanced integrator for detection of liberated hippuric acid (A). Hippuric acid standard was used to calibrate the standard curve and retention time. The percentage inhibition was calculated by:

$$\%\ \text{inhibition} = \frac{(A_{\text{control}} - A_{\text{extract}})}{(A_{\text{control}} - A_{\text{blank}})} \times 100$$

5.1.3.10 Protein Assay

Protein content was measured by the method of Bradford assay (Bradford 1976). The dye reagent concentrate (Bio-Rad protein assay kit II, Bio-Rad Laboratory, Hercules, Calif., USA) was diluted 1:4 with distilled water. Five mL of diluted dye reagent was added to 100 μL of fermented supernatant. After vortexing and incubating for 5 min, the absorbance was measured at 595 nm against a 5 mL reagent blank and 100 μL buffer using a UV-VIS Genesys spectrophotometer (Milton Roy, Inc., Rochester, N.Y., USA).

5.1.3.11 HPLC Determination of Lactic Acid Content

Five μl of sample was injected using Agilent ALS 1100 autosampler into Agilent 1100 series HPLC (Agilent Technologies) 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, 25 min). The analytical column used was Agilent Zorbax SB-C18, 250 × 4.6 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 210 nm and integrated using Agilent Chemstation enhanced integrator. Pure standards of lactic acid (purchased from Sigma Chemical Co. in 100% methanol were used to calibrate the standard curve and retention times.

5.1.3.12 High Performance Liquid Chromatography (HPLC) Analysis of Phenolic Profiles

The extracts (2 mL) were filtered through a 0.2 μm filter. A volume of 5 μL of extract was injected using an Agilent ALS 1100 auto sampler into an Agilent 1100 series HPLC (Agilent Technologies, Palo Alto, CA) equipped with a 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, 25 min). The analytical column used was Agilent Zorbax SB-C_{18}, 250 x 4.6 mm i.d., with packing material of 5 μm particle size at a flow rate of 1 mL/min at room temperature. During each run the absorbance was recorded at 226 and 306 nm and the chromatogram was integrated using Agilent Chemstation enhanced integrator. Pure standards of gallic acid, catechin,

epicatechin, quercetin, p-coumaric acid, caffeic acid and resveratrol in 100% methanol were used to calibrate the standard curves and retention times.

5.1.3.13 Preparation of Starter Culture of *Helicobacter pylori*

Helicobacter pylori were cultured according to Stevenson *et al.,* (2000). Standard plating medium (*H. pylori* agar plates) were prepared by using 10 g of special peptone (Oxoid Ltd., Basingstoke, England) per liter, 15 g of granulated agar (Difco Laboratories, Becton, Dickinson and Co., Sparks, MD) per liter, 5 g of sodium chloride (EM Science, Gibbstown, NJ) per liter, 5 g of yeast extract (Difco) per liter, 5 g of beef extract (Difco) per liter of water.

Broth media were prepared by 10 g of special peptone (Oxoid Ltd., Basingstoke, England) per liter, 5 g of sodium chloride (EM Science, Gibbstown, NJ) per liter, 5 g of yeast extract (Difco) per liter, 5 g of beef extract (Difco) per liter of water. A volume of 1 mL of stock *H. pylori* was added into test tubes containing 10 mL of sterile broth media. They were incubated at 37 °C for 24 h before being used for inoculating by the spread plate technique. The active culture was then spread on *H. pylori* agar plates to make bacterial lawn for the agar-diffusion assay.

5.1.3.14 Agar-Diffusion Assay

The antimicrobial activity of sample extracts was analyzed by the agar-diffusion method. The assay was done aseptically using sterile 12.7 mm diameter paper disks (Schleicher & Schuell, Inc., Keene, NH) to which 100 µL of test extracts were added. Saturated disks were placed onto the surface of seeded agar plates. Controls consisted of disks with distilled water only. 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). The diameter of clear zone (no growth) surrounding each disk was measured and the zone of inhibition was determined and expressed in mm. For the dose dependency studies, 50 µL and 75 µL of the sample were used. Each experiment was repeated 2 times and consisted of three replicates each time (three disks per sample or treatment).

5.1.3.15 Proline Growth Response Assay

A model for mode of action of phenolic phytochemicals was developed based on the rationale that small phenolics could behave as proline analogs or proline analog mimics and likely inhibit proline oxidation via proline dehydrogenase (Shetty and Wahlqvist, 2004). Further, the likely inhibitory effects of phenolic phytochemicals should be overcome by proline if the site of action is proline dehydrogenase.

Bacterial lawns of *H. pylori* were prepared as described previously. Plating media were prepared by using standard plating medium as described in agar-diffusion assay with some modifications. Proline (Sigma, Louis, Mo) was added into the medium to a final concentration of 0.5 and 5 mM. Then a similar protocol as mentioned in the agar diffusion assay was followed.

5.1.3.16 Lactic Acid Bacterial Proliferation Assay

Initially 100 µL of frozen stock from the lactic acid bacterial strain *Bifidobacterium longum* were inoculated into 10 mL MRS broth (Difco) and incubated

for 24 h at 37 °C. Then, 100 µL of the 24 h grown strain was re-inoculated into 10 mL MRS broth for 24 h at 37 ^{o}C. Sample volumes of one mL were filter sterilized using sterile filters Millex GP 0.22 µm (Millipore Corp., Bedford, MA). Filter sterilized sample extracts (1 mL) and 100 µL of the 48 h grown strain (diluted 100 times in sterile distilled water) were added into 9 mL of MRS broth tubes and incubated at 37 °C for 24 h. Control with 1 mL of sterile distilled water instead of sample extract was also included. 100 µL of the serially diluted samples were plated in triplicates every 0, 6, 9, 12, and 24 h on MRS agar (Difco) plates and incubated in anaerobic BBL GasPak jars (Becton, Dickinson and Co., Sparks, MD) with BD GasPak EZ anaerobe container system sachets (Becton, Dickinson and Co., Sparks, MD) at 37 °C for 48 h to determine the CFU/mL.

5.1.3.17 Statistical Analysis

All experiments were performed at least in duplicate or triplicate. Analysis at every time point from each experiment was carried out in duplicate or triplicate. Means, standard errors and standard deviations were calculated from replicates within the experiments and analyzed using Microsoft Excel XP. Significant differences were determined using the student *t*-test and valued at $p<0.05$.

5.1.4 Results and Discussion

5.1.4.1 Absorbance and Colony Counts

Both absorbance and plate counts represented the bacterial growth curve over a period of 72 h (Fig 17). Fermentation started at 0 h with CFU/mL around 7 logs. At 24 h it increased approximately by half a log representing the log phase of the bacterial curve. Then at 48 h the numbers either stayed the same or decreased slightly representing the stationary phase. At 72 h the numbers were reduced for all samples but for most samples the final numbers were more than at 0 h. This suggested that pH adjusted whole apple juice is a good substrate for *Lactobacillus helveticus* fermentation.

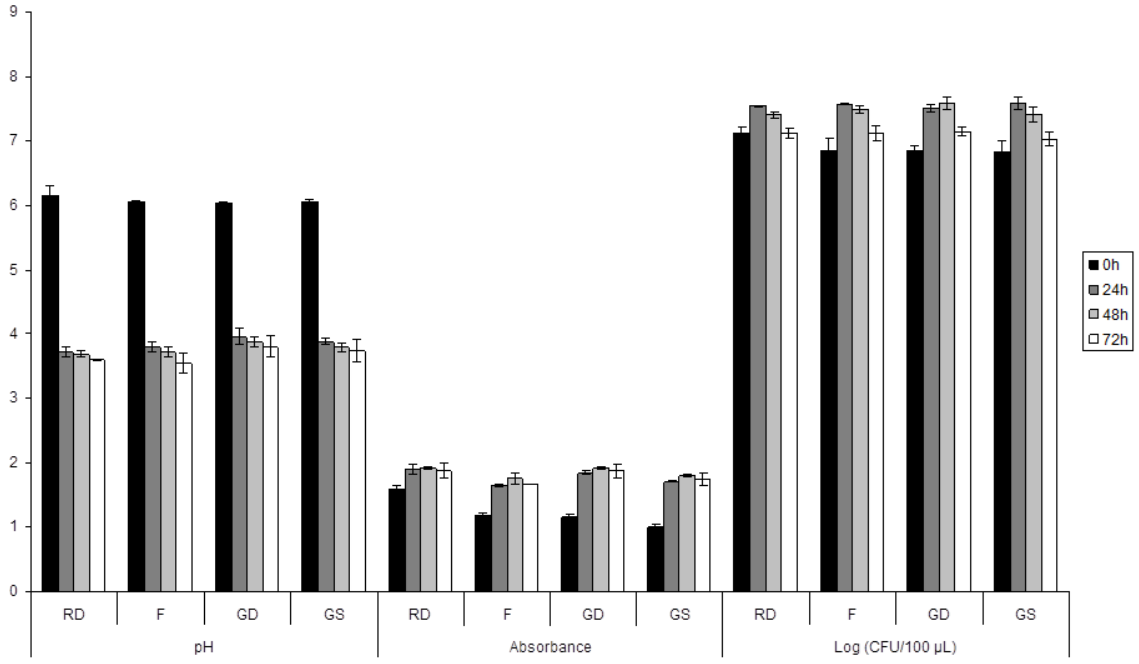

Figure 17: pH, absorbance and colony counts of fermented apple juice extracts.

5.1.4.2 Total Soluble Phenolics

Total phenolics were determined for all samples using Folin-Ciocalteu method. At 0 h Red delicious had the highest phenolics (0.38 mg/mL) whereas Granny smith had the lowest (0.23 mg/mL) (Fig 18). For pH adjusted samples total phenolics decreased significantly ($p<0.05$) at 24 h for all samples. At 48 h, total phenolics decreased significantly ($P<0.05$) for Red delicious and Fuji but not for Golden delicious and Granny smith. This difference could be because of different skin color of the apple resulting in different spectrum of phenolic compounds in the fermenting substrate. At 72 h, total phenolics decreased for Fuji but for all other samples it did not change.

For fermented acidic pH samples a similar overall effect was seen. Total phenolics decreased significantly ($P<0.05$) for all samples at 24 h. For Red delicious total phenolics stayed almost same at 48 h whereas for Fuji it decreased.

For the green colored skin-cultivars the total phenolic increased at 48 h. For 72 h the values decreased again for all samples however this was only significant in case of Fuji.

McCue and Shetty (2005), reported that the total phenolics present in the fermented substrate is a result of phenolic mobilization linked to a flux between the formation/degradation of polymeric phenolics and the liberation of free phenolics in soymilk bioprocessing using mixed Kefir cultures. Decrease in total phenolics during fermentation was further supported by the work of Apostolidis *et al.*, (2007). The decrease in soluble phenolic content could result from polymerization of phenolic compounds. The decrease could also result from degradation of phenolic compounds as a possible detoxification mechanism for lactic acid bacteria.

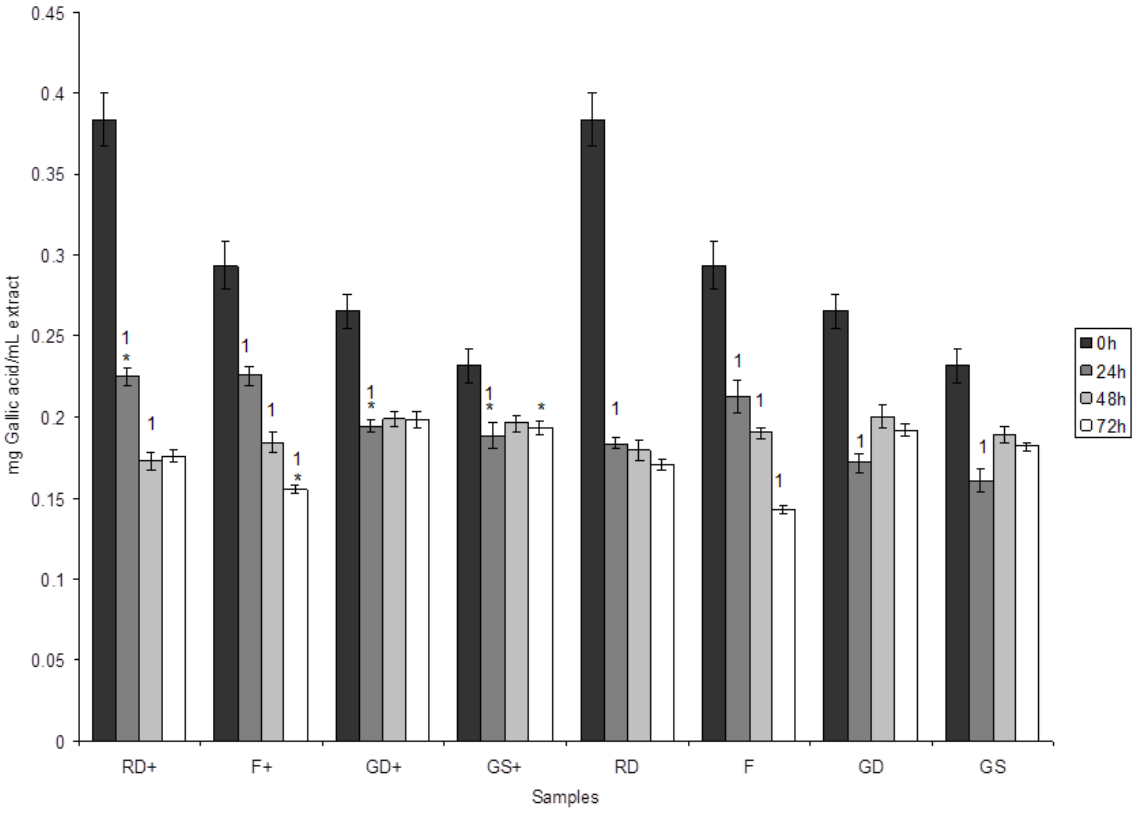

Figure 18: Total phenolic content of extracts of apple juice fermented using *Lactobacillus helveticus*.

The significant increase in total phenolic content at 48 h for acidified (fermented acidic pH) samples of Golden delicious and Granny smith could be because of degradation of large polymeric phenolic structures present in the peel of these green colored cultivars. Further investigation of the enzymes related to these biochemical changes would be the focus of our future studies.

The effect of pH on phenolic changes is summarized by Friedman and Jurgens (2000). They suggested that the spectrum of monocyclic phenolic compounds such as caffeic, cholorogenic, and gallic acid could be irreversibly changed between pH 7-11 and that the extents of these spectral changes are related to pH in that range. Multi-ring aromatic structures such as catechin, epicatechin and rutin are more stable to such changes. Changes in spectra would mean a change in the chemical structure; however the nature of such chemical transformations were not discussed. Although the pH range tested in their research was high, these results might explain the difference in total phenolics in pH adjusted and not adjusted samples.

5.1.4.3 Antioxidant Activity by DPPH Radical Inhibition Assay

Measurement of antioxidant activity using discoloration of 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals has been widely used due to its stability, simplicity, and reproducibility (Katsube *et al.,* 2004). The DPPH-linked free radical scavenging activity was moderately high for all samples at 0 h indicating whole apple juice has good potential for free radical inhibition (Fig 19). For pH adjusted samples, antioxidant activity decreased at 24 h for all samples.

At 48 h, antioxidant activity increased, however this increase was significant ($P<0.05$) only in case of Fuji and Golden delicious. At 72 h, the antioxidant activity decreased for all samples except Red delicious.

For fermented acidic pH samples a similar trend was observed. At 24 h, the antioxidant activity decreased for all samples except Golden delicious. This decrease was significant ($P<0.05$) only in case of Red delicious. The antioxidant activity at 48 h

increased for all samples. At 72 h, like in pH adjusted samples, the antioxidant activity decreased in all samples.

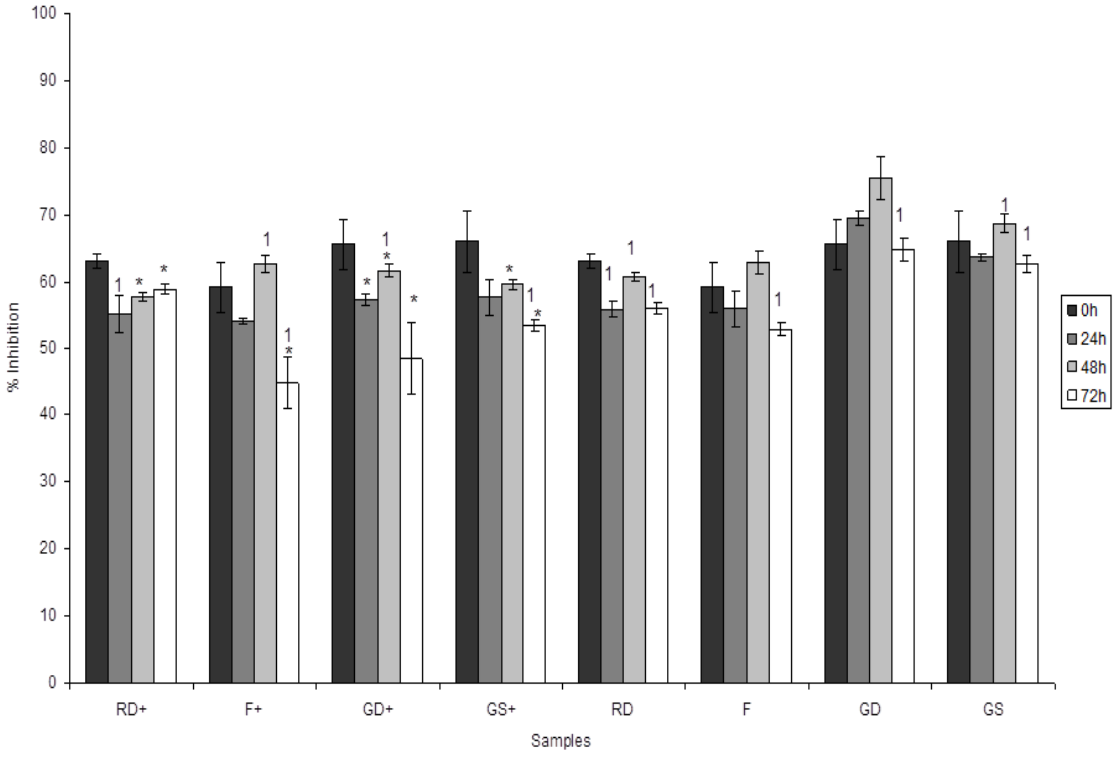

Figure 19: DPPH scavenging activity (% Inhibition) of extracts of fermented apple juice using *Lactobacillus helveticus*.

In most cases a significant difference was observed at 48 and 72 h, fermented acidic pH samples were seen to have a higher antioxidant activity. This suggests that the structural changes caused by adjusting the pH might be responsible for the change in potential antioxidant activity. A moderate correlation was observed for pH adjusted

samples (0.49-0.87) whereas a weak correlation was observed for fermented acidic pH samples (-0.32-0.78). An inverse relationship has been reported during soymilk fermentation; possibly due to mobilization and likely polymerization of phenolics (McCue and Shetty, 2005; Apostolidis *et al.*, 2007). Wang *et al.*, (2006) reported an increase in the antioxidant activity during soymilk fermented with lactic acid bacteria and bifidobacteria. They suggest liberation of isoflavone aglycones in soymilk through catalytic action of β-glucosidase activity and also intracellular antioxidants of starter organisms. The increase or decrease in DPPH radical scavenging linked antioxidant activity at a time point may depend on either one of these factors or it may be possible that these factors work in combination.

5.1.4.4 α-Glucosidase Inhibition Assay

Consumption of apples has been inversely linked to the risk of type 2 diabetes (Boyer and Liu, 2004). High α-glucosidase inhibitory activity was observed for all samples at 0 h which indicates whole apple juice has good potential to reduce post-prandial rise in blood glucose. For pH adjusted samples, at 24 h α-glucosidase inhibitory activity decreased for all samples (Fig 20). At 48 h, inhibitory activity increased slightly for Red delicious, Fuji and Granny smith whereas it increased significantly ($P<0.05$) for Golden delicious. The activity decreased for all samples at 72 h. Dose dependency studies using 10 µL and 25 µL indicated a dose dependent response. Dose dependency studies yielded a similar pattern of inhibitory activity overall for different doses.

For fermented acidic pH samples, after 0 h the inhibitory activity increased for all samples at all time points (Fig 21). The inhibitory activity for fermented acidic pH samples were significantly ($P<0.05$) higher than the pH adjusted ones at all time points after 0 h. At 24 h, Golden delicious showed a significant ($P<0.05$) increase. At 48 h, the increase in activity was significant ($P<0.05$) for all samples except Red delicious. At 72 h, only Red delicious showed a significant increase ($P<0.05$).

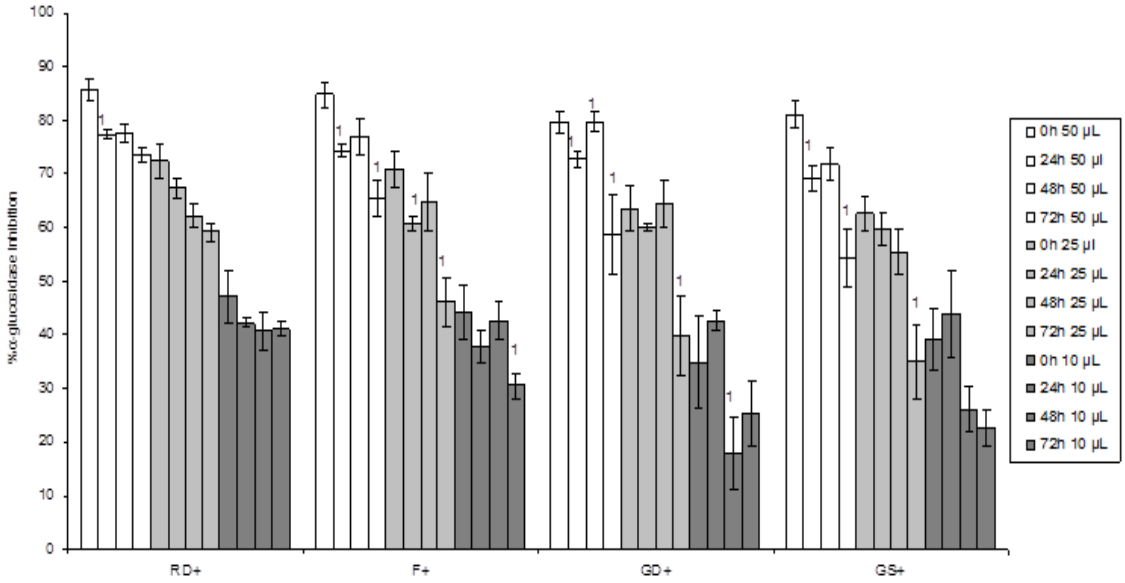

Figure 20: Dose-dependent changes in percentage α-glucosidase inhibitory activity of pH adjusted extracts of fermented apple juice using *Lactobacillus helveticus*.

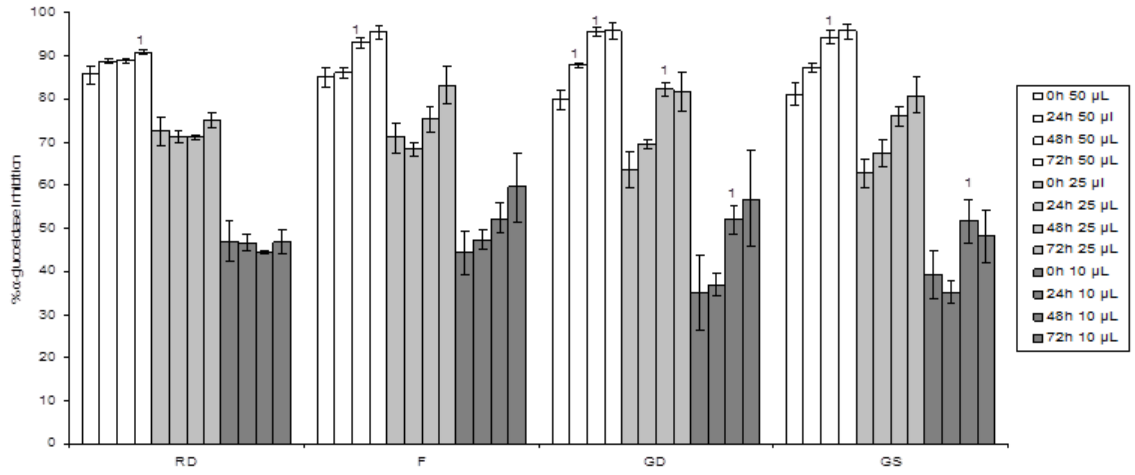

Figure 21: Dose-dependent changes in percentage α-glucosidase inhibitory activity of pH not adjusted extracts of fermented apple juice using *Lactobacillus helveticus*.

This suggests that fermenting whole apple juice with *Lactobacillus helveticus* can enrich the juice with compounds that can potentially have relevance for countering hyperglycemia linked to type 2 diabetes.

α-Glucosidase inhibitory activity in the fermented samples seems to be a combined phenolic and pH effect. In the pH adjusted samples the decrease in α-glucosidase inhibitory activity could be because of degradation of simple phenolics which have α-glucosidase inhibitory activity (Apostolidis *et al.*, 2006b; Kwon *et al.*,

2006b) or it could be because the compounds formed because of polymerization of simple phenolics may not be equally effective in inhibiting α-glucosidase. The increase in 48 h sample may be because of formation of free phenolic because of degradation of large polymeric phenolics (Apostolidis *et al.,* 2007). This increase however was not significant except for Golden delicious.

For the fermented acidic pH samples, the increase in the α-glucosidase inhibitory activity at all time points could also be a contribution from the pH effect. For all cultivars, a high correlation (r =0.95-0.99) between lactic acid and α-glucosidase inhibition was observed. Although the phenolic effect may modulate overall inhibitory activity based on the type of phenolics, the pH effect may further enhance inhibitory activity. This was confirmed by the observation that at a given time point when pH was acidic the total inhibitory activity was higher than when the pH was adjusted reflecting the contribution of both phenolics and lactic acid. In a similar study Apostolidis *et al.,* (2007), also reported an increase in inhibitory activity during fermentation of milk and soymilk where the pH of the extracts was acidic.

5.1.4.5 α-Amylase Inhibition Assay

α-Amylase catalyzes hydrolysis of starch and inhibition of α-amylase is one of the mechanisms by means of which commercial drugs help reduce blood glucose level after a high carbohydrate meal. Because of high sugar content, the samples were diluted before the assay by 1:10. Fermentation at 24 h increased or maintained same inhibition for all pH adjusted samples (Fig 22). At 48 h, inhibitory activity decreased for Red

delicious and Granny smith whereas increased inhibition was observed in Fuji and Golden delicious samples. At 72 h, activity decreased for all samples except Red delicious. There was not a clear difference between the pH adjusted and fermented acidic pH samples.

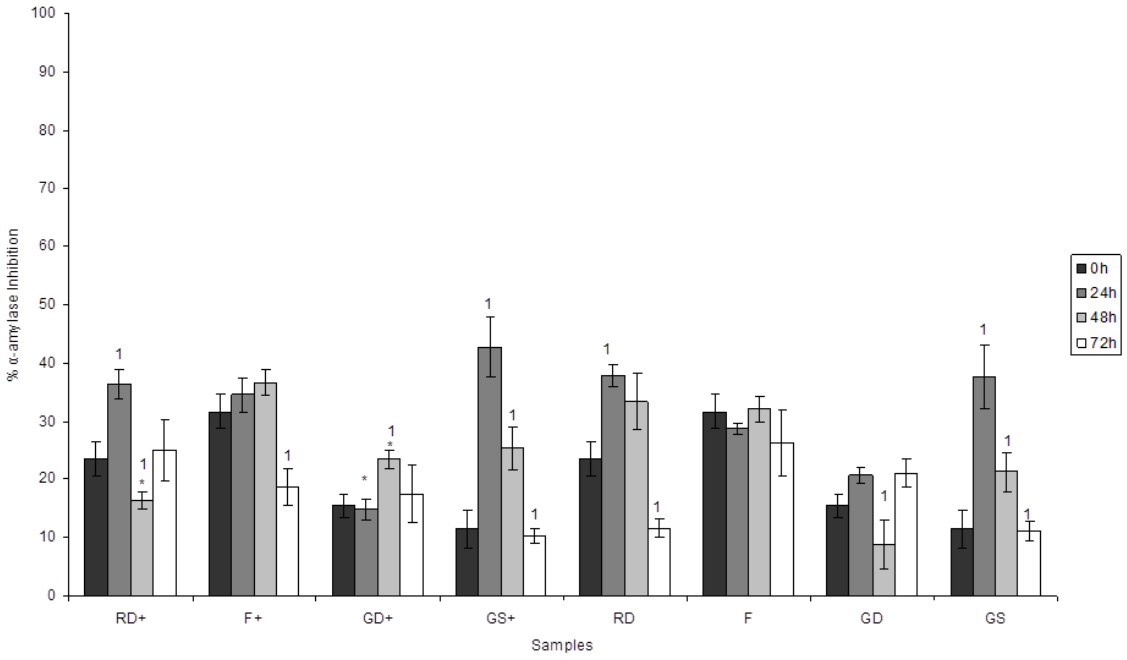

Figure 22: Changes in percentage α-amylase inhibitory activity of both pH adjusted and pH not adjusted extracts of fermented apple juice using *Lactobacillus helveticus*.

For fermented acidic pH samples, a similar trend was observed at 24, 48 and 72 h as the pH adjusted samples except for Fuji 24 h, where the value decreased and for Golden delicious where the inhibitory activity was found to decrease at 48 h and then increase at 72 h. Overall, fermentation maintained the inhibition constant or caused an

overall decrease in α-amylase inhibitory activity in most cases. Excessive inhibition of α-amylase could result in problems arising from abnormal bacterial fermentation of undigested starch in the colon (Puls *et al.,* 1977; McCue *et al.,* 2005a). So mild inhibition of α-amylase is good for food-based therapeutic uses for potentially managing early stages of hyperglycemia linked to on-set of type 2 diabetes.

α-Amylase inhibitory activity seems to be only a phenolic effect. At most time points there was not a significant difference in the pH adjusted and fermented acidic pH samples. Apostolidis *et al.,* (2007), reported that only lactic acid does not have any significant α-amylase inhibitory properties. The inhibition could be only related to the flux between the formation and degradation of polymeric phenolics and the liberation of free phenolics as discussed previously.

5.1.4.6 ACE Inhibition Assay

Inhibition of ACE, an important enzyme in maintaining vascular tension, is considered a useful therapeutic approach in the treatment of high blood pressure in both diabetic and non-diabetic patients (McCue *et al.,* 2005b). The 0 h values for ACE inhibitory activity were high for whole apple juice indicating it has a good potential in managing high blood pressure (Fig 23). At 24 h, for pH adjusted samples, the inhibitory potential decreased for all samples except for Red delicious, in which case it increased slightly. At 48 h, the values decreased for all samples, however the decrease was significant ($P<0.05$) only in case of Red delicious. At 72 h, the inhibitory activity decreased for all samples except Golden delicious where it increased slightly.

For the fermented acidic pH samples, the inhibitory activity increased at 24 h for all samples, however it was only significant in case of Golden delicious and Granny smith. At 48 h, the inhibitory activity decreased for all samples except Granny smith where it was the same. At 72 h, the values did not change for Red delicious and Fuji, whereas it slightly reduced for Granny smith and it increased significantly for Golden delicious. At all time points the fermented acidic pH samples of Golden delicious and Granny smith had significantly ($P<0.05$) higher inhibitory activity than their pH adjusted samples of the same cultivar, whereas Red delicious was only significantly ($P<0.05$) higher at the last two time points. Fuji was not different for pH adjusted and fermented acidic pH samples at all time points.

The changes in ACE inhibitory potential could be a combined phenolic and pH effect. Lactic acid is suggested to have significant relevance in ACE inhibition. An elevation in *in vivo* lactate production has been observed during administration of ACE inhibitors to humans (Frossard *et al.*, 2000). For pH adjusted samples, ACE inhibitory activity decreased indicating the lactic acid effect may be neutralized by pH adjustment. For fermented acidic pH samples, ACE inhibitory activity increased at 24 h and then subsequently remained constant or decreased at all time points except Golden delicious where it increased at 72 h.

Apostolidis *et al.*, (2007) reported that although lactic acid has a significant effect on ACE inhibition this effect was more pronounced when lactic acid is present at 0.5% or 1%, and no effect was observed when lactic acid was present at 1.5% and 3%. This explains why although lactic acid amount increased in the samples during fermentation, ACE inhibitory activity did not further increase or decrease. The decrease

in inhibitory activity may be because of degradation of small phenolics which had ACE inhibitory potential. The increase in inhibitory activity at 72 h for Golden delicious cannot be explained at this time.

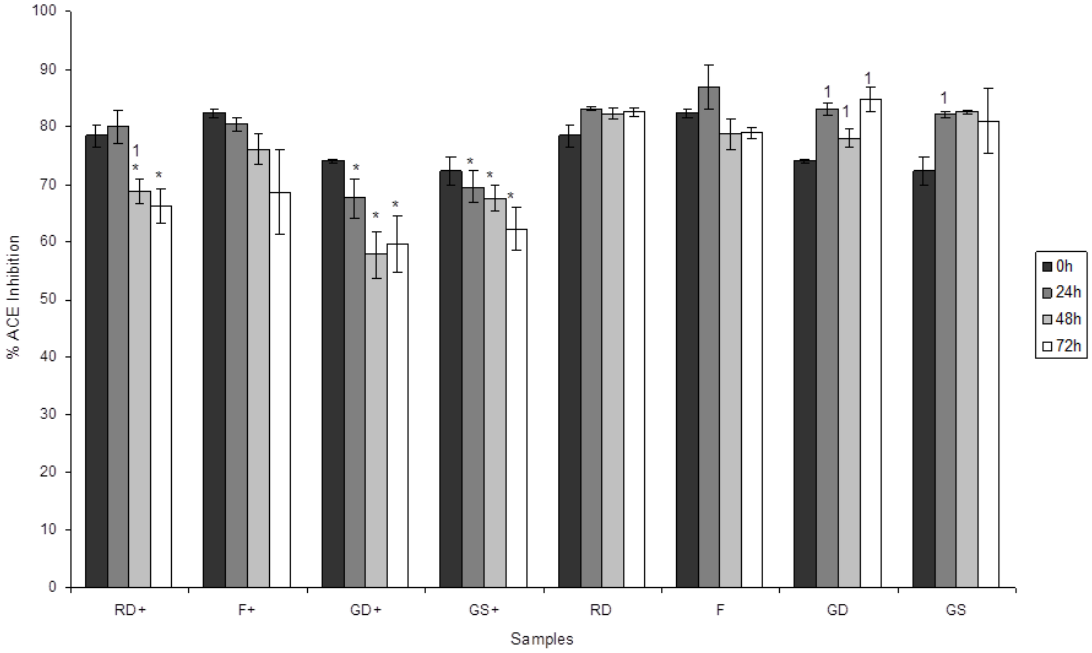

Figure 23: Percentage ACE inhibitory activity of both pH adjusted and pH not adjusted extracts of fermented apple juice using *Lactobacillus helveticus*.

5.1.4.7 Protein Assay

Protein content was measured using the Bradford assay (Bradford, 1976). The protein content decreased for both pH adjusted and fermented acidic pH samples for all time points (Fig 24). The pH adjusted samples were higher in protein content than the fermented acidic pH samples. However, this difference was significant ($P<0.05$) only for Red delicious and Fuji at 24 and 48 h and Golden delicious at 24 h. Lactic acid bacteria depend strongly on exogenous sources of nitrogen (Yamamoto *et al.,* 1993), indicating the action of nonspecific proteinases secreted during fermentation might cause liberation of peptides which explains the decrease in protein content during fermentation. The decrease in protein content could also be because of denaturation of protein because of pH changes during fermentation. The lower protein content in fermented acidic pH samples could be because adjusting the pH would lead to refolding of some proteins and which was better reflected in the Bradford assay.

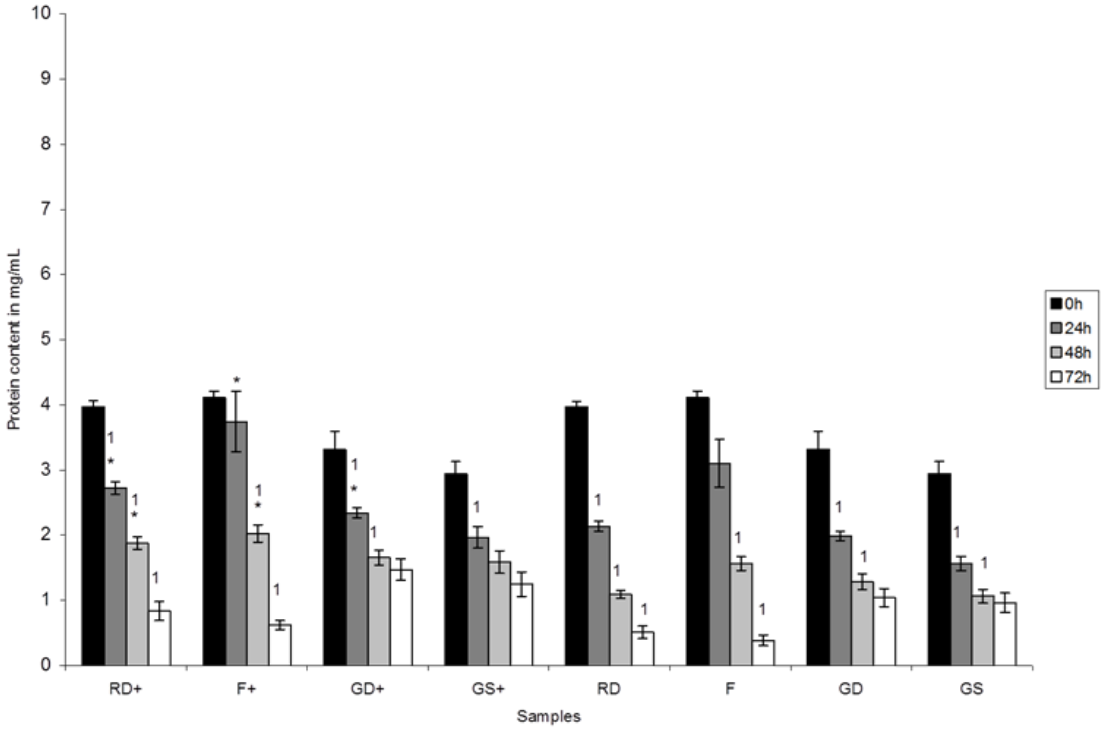

Figure 24: Protein content (mg/mL) as measured by the Bradford assay of both pH adjusted and pH not adjusted extracts of fermented apple juice using *Lactobacillus helveticus*.

5.1.4.8 Lactic Acid Content Measured by HPLC

Lactic acid in the sample was measured by using HPLC. At 0 h some amount of lactic acid was present in the sample (Fig 25). At 24 h, the lactic acid content increased for all samples significantly ($P<0.05$) as expected. However at 48 h, the lactic acid content decreased for all samples except Granny smith. At 72 h, it remained constant for Red delicious and Fuji whereas it increased for Golden delicious and decreased for

Granny smith. The reasons for change in the amount of lactic acid after 24 h are not clear. It may be possible that other organic acids like malic acid are being detected by HPLC or adjusting the pH is causing the salt of the acid not being detected by the HPLC under the conditions used.

For fermented acidic pH samples, the amount of lactic acid for all samples at all time points increased. Significant difference in time point is indicated (Fig 25). There was no significant ($P>0.05$) difference in the pH adjusted and the fermented acidic pH at any time point for any sample.

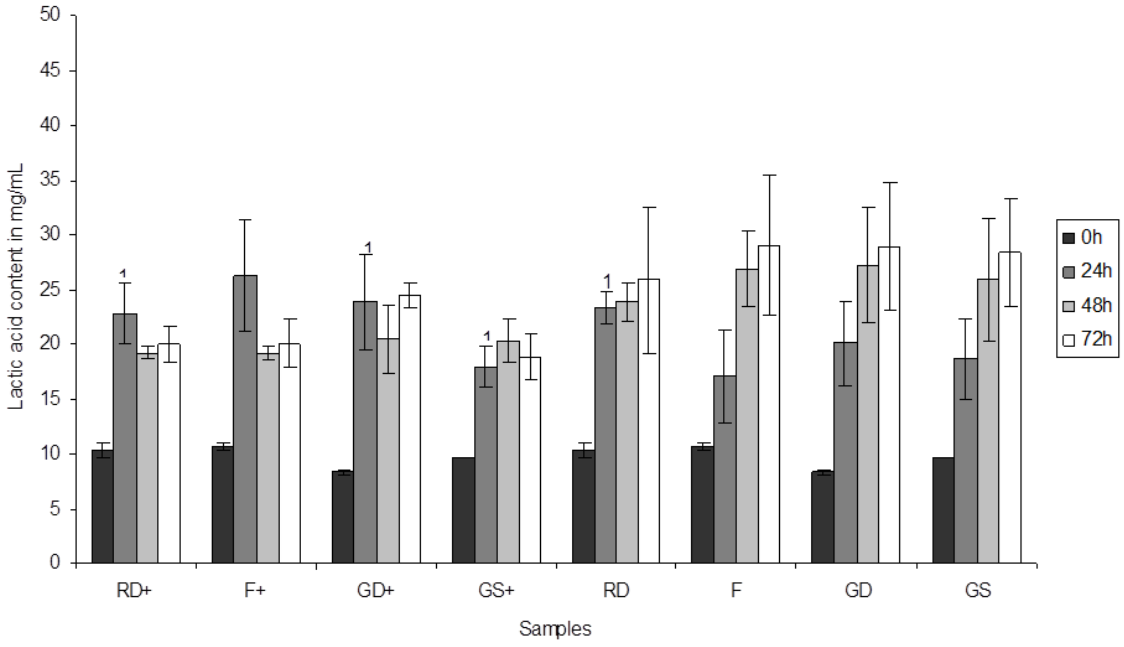

Figure 25: Lactic acid content (mg/mL) as measured by HPLC of both pH adjusted and pH not adjusted extracts of fermented apple juice using *Lactobacillus helveticus*.

5.1.4.9 High Performance Liquid Chromatography (HPLC) Analysis of Phenolic Profiles

Apple is a major source of phenolic compounds in the American diet. It is estimated that in USA about 22% of the total phenolic compounds from fruits is contributed by apple alone (Vinson *et al.,* 2001). It is reported that phenolic compounds are transformed by the gut bacteria before absorption and also this modulates the biological activity of these compounds (Selma *et al.,* 2009).

Table 3: HPLC Analysis of Individual Phenolic compounds of Fermented Extracts of Red Delicious (µg/mL).

Phenolic compound	pH adjusted				pH not adjusted			
	0 h	24 h	48 h	72 h	0 h	24 h	48 h	72 h
Gallic acid	0.255 ± 0.002	0.265 ± 0.002	0.34 ± 0.09		0.255 ± 0.002	0.23 ± 0.012	0.35 ± 0.06	0.24 ± 0.005
Catechin	1.02 ± 0.017	0.795 ± 0.08	0.54 ± 0.01	0.56 ± 0.064	1.02 ± 0.017	0.98 ± 0.05	1 ± 0.02	0.96 ± 0.005
Epicatechin	28.1 ± 2.8	23.3 ± 0.6	19.5 ± 3	13.2 ± 0.18	28.1 ± 2.8	17.6 ± 1.1	17.6 ± 2.1	
Quercetin derivatives	0.07 ± 0.01	0.033 ± 0.01			0.07 ± 0.01			
p-coumaric acid	0.054 ± 0.006	0.041 ± 0.01			0.054 ± 0.006			
Caffeic acid			0.22 ± 0.03					
Resveratrol		0.028 ± 0.001				0.037 ± 0.002	0.13 ± 0.001	0.147 ± 0.005

Table 4: HPLC Analysis of Individual Phenolic compounds of Fermented Extracts of Fuji (µg/mL).

Phenolic compound	pH adjusted				pH not adjusted			
	0 h	24 h	48 h	72 h	0 h	24 h	48 h	72 h
Gallic acid	0.236 ± 0.001	0.344 ± 0.05	0.46 ± 0.09	0.55 ± 0.023	0.236 ± 0.001	0.21 ± 0.001	0.314 ± 0.045	0.39 ± 0.05
Catechin	0.806 ± 0.09	0.91 ± 0.01	1.03 ± 0.08	0.76 ± 0.01	0.806 ± 0.09	0.75 ± 0.03	0.89 ± 0.066	0.8 ± 0.02
Epicatechin	28.2 ± 3.8	40.6 ± 3	26.4 ± 5	16.8 ± 0.23	28.2 ± 3.8	25 ± 2	26.4 ± 5	16.9 ± 0.75
Quercetin derivatives	0.098 ± 0.01	0.044 ± .0007	0.11 ± 0.005	0.1 ± 0.003	0.098 ± 0.01	0.11 ± 0.01	0.17 ± 0.03	0.13 ± 0.01
p-coumaric acid		0.043 ± 0.001	0.038 ± .001	0.035 ± 0.004		0.043 ± 0.001	0.06 ± 0.01	
Protocatecheuic acid			0.015 ± 0.002					

Table 5: HPLC Analysis of Individual Phenolic Compounds of Fermented Extracts of Golden Delicious (µg/mL)

Phenolic compound	pH adjusted				pH not adjusted			
	0 h	24 h	48 h	72 h	0 h	24 h	48 h	72 h
Gallic acid	0.236 ± 0.001	0.344 ± 0.05	0.46 ± 0.09	0.55 ± 0.023	0.236 ± 0.001	0.21 ± 0.001	0.314 ± 0.045	0.39 ± 0.05
Catechin	0.806 ± 0.09	0.91 ± 0.01	1.03 ± 0.08	0.76 ± 0.01	0.806 ± 0.09	0.75 ± 0.03	0.89 ± 0.066	0.8 ± 0.02
Epicatechin	28.2 ± 3.8	40.6 ± 3	26.4 ± 5	16.8 ± 0.23	28.2 ± 3.8	25 ± 2	26.4 ± 5	16.9 ± 0.75
Quercetin derivatives	0.098 ± 0.01	0.044 ± .0007	0.11 ± 0.005	0.1 ± 0.003	0.098 ± 0.01	0.11 ± 0.01	0.17 ± 0.03	0.13 ± 0.01
p-coumaric acid		0.043 ± 0.001	0.038 ± .001	0.035 ± 0.004		0.043 ± 0.001	0.06 ± 0.01	
Protocatecheuic acid			0.015 ± 0.002					

Table 6: HPLC Analysis of Individual Phenolic Compounds of Fermented Extracts of Granny Smith (µg/mL)

Phenolic compound	pH adjusted				pH not adjusted			
	0 h	24 h	48 h	72 h	0 h	24 h	48 h	72 h
Gallic acid	0.227 ± 0.003	0.34 ± 0.02	0.3 ± 0.03	0.4 ± 0.09	0.227 ± 0.003	0.229 ± 0.02	0.3 ± 0.03	0.3 ± 0.042
Catechin	1.12 ± 0.04	0.93 ± 0.06	0.76 ± 0.08	0.65 ± 0.04	1.12 ± 0.04	0.82 ± 0.03	0.99 ± 0.044	0.83 ± 0.005
Epicatechin	30.2 ± 2.7	15.7 ± 2	9.2 ± 2.2	12.4 ± 0.9	30.2 ± 2.7	14.2 ± 1.5	12.1 ± 0.88	
Quercetin derivatives	0.083 ± 0.016	0.22 ± 0.01	0.29 ± 0.002	0.21 ± 0.027	0.083 ± 0.016	0.23 ± 0.01	0.34 ± 0.023	0.34 ± 0.02
p-coumaric acid		0.092 ± 0.006				0.071 ± 0.001	0.083 ± 0.005	
Caffeic acid			0.312 ± 0.013					
Resveratrol		0.072 ± 0.005				0.009 ± 0.001	0.095 ± 0.005	0.085 ± 0.002

Gallic acid, catechin, epicatechin and quercetin derivatives were the major phenolic compounds found in fresh samples as well as the fermented extracts although the amounts changed as fermentation proceeded (Tables 3,4,5,and 6). *p*-Coumaric acid, caffeic acid, resveratrol and protocatechuic acid were also found in some samples at certain time points. The peaks were identified taking into account the retention time and the UV absorption spectra of the corresponding standards.

At 24 h gallic acid content slightly increased for pH adjusted samples whereas it slightly decreased fermented acidic pH samples as compared to 0 h. After that the content increased in both pH adjusted and fermented acidic pH samples except pH adjusted sample of Red delicious at 72 h where no gallic acid was detected.

Catechin content decreased for all samples over a period of 72 h. For most samples there was an initial decrease at 24 h and after that the amount remained constant or it decreased slightly. Only for Golden delicious pH adjusted samples the catechin content increased and then at 72 h it decreased significantly. Decrease in catechin content was highest for Red delicious pH adjusted.

Epicatechin content decreased steadily for Red delicious and Granny smith over a period of 72 h whereas for Fuji and Golden delicious the amount slightly increased at 24 h, then decreased and then increased again. Epicatechin was not detected in Red delcious and Granny smith pH adjusted sample at 72 h.

The trend for quercetin content was different for different varieties. For Red delicious, quercetin content decreased rapidly whereas for Fuji it decreased steadily over a period of 72 h. For Golden delicious, quercetin content slightly decreased and

then increased steadily. For Granny smith, the increase was rapid until 48 h, and then it decreased for pH adjusted sample whereas it remained constant for fermented acdic pH sample. No quercetin was detected for pH adjusted sample after 24 h and after 0 h for fermented acidic pH sample.

p-Coumaric acid was initially present in Red delicious and Fuji however, it was not detected after 24 h. In Golden delicious and Granny smith it was not present initially but it was detected at 24 h as a result of fermentation, it decreased at 48 h and then at 72 h it was not detected except in pH adjusted sample of Golden delicious.

Caffeic acid was detected only in Red delicious 48 h, and then pH adjusted samples of Granny smith 48 h and fermented acidic pH samples of Red delicious 72 h. Resveratrol was detected in Red delicious and Granny smith at 24 h but not at 0 h, indicating that this is a result of fermentation. It was further detected in fermented acidic pH samples of Granny smith at 48 and 72 h. Protocatechuic acid was detected only in pH adjusted sample of Golden delcious at 48 h.

The increase or decrease in a phenolic compound detected by the HPLC method used, at a specific time point is likely determined by the flux between formation of simple phenolics by degradation of complex polymeric phenolic compounds and complete degradation of simple phenolics with possibilities of re-formation of more complex biphenolics and flavonoid moieties.

5.1.4.10 *Helicobacter pylori* Inhibition

Potential of fermented whole apple juice extracts on *H. pylori* inhibition were evaluated. None of the pH adjusted samples had inhibition. For the fermented acidic pH samples only 48 h and 72 h fermented samples had inhibitory activity (Fig 26). Golden delicious 72 h showed the highest inhibitory activity (17.0 mm) followed by Fuji 72 h (16.4 mm). For all the samples 75 µL had inhibitory activity however, 50 µL failed to produce any zone of inhibition.

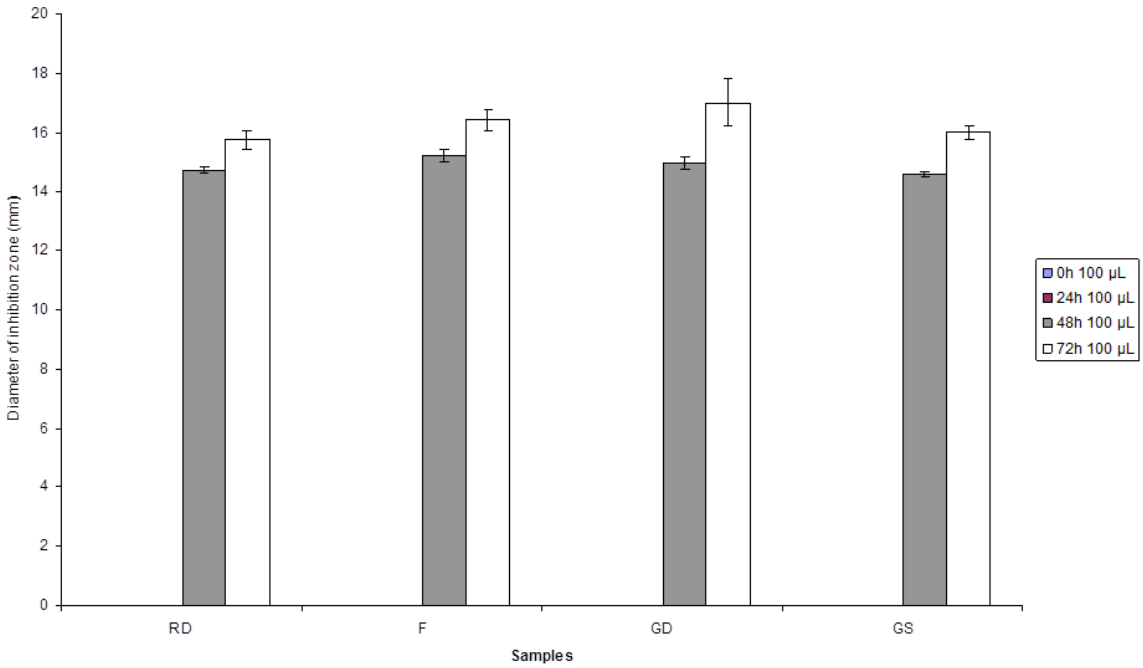

Figure 26: *Helicobacter pylori* inhibition of pH not adjusted samples of fermented apple juice at different sample volumes (50, 75, 100 µL).

Some of the findings reported by other authors (Midolo *et al.,* 1995; Alakomi *et al.,* 2000) support our results. Midolo *et al.,* (1995), reported that a 0.33 mol l^{-1} lactic acid solution lost its ability to inhibit *H. pylori* when the pH was adjusted from its natural pH range of 2.3 to 5.3. A much higher concentration of sodium lactate was required to produce a comparable zone of inhibition as lactic acid. Similar results were reported by Alakomi *et al.,* (2000), who found that the dissociated potassium lactate at neutral pH had no permeabilizing activity on gram-negative bacteria and as pH increased, the dissociated form of lactic acid increased. So adjusting the pH caused lactic acid to loose its *H. pylori* inhibiting property or it could also be that the concentration of the corresponding salt formed was not high enough to inhibit this pathogen.

Although for the 48 h fermented acidic pH samples there was no significant difference in the total phenolics or lactic acid compared with the 24 h samples for Red delicious and Fuji, the 24 h samples failed to inhibit *H. pylori.* The reasons for this are not clear. Alakomi *et al.,* (2000), suggest that lactic acid permeabilizes gram-negative bacteria by disrupting the outer membrane and as such permeabilizers may not be bactericidal or bacteriostatic but it may increase susceptibility by enabling other compounds such as antibiotics and bacteriocins to penetrate and inhibit the gram-negative bacterium. If lactic acid alone is not responsible for inhibition then it might explain the difference in inhibition between the 24 h and the 48 h fermented acidic pH samples. It could be because in the 48 h sample greater amount of other bioactive compounds, which may work in combination with lactic acid in inhibiting gram-

negative *H. pylori* may be present. This is further supported by the fact that the zone of inhibition produced by the 72 h samples were significantly ($P<0.05$) higher than the 48 h samples, although there were no significant difference in the total phenolics and lactic acid between the two time points in the fermented acidic pH samples.

Previous work in our laboratory has provided some insights that in animal cell and bacterial model systems phenolics could regulate cellular redox response through proline- linked pentose phosphate pathway (Shetty and Wahlqvist, 2004; Lin *et al.*, 2005). The proline growth response assay is based on the rationale that small phenolics could behave as proline mimics and can inhibit proline oxidation via proline dehydrogenase (PDH) at the plasma membrane level in prokaryotic cell disrupting the oxidative phosphorylation linked proton motive force resulting in inhibition of the bacterium. If so, then addition of proline could overcome inhibition of the bacterium by proline analog-like phenolic structures present in the fermented extract (Lin *et al.*, 2005). The results obtained in this study indicate that *H. pylori* inhibition by fermented acidic pH samples of whole apple juice extract may not associated to the proline dehydrogenase based oxidative phosphorylation (Fig 27). Even when high doses of proline were used, complete or even partial reversal was not observed indicating other mechanisms could be involved in *H. pylori* inhibition.

Our results here and previous reports published by other authors (Midolo *et al.*, 1995; Alakomi *et al.*, 2000) suggests that lactic acid might aid the antimicrobial activity of other polyphenolic compounds present in the fermented extracts. Lactic acid by releasing lipopolysaccharide or other components can abolish the integrity of the outer membrane (Alakomi *et al.*, 2000). This can enable phenolic antioxidants to quench

electrons from electron transport chain (ETC) along the bacterial membrane and act as antimicrobials by disrupting oxidative phosphorylation or inhibit dehydrogenases linked proton efflux by interfering with the flow of electrons at the level of cytochromes (Vattem *et al.,* 2005a).

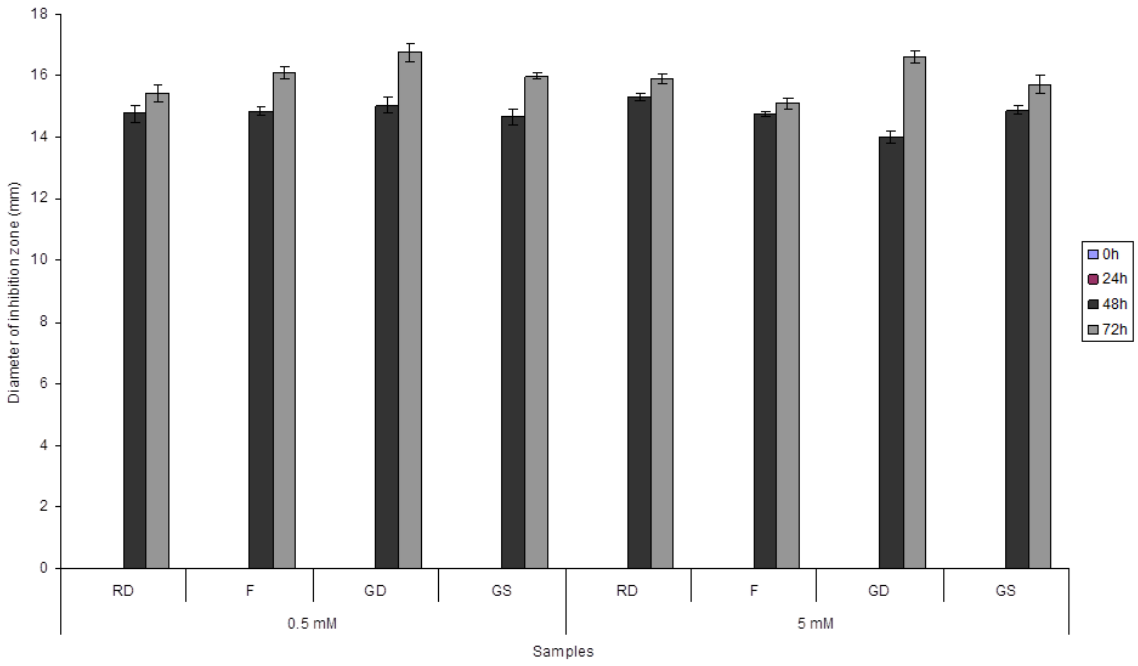

Figure 27: Effect of different concentrations of pH not adjusted samples of fermented apple juice with proline (0.5 mM and 5 mM) on anti-*Helicobacter pylori* activity.

It is also possible that simple phenolics can disrupt the H^+-ATPase required for ATP synthesis by causing hyper-acidification via proton donation at the plasma membrane and intra-cellular cytosolic acidification (Shetty and Labbe, 1998; Shetty and Wahlqvist, 2004; Kwon *et al.,* 2007b) and this is possible even in microaerophilic *H. pylori*. Ikigai *et al.,* (1993) reported that the bactericidal effects of catechins is by damage to bacterial membranes. Catechins and Epicatechins present in our fermented extracts may cause inhibition by damage to bacterial membrane after lactic acid present in the pH fermented acidic pH samples disrupts the outer membrane. Quercetin, a phenolic compound present in our extract is known to cause DNA damage in bacteria, and has been shown to inhibit *E. coli* CM 871, a mutant which lacked DNA repair mechanisms (Puupponen-Pimia *et al.,* 2001). Phenolic phytochemicals including catechins have been shown to inhibit ureases, an important enzyme which helps *H. pylori* grow in extremely acidic environment by releasing ammonia (Matsubara *et al.,* 2003; Lin *et al.,* 2005). Catechins may also bring about the inhibition by restricting the supply of ions Fe^{3+} and Ni^{2+} essential for bacterial growth by forming complexes with these metal ions (Kumamoto *et al.,* 2001; Montecucco and Rappuoli, 2001).

5.1.4.11 Lactic Acid Bacterial Proliferation Assay

Extracts which showed inhibition against *H. pylori* were further investigated for their effects on lactic acid bacteria to determine relevance for positive health benefits linked to probiotics. Evaluation was carried out by determining the plate counts of a specific probiotic bacterium, *Bifidobacterium longum* at 6, 9, 12 and 24 h of growth in

liquid medium. The concentrations of extracts used in this study were similar to those used in *H. pylori* inhibition studies. No inhibitory effects of the extracts were observed on probiotic strain of *Bifidobacterium longum* as compared to control (Fig 28 and Fig 29). Some stimulatory effects were observed with 48 h fermented samples at 12 h of probiotic bacterial growth. Except for Golden delicious, all the other samples showed significant ($P<0.05$) stimulation in growth as compared to the control. This difference in growth was not observed at 24 h of probiotic bacterial growth but there was no inhibition either. This suggests that the fermented extracts might be able to inhibit *H. pylori* without inhibiting or even slightly stimulating the intestinal bacteria to some extent.

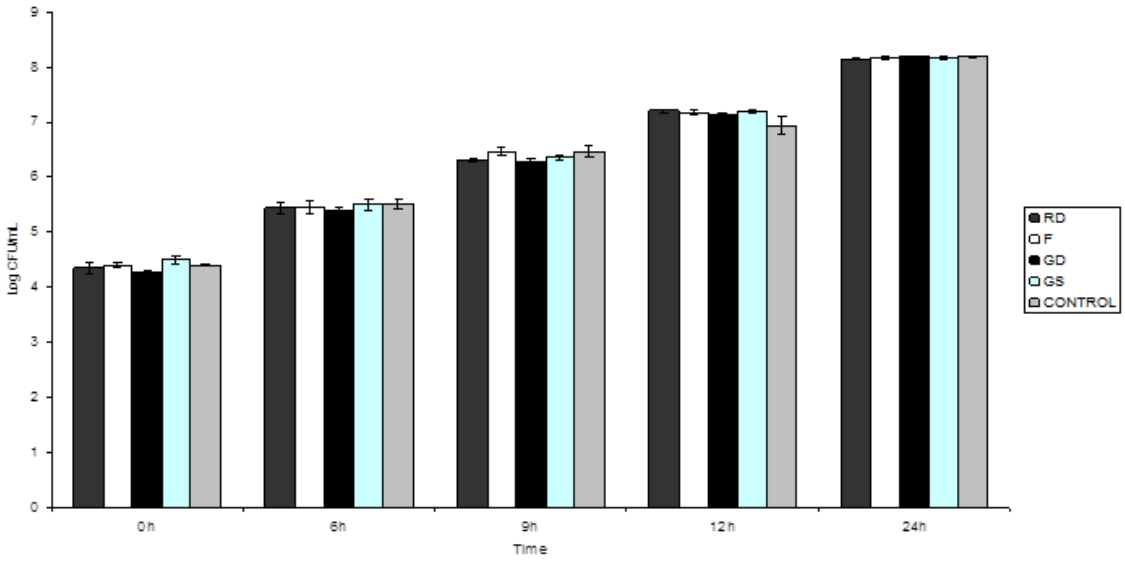

Figure 28: Effect of 48 h pH not adjusted samples of fermented apple juice on *Bifidobacterium longum* proliferation (CFU/mL).

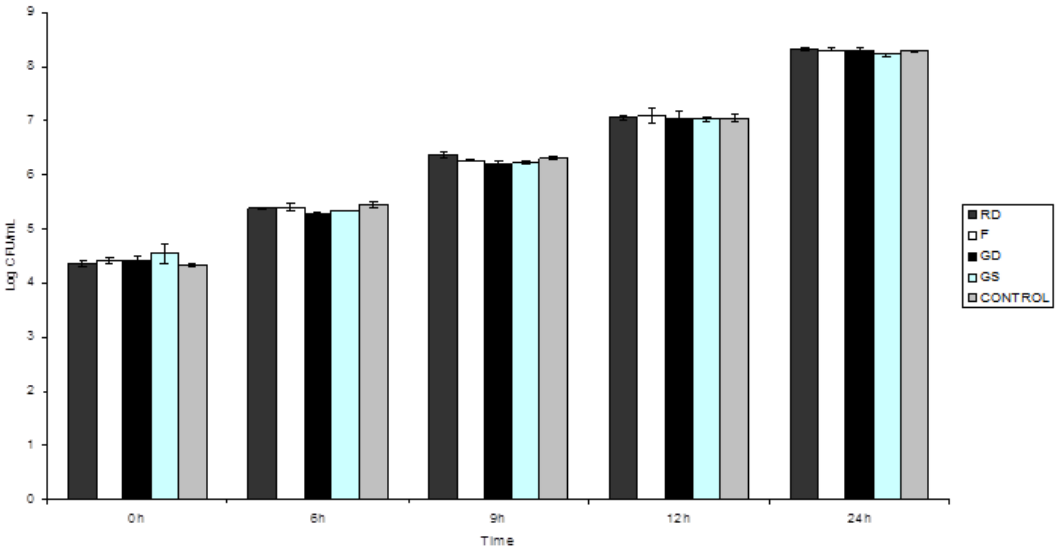

Figure 29: Effect of 72h pH not adjusted samples of fermented apple juice on *Bifidobacterium longum* proliferation (CFU/mL).

Puupponen-Pimia *et al.,* (2001), reported that lipophilic phenolics like quercetin and kaempferol were unable to inhibit lactic acid bacteria whereas more hydrophilic phenolics like myricetin were completely inhibitory to lactic acid bacteria of human gastrointestinal tract origin. The difference in dependency on partial oxidative phosphorylation in microaerobic bacteria such as *H. pylori* may be another reason for inhibition and lack of inhibition in lactic acid probiotic bacteria that use substrate level phosphorylation for energy generation.

5.1.4.12 Sensory Properties

The fermented apple juice had a sour taste with a typically fermented flavor but with a pleasant smell. pH of natural apple juice is not very different from the final fermented juice however there seems to be change in the flavor profile due to lactic acid being formed in the juice. Although the taste of the fermented juice may not measure up to its unfermented counterpart it was good enough to be edible considering the health beneficial properties enhanced by enrichment of phenolic bioactives as well the presence of viable probiotic bacterium. Consistency as expected had not changed during fermentation however the juice had become cloudy due to the growth of the probiotic bacterium.

5.1.5 Conclusion

Fruit and fruit juices have been shown to have potential in managing oxidation linked chronic and infectious diseases. Apple is one of the most consumed fruit in the United States. In this study, we fermented apple juice from four different cultivars and investigated the changes in functionality linked to potential dietary management of early stages of hyperglycemia linked to type 2 diabetes. Total phenolics and DPPH linked antioxidant activity decreased over a period of 72 h. Fermentation enhanced the ability of the juice to inhibit intestinal glucose and related soluble sugar uptake enzyme α-glucosidase and hypertension relevant ACE at its fermented acidic pH. Also at 48 and 72 h, fermented apple juice was able to inhibit *H. pylori* which has been linked to stomach related diseases without affecting the beneficial lactic acid bacteria with probiotic potential.

5.2 Phenolic Bioactive Modulation by *Lactobacillus helveticus* Mediated Fermentation of Cherry Extracts for Anti-Diabetic Functionality, *Helicobacter pylori* Inhibition and Probiotic *Bifidobacter longum* Stimulation.

5.2.1 Abstract

Cherry juice from one cultivar was fermented for 0, 24,48 and 72 h using *Lactobacillus helveticus* and its effects related to management of hyperglycemia, hypertension, inhibition of *Helicobacter pylori* and proliferation of probiotic *Bifidobacterium longum* were evaluated using *in vitro* models. Cherry extract was fermented by initially adjusting the pH to 6.0 and also not adjusting the pH. Further, analysis was carried out by adjusting the pH and at fermented acidic pH at each time point from each of the two samples. Total soluble phenolics decreased over a period of 72 h for initial pH adjusted samples whereas it increased or remained constant for samples where initial pH was not adjusted. DPPH linked antioxidant activity and α-glucosidase inhibition decreased for samples where final pH was adjusted whereas it increased for samples where final pH was not adjusted. Fermentation led to a decrease in hypertension-relevant ACE inhibition for all samples. Samples where pH was not adjusted had *Helicobacter pylori* inhibition at 24, 48 and 72 h. Based on the rationale that simple phenolics in cherry could behave as proline analogs, the potential recovery of the pathogen was evaluated with of addition of 0.5 mM proline in the medium. A proline induced growth recovery was observed indicating that the mechanism of inhibition is related to proline dehydrogenase based oxidative phosphorylation. Overall no inhibition was observed when samples that had *H. pylori* inhibition were further evaluated for their effect on probiotic *Bifidobacterium longum*.

5.2.2 Introduction

There are more than 30 species of cherries available mostly indigenous to Europe and Asia; however in the United States only two types are commercially produced: sweet and tart cherries (Iezzoni, 2008; Agricultural Marketing resource Centre, www. agmrc.org). The two main cultivars of cherries commercially grown in the USA are reported to have been originated near an area that includes Asia Minor, Iran, Iraq and Syria (Vavilov, 1951). USA is the second largest producer of cherries and in 2008, the average per capita fresh consumption in the USA was one lb (Agricultural Marketing resource Centre, www. agmrc.org). Cherries contain anthocyanins which have been shown to have potential in reducing oxidation linked chronic diseases including CVD, cancer and hyperlipidemia (Gao and Mazza, 1995; De Pascual-Teresa and Sanchez-Ballesta, 2008). Potent α-glucosidase and α-amylase inhibitory activity, which can be of potential in managing type 2 diabetes, of acylated anthocyanins from some plant sources has been demonstrated (Matsui *et al.*, 2001). Cherry phenolics have been shown to protect neuronal cells from cell-damaging oxidative stress (Kim *et al.*, 2005) and it has also been reported that tart cherry anthocyanins and cyanidin may reduce the risk of colon cancer (Kang *et al.,* 2003). Flavonols like catechin, epicatechin, quercetin glycosides and hydroxycinnamates like neochlorogenic acid and *p*-coumaroylquinic acid have been reported in sweet and tart cherries (Gao and Mazza, 1995; Chaovanalikit and Wrolstad, 2004; Goncalves *et al.*, 2004).

Oxidation linked diseases such as type 2 diabetes, CVD, cancer and other chronic health related diseases are on a rise. It is mostly attributed to unhealthy diets low in fruits and vegetables, high in refined calories, further complicated by stress and

environmental factors such as pollution and smoking. The drug strategies to counter type 2 diabetes by mildly inhibiting starch breakdown enzymes; mainly pancreatic α-amylase and intestinal α-glucosidase is associated with side effects including flatulence, abdominal distension and possible diarrhea (Bischoff, 1994; Kwon *et al.,* 2006b). Fruits and vegetables offer a simple, cheap and overall a more attractive strategy in controlling post-prandial rise in blood glucose without the associated side effects by providing naturally occurring α-amylase and α-glucosidase inhibitors (Matsui *et al.,* 2001; McDougall and Stewart, 2005).

Helicobacter pylori is a gram-negative, curve shaped, microaerophilic pathogen that survives in the low pH of the stomach and has been identified as an etiological agent of many stomach related diseases including gastritis, peptic ulcer, and rare cases of gastric cancer (Warren and Marshall, 1983; Mitchell, 1999; You *et al.,* 2000; Uemura *et al.,* 2001). Natural dietary ingredients from fermented milk (Felley *et al.,* 2001), wine (Daroch *et al.,* 2001; Ruggiero *et al.,* 2007), and fruits including berries (Kubo *et al.,* 1999; Malekzadeh *et al.,* 2001; Puupponen-Pimia *et al.,* 2001; Chatterjee *et al.,* 2004; Lin *et al.,* 2005; Vattem *et al.,* 2005a) have been shown to inhibit *H. pylori* both *in vitro* and *in vivo* and offer a good alternative to rigorous antibiotic therapies associated with side effects.

Lactic acid bacteria colonize the intestine and may confer several benefits to the host including better absorption of nutrients from food, decrease in lactose intolerance in some individuals, control of diseases originating from intestinal infections, potential control of some types of cancer and stimulation of host immune response (Gilliland, 1990; Perdigón *et al.,* 2001; Rafter, 2002).

In this study, we fermented juice extracted from whole cherries using *Lactobacillus helveticus* over a period of 72 h and investigated the changes in total phenolics, antioxidant activity, and enzymes related to type 2 diabetes using *in vitro* assays. Fermented extracts were further tested for *H. pylori* inhibition and lactic acid bacteria proliferation potential. Analysis was carried out at 0, 24, 48 and 72 h by adjusting the pH and at fermented acidic pH of the extract. Furthermore, investigations on the mode of action of *H. pylori* inhibition by fermented cherry juice were carried out on the basis of the rationale that simple phenolics may mimic proline analogs and may exhibit antimicrobial activity by inhibition of proline oxidation via proline dehydrogenase, at the same time these extracts would not affect beneficial lactic acid bacteria such as *Bifidobacterium longum* (Shetty and Wahlqvist, 2004; Lin *et al.*, 2005; Kwon *et al.*, 2007b; Apostolidis *et al.*, 2008).

5.2.3 Materials and Methods

5.2.3.1 Sample Extraction

One cultivar of Cherry was purchased from Stop and Shop, Hadley, MA, USA. Whole Cherries were washed with water, cut into small pieces and pits were removed. These small pieces were homogenized using a Waring blender for 5 min. It was then centrifuged for 15 min at 15,000 g, supernatants were collected and kept at -20 ^{o}C during the period of the study.

5.2.3.2 Bacterial Strains

The lactic acid bacteria strains used in this study were the following: *Lactobacillus helveticus* was provided by Rosell Institute Inc. Montreal, Canada (Lot#: XA 0145, Seq#:00014160), *Bifidobacterium longum* was isolated in a previous study (Apostolidis *et al.*, 2007), *Helicobacter pylori* (strain ATCC 43579, which originated from human gastric samples) was obtained from the American Type Culture Collection (Rockville, MD).

5.2.3.3 Fermentation

Initially 100 µL of frozen *Lactobacillus helveticus* stock were inoculated into 10 mL MRS broth (Difco) and incubated for 24 h at 37 °C. Then, 100 µL of the 24 h grown strain was re-inoculated into 10 mL MRS broth for 24 h at 37 ^{o}C. Ninety mL of the juice was put into a 125 mL Erlenmeyer flask; for one of the sample the pH adjusted to 6 using NaOH and another sample was fermented at its natural pH of 4.2. Samples were inoculated using 10 mL of the broth culture. Fermentation was carried out at 37 ^{o}C and 12.5 mL samples were taken out at 0, 24, 48 and 72 h. At every time point except for 0 h, two 12.5 mL samples were taken out, for one sample pH was adjusted to 6 by adding drops of 0.1M NaOH and for the other non-pH adjusted sample; same number of drops of distilled water was added to keep the volume same. The samples were centrifuged at 15,000 g for 15 min and then used for the assays.

5.2.3.4 Absorbance of Sample and Colony Counts

Growth of bacteria in the sample was estimated using absorbance at 600 nm. More accurate estimations of the bacterial numbers were made by plate count technique. At 0, 24, 48 and 72 h, CFU/mL was determined by pipetting 100 µL of the sample, serially diluting and plating on MRS medium. The plates were incubated anaerobically at 37 ^{o}C for 24 h and individual colonies were counted. The pH of the samples was also measured at 0, 24, 48 and 72 h.

5.2.3.5 Total Phenolics Assay

The total phenolics in all samples were determined by using a method modified by Shetty *et al.*, (1995). In brief, 0.5 mL of sample extract was added to a test tube and mixed with 0.5 mL of 95% ethanol and 5 mL of distilled water. To each sample, 0.5 mL of 50% (vol/vol) Folin-Ciocalteu reagent was added and mixed. The absorbance was read at 725 nm using a spectrophotometer (Genesys UV/Visible, Milton Roy, Inc., Rochester, NY). Different concentrations of gallic acid were used to develop a standard curve. Results were expressed as mg of gallic acid/mL of sample.

5.2.3.6 Antioxidant Activity by DPPH Radical Inhibition Assay

The antioxidant activity was determined by the DPPH radical scavenging method modified from Kwon *et al.*, (2006b). A 250-µL aliquot of the sample extract was mixed with 1,250 µL of DPPH (60 µM in ethanol). Absorbance was measured at 517 nm using the Genesys UV/Visible spectrophotometer. The readings were compared

with the controls, containing 95% ethanol instead of sample extract. The percentage inhibition was calculated by:

$$\%\ \text{inhibition} = \frac{(Absorbance_{\text{control}} - Absorbance_{\text{extract}})}{Absorbance_{\text{control}}} \times 100$$

5.2.3.7 α-Glucosidase Inhibition Assay

The α-glucosidase inhibitory activity was determined by an assay modified from McCue *et al.*, (2005a). α-Glucosidase was assayed by using 50 μL of sample extracts and 100 μL of 0.1 M phosphate buffer (pH 6.9) containing α-glucosidase solution (1 U/mL) and was incubated in 96-well plates at 25 °C for 10 min. After preincubation, 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 incubation, absorbance readings were recorded at 405 nm by a microplate reader (Thermomax, Molecular Devices Co., Sunnyvale, CA) and compared to a control that had 50 μL of buffer solution in place of the extract. The α-glucosidase inhibitory activity was expressed as percentage inhibition and was calculated with the same equation as for percentage inhibition in the DPPH radical inhibition assay. Dose dependency was tested using 25 μL and 10 μL of the sample, the volume made up to 50 μL using 0.1 M phosphate buffer (pH 6.9) and same protocol was followed.

5.2.3.8 α-Amylase Inhibition Assay

The α-amylase inhibitory activity was determined by an assay modified from McCue *et al.,* (2005a). A total of 500 µL of 1:20 dilution (due to the high sugar content) of sample extract and 500 µL of 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M NaCl) containing α-amylase solution (0.5 mg/mL) were incubated at 25 °C for 10 min. After preincubation, 500 µL of a 1% starch solution in 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M NaCl) was added to each tube at timed intervals. The reaction was stopped with 1.0 mL of dinitrosalicylic acid color reagent. The test tubes were incubated in a boiling water bath for 5 min and cooled to room temperature. The reaction mixture was then diluted after adding 15 mL of distilled water, and the absorbance was measured at 540 nm using the Genesys UV/Visible spectrophotometer. The readings were compared with the controls, containing buffer instead of sample extract. The percentage α-amylase inhibitory activity was calculated with the same equation as for percentage inhibition in the DPPH radical inhibition assay.

5.2.3.9 ACE Inhibition Assay

ACE inhibition was assayed by a method modified by Kwon *et al.,* (2006b). The substrate hippuryl-histidyl-leucine (HHL) and the enzyme ACE-I from rabbit lung (EC 3.4.15.1) were used. Fifty µL of sample extracts were incubated with 100 µL of 1 M NaCl-borate buffer (pH 8.3) containing 2 mU of ACE-I solution at 37 °C for 10 min. After preincubation, 100 µL of a 5 mU substrate (HHL) solution was added to the reaction mixture. Test solutions were incubated at 37 °C for 1 h. The reaction was

stopped with 150 μL of 0.5 N HCl. Five μL of the sample was injected in a high-performance liquid chromatography (HPLC) apparatus (Agilent 1100 series equipped with autosampler and DAD 1100 diode array detector, Agilent Technologies, Palo Alto, CA). The solvents used for gradient were (1) 10 mM phosphoric acid (pH 2.5) and (2) 100% methanol. The methanol concentration was increased to 60% for the first 8 min and to 100% for 5 min and then was decreased to 0% for the next 5 min (total run time, 18 min). The analytical column used was an Agilent Nucleosil 100-5C18, 250 mm × 4.6 mm inside diameter, with packing material of 5 μm particle size at a flow rate of 1 mL/minute at ambient temperature. During each run, the absorbance was recorded at 228 nm, and the chromatogram was integrated using the Agilent Chemstation (Agilent Technologies) enhanced integrator for detection of liberated hippuric acid (A). Hippuric acid standard was used to calibrate the standard curve and retention time. The percentage inhibition was calculated by:

$$\%\ \text{inhibition} = \frac{(A_{\text{control}} - A_{\text{extract}})}{(A_{\text{control}} - A_{\text{blank}})} \times 100$$

5.2.3.10 Protein Assay

Protein content was measured by the method of Bradford assay (Bradford, 1976). The dye reagent concentrate (Bio-Rad protein assay kit II, Bio-Rad Laboratory, Hercules, Calif., USA) was diluted 1:4 with distilled water. Five mL of diluted dye reagent was added to 100 μL of fermentation supernatant. After vortexing and incubating for 5 min, the absorbance was measured at 595 nm against a 5 mL reagent

blank and 100 µL buffer using a UV-VIS Genesys spectrophotometer (Milton Roy, Inc., Rochester, N.Y., USA).

5.2.3.11 HPLC Determination of Lactic Acid Content

Five µl of sample was injected using Agilent ALS 1100 autosampler into Agilent 1100 series HPLC (Agilent Technologies) 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, 25 min). The analytical column used was Agilent Zorbax SB-C18, 250 × 4.6 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 210 nm and integrated using Agilent Chemstation enhanced integrator. Pure standards of lactic acid (purchased from Sigma Chemical Co.) in 100% methanol were used to calibrate the standard curve and retention times.

5.2.3.12 High Performance Liquid Chromatography (HPLC) Analysis of Phenolic Profiles

The extracts (2 mL) were filtered through a 0.2 µm filter. A volume of 5 µL of extract was injected using an Agilent ALS 1100 auto sampler into an Agilent 1100 series HPLC (Agilent Technologies, Palo Alto, CA) equipped with a 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, 25 min). The analytical column used was Agilent Zorbax SB-C_{18}, 250 x 4.6 mm i.d., with packing material of 5 μm particle size at a flow rate of 1 mL/min at room temperature. During each run the absorbance was recorded at 226 and 306 nm and the chromatogram was integrated using Agilent Chemstation enhanced integrator. Pure standards of gallic acid, catechin, epicatechin, quercetin, p-coumaric acid, caffeic acid and resveratrol in 100% methanol were used to calibrate the standard curves and retention times.

5.2.3.13 Preparation of Starter Culture of *Helicobacter pylori*

Helicobacter pylori were cultured according to Stevenson *et al.*, (2000). Standard plating medium (*H. pylori* agar plates) were prepared by using 10 g of special peptone (Oxoid Ltd., Basingstoke, England) per liter, 15 g of granulated agar (Difco Laboratories, Becton, Dickinson and Co., Sparks, MD) per liter, 5 g of sodium chloride (EM Science, Gibbstown, NJ) per liter, 5 g of yeast extract (Difco) per liter, 5 g of beef extract (Difco) per liter of water.

Broth media were prepared by 10 g of special peptone (Oxoid Ltd., Basingstoke, England) per liter, 5 g of sodium chloride (EM Science, Gibbstown, NJ) per liter, 5 g of yeast extract (Difco) per liter, 5 g of beef extract (Difco) per liter of water. A volume of 1 mL of stock *H. pylori* was added into test tubes containing 10 mL of sterile broth media. They were incubated at 37 °C for 24 h before being used for inoculating by the spread plate technique. The active culture was then spread on *H. pylori* agar plates to make bacterial lawn for the agar-diffusion assay.

5.2.3.14 Agar-Diffusion Assay

The antimicrobial activity of sample extracts was analyzed by the agar-diffusion method. The assay was done aseptically using sterile 12.7 mm diameter paper disks (Schleicher & Schuell, Inc., Keene, NH) to which 100 µL of test extracts were added. Saturated disks were placed onto the surface of seeded agar plates. Controls consisted of disks with distilled water only. 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). The diameter of clear zone (no growth) surrounding each disk was measured and the zone of inhibition was determined and expressed in mm. For the dose dependency studies, 50 µL and 75 µL of the sample were used. Each experiment was repeated 2 times and consisted of three replicates each time (three disks per sample or treatment).

5.2.3.15 Proline Growth Response Assay

A model for mode of action of phenolic phytochemicals was developed based on the rationale that small phenolics could behave as proline analogs or proline analog mimics and likely inhibit proline oxidation via proline dehydrogenase (Shetty and Wahlqvist, 2004). Further, the likely inhibitory effects of phenolic phytochemicals should be overcome by proline if the site of action is proline dehydrogenase.

Bacterial lawns of *H. pylori* were prepared as described previously. Plating media were prepared by using standard plating medium as described in agar-diffusion assay with some modifications. Proline (Sigma, Louis, Mo) was added into the medium

to a final concentration of 0.5 mM. Then a similar protocol as mentioned in the agar diffusion assay was followed.

5.2.3.16 Lactic Acid Bacterial Proliferation Assay

Initially 100 µL of frozen stock from the lactic acid bacterial strain *Bifidobacterium longum* were inoculated into 10 mL MRS broth (Difco) and incubated for 24 h at 37 °C. Then, 100 µL of the 24 h grown strain was re-inoculated into 10 mL MRS broth for 24 h at 37 ^{o}C. Sample volumes of one mL were filter sterilized using sterile filters Millex GP 0.22 µm (Millipore Corporation, Bedford, MA). Filter sterilized sample extracts (1 mL) and 100 µL of the 24 h grown strain (diluted 100 times with sterile distilled water) were added into 9 mL of MRS broth tubes and incubated at 37 °C for 24 h. A control with 1 mL of sterile distilled water instead of sample extract was also included. 100 µL of the serially diluted samples were plated in triplicates every 0, 6, 9, 12, and 24 h on MRS agar (Difco) plates and incubated in anaerobic BBL GasPak jars (Becton, Dickinson and Co., Sparks, MD) with BD GasPak EZ anaerobe container system sachets (Becton, Dickinson and Co., Sparks, MD) at 37 °C for 24 h to determine the CFU/mL.

5.2.3.17 Statistical Analysis

All experiments including analysis at every time point were performed at least in duplicate or triplicate. Means, standard errors and standard deviations were calculated from replicates within the experiments and analyzed using Microsoft Excel. Effect of

different treatments was determined by analysis of variance (ANOVA) of SAS (version 8.2; SAS Institute, Cary, NC). Differences among different treatments were determined by Fisher's Least significant difference (LSD) test at the 0.05 probability level.

5.2.4 Results and Discussion

5.2.4.1 Absorbance and Colony Counts

Plate counts represented a bacterial growth curve over a period of 72 h (Fig 30). Fermentation started at 0 h with CFU/mL around 7 log. At 24 h it increased representing the log phase of the bacterial curve. Then at 48 h the numbers decreased slightly representing the stationary phase. At 72 h the numbers decreased for all samples and the final numbers were lower than 0 h. Absorbance which is an indicator of the growth of bacteria in the fermenting substrate increased for all samples after 0 h. pH decreased at 24 h and after that it remained constant. This suggests that cherry juice; pH adjusted or at its natural pH is a good substrate for *Lactobacillus helveticus* fermentation.

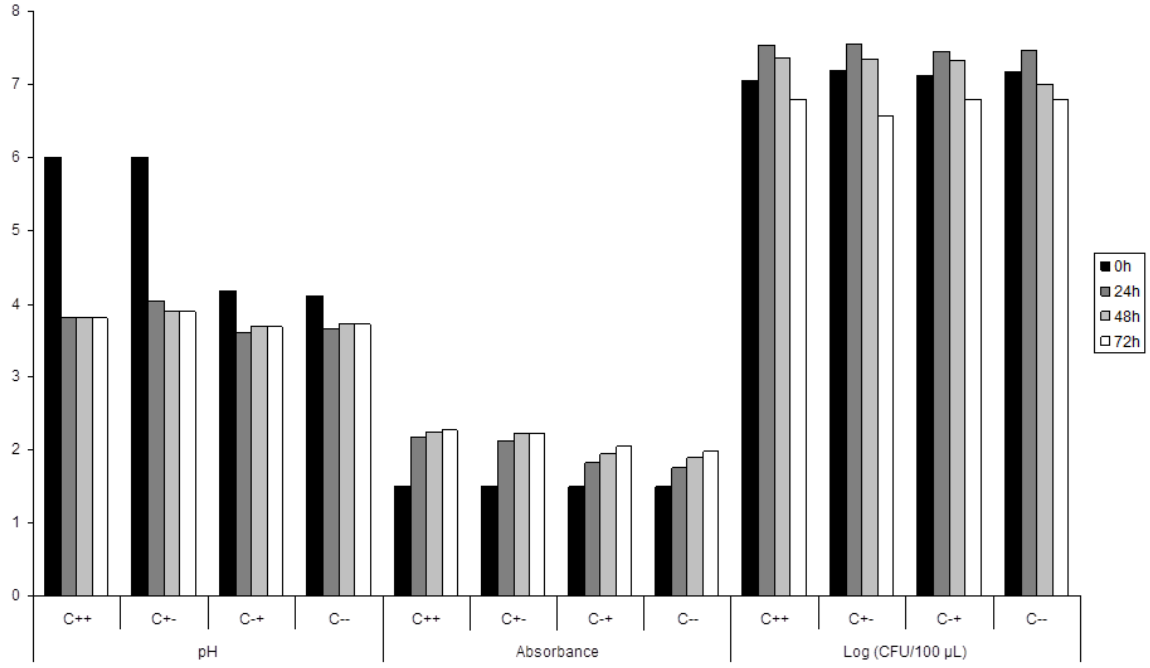

Figure 30: pH, absorbance and colony counts of fermented cherry juice extracts at various time points.

5.2.4.2 Total Soluble Phenolics

Total phenolics was determined for fermented cherry extract at 0, 24, 48 and 72 h using Folin-Ciocalteu assay. At 0 h the initial pH adjusted samples (C++, C+-) had more total soluble phenolics (0.65 mg/mL) than samples where initial pH was not adjusted (C-+, C-- ; 0.63 mg/mL) (Fig 31). The 0 h value for total phenolic content is in reasonable agreement with previously published reports (Kim and Padilla-Zakour, 2006; Usenik *et al.,* 2008). At 24 h total phenolics decreased for initial pH adjusted samples and for initial pH not adjusted samples it increased. After 24 h, there was no significant change ($P>0.05$) in total phenolics for any sample. The contrasting trend

between the initial pH adjusted (C++, C+-) and not adjusted samples (C-+, C--) could be because of the difference in activities of the enzymes involved in fermentation.

As indicated by the difference in total phenolic content between same samples but different final pH (C++ vs C+-, and C-+ vs C--), a significantly higher ($P<0.05$) value was obtained by adjusting the pH at the point of analysis as compared to analysis at fermented acidic pH. However, this difference was not significant at 0 h ($P>0.05$). Friedman and Jurgens, (2000), suggested that a pH of greater than 7 could cause changes in the spectrum of phenolic compounds. Multiring aromatic structures such as catechin, epicatechin and rutin are more resistant to such changes. Although the pH range tested in their work was high, these results might explain the difference in total phenolics assayed at fermented acidic pH vs final pH adjusted samples. Although the values obtained at different time points in case of similar initial pH treatments (C++ vs C+-; C-- vs C-+) were different the overall trend in the changes during fermentation were quite similar.

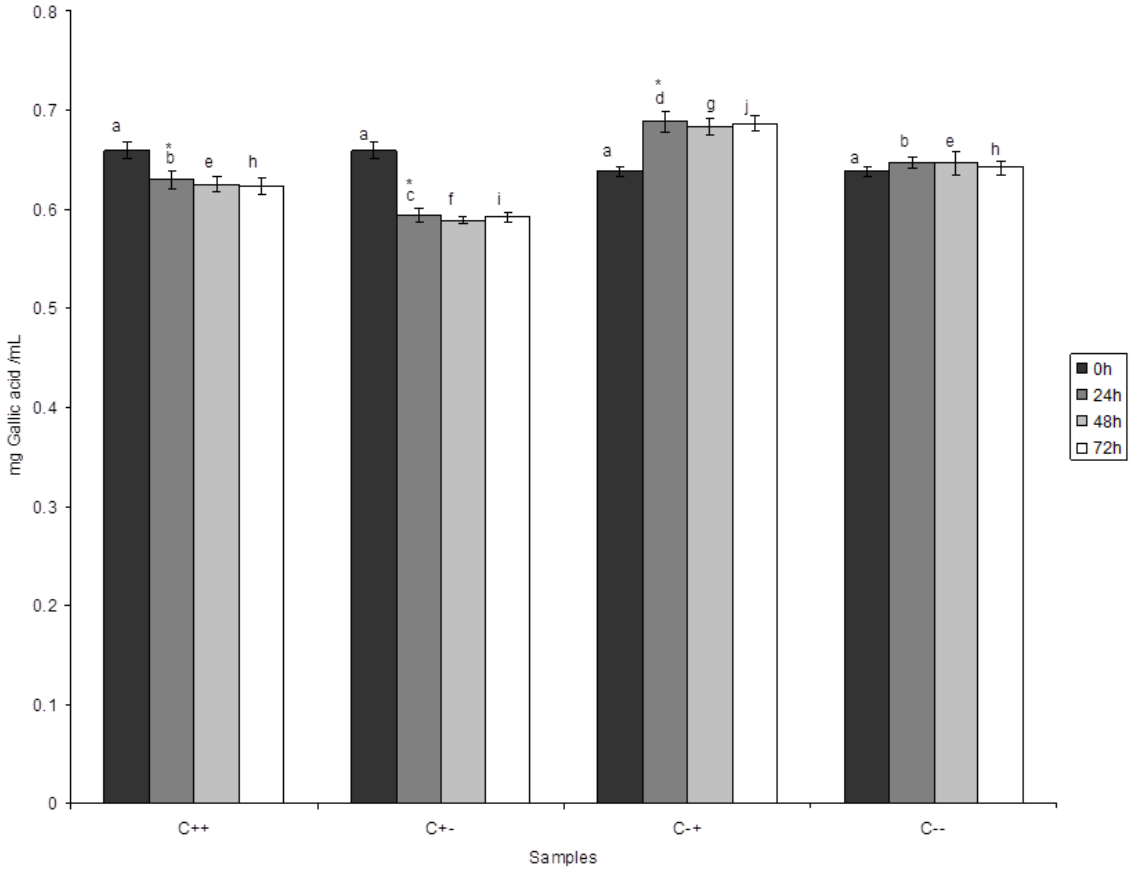

Figure 31: Effect of *Lactobacillus helveticus* fermentation on total phenolic content of cherry juice with different treatments in pH at 0, 24, 48 and 72 h of fermentation.

Total phenolics present in a fermented substrate are a result of phenolic mobilization linked to a flux between the formation/degradation of polymeric phenolics and degradation of simple phenolics as a possible detoxification mechanism for bacteria and yeast (McCue and Shetty, 2005). The decrease in phenolics obtained in initial pH adjusted samples (C++, C+-) is supported by the findings of Apostolidis *et al.*, (2007), where they fermented milk and soymilk with *L. helveticus* and observed a decrease in

total phenolics. Milk and soymilk have a pH greater than 6 which is comparable to our initial pH adjusted samples (C++, C+-). The decrease in soluble phenolic content could result from polymerization of phenolic compounds or it could also be because of degradation of phenolic compounds as a possible detoxification mechanism for *L. helveticus*. The increase in phenolics in initial pH not adjusted samples (C-+, C--) could be because of degradation of large polymeric phenolics. Further investigation into the activities of the enzymes related to these biochemical changes would be useful.

5.2.4.3 Antioxidant Activity by DPPH Radical Inhibition Assay

Antioxidant activity was measured by DPPH-linked free radical scavenging activity assay at 0, 24, 48 and 72 h of fermentation time. 0 h values for the DPPH assay for both initial pH adjusted samples (82%) and initial pH not adjusted samples (87%) was high, indicating cherry juice has good potential for free radical inhibition (Fig 32). Investigations by other authors (Kim and Padilla-Zakour, 2006; Usenik *et al.*, 2008) imply cherry has good antioxidant potential and our findings here are in conformity to these findings. As indicated by the difference in % DPPH inhibition between same samples but different final pH (C++ vs C+-, and C-+ vs C--), a significantly lower ($P<0.05$) value was obtained by adjusting the pH at the point of analysis. At 24 h, when final pH was adjusted (C++, C-+) the antioxidant activity decreased whereas when final pH was not adjusted (C+-, C--) the activity increased or remained constant irrespective of the initial pH adjustment. At 48 h, a slight change was observed in the antioxidant activity for all samples. At 72 h, the antioxidant activity significantly ($P<0.05$) increased for all samples.

A high correlation between total phenolics and DPPH was found for sample where initial and final pH was adjusted (C++ = 0.91). This indicates when pH is not a factor total phenolics and DPPH inhibition are related. A weakly negative correlation was found for sample where initial and final pH were not adjusted (C-- = -0.37) and a strongly negative correlation was found for the other two samples (C+- = -0.87, C-+ = -0.95).

DPPH inhibition seems to be a combined pH and phenolic effect. An increase in antioxidant activity was reported by Apostolidis *et al.,* (2007), where the analysis was carried out at fermented acidic pH which is in agreement with the results obtained in this study. An inverse relationship between total phenolics and DPPH inhibition has been reported during soymilk fermentation (McCue and Shetty, 2005; Apostolidis *et al.,* 2007). An increase in the antioxidant activity by liberation of isoflavone aglycones during soymilk fermentation through catalytic activity of β-glucosidase and intracellular antioxidants of starter organisms has been suggested (Wang *et al.,* 2006).

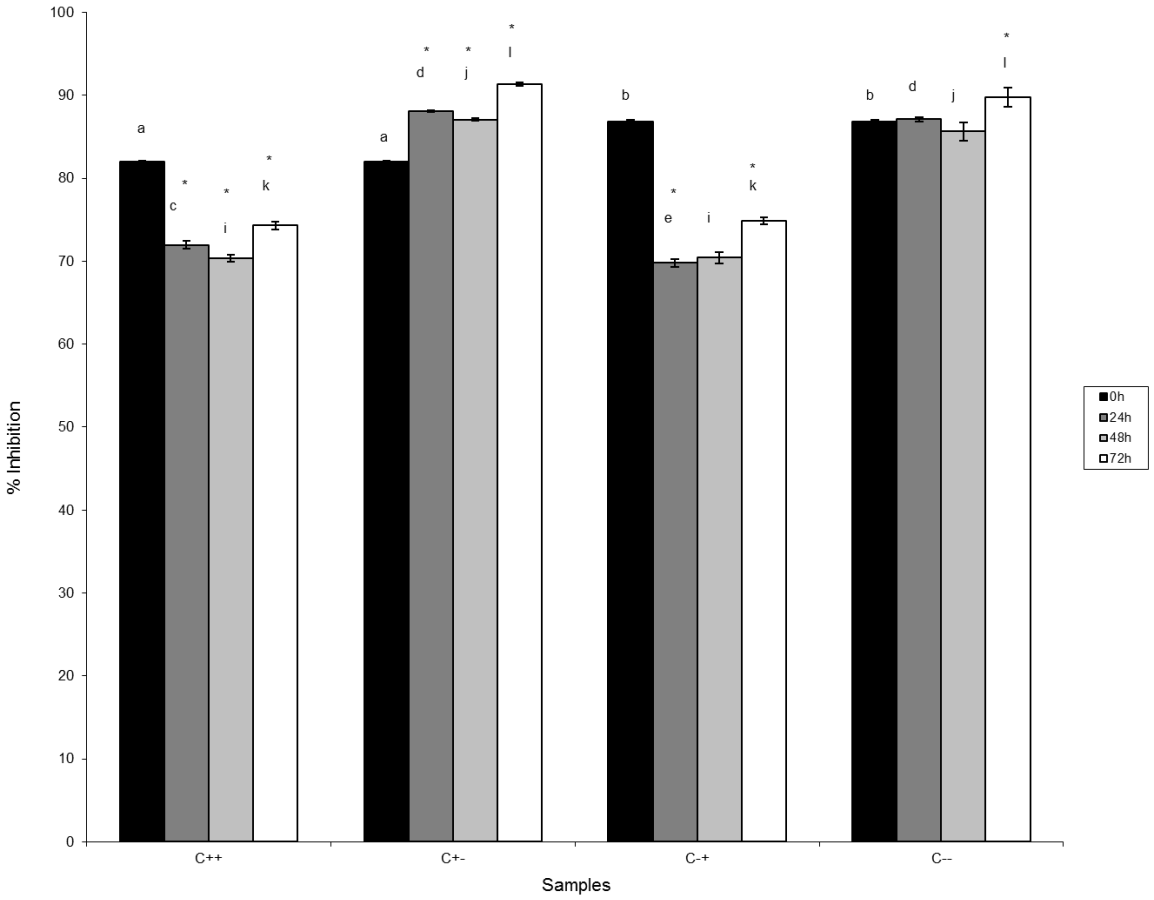

Figure 32: Changes in the DPPH scavenging activity (% Inhibition) of extracts of fermented cherry juice using *Lactobacillus helveticus* at 0, 24, 48 and 72 h of fermentation.

5.2.4.4 α-Glucosidase Inhibition Assay

Cherries are reported to have anthocyanins, and acylated anthocyanins from plant sources have been shown to have α-glucosidase activity (Gao and Mazza, 1995; Matsui *et al.,* 2001). High α-glucosidase inhibitory activity was observed for all samples at 0 h which indicates whole cherry juice has good potential to reduce post-prandial rise in blood glucose through α-glucosidase inhibition. At 0 h, initial pH adjusted samples (C++, C+-) had significantly lower ($P<0.05$) inhibition (88%) than initial pH not adjusted samples (C-+, C--; 92%) (Fig. 33). Analysis at fermented acidic pH increased the potential of the extract to inhibit the intestinal starch metabolizing enzyme, although it was not significant for all the time points. At 24 h, the inhibitory activity increased for samples where final pH was not adjusted (C+-, C--) whereas it decreased for samples where the final pH was adjusted (C++, C-+). At 48 h, no significant changes in the activity were observed for any samples. At 72 h, the inhibitory activity decreased for all samples however it was significant ($P<0.05$) only in case of C-+. Dose dependency studies using 10 µL and 25 µL indicated a dose dependent response. Dose dependency studies yielded a similar pattern of inhibitory activity overall for different doses.

Changes during fermentation were both a combined phenolic and pH effect however the pH effect was more dominant. The decrease in activity at 24 h in the pH adjusted samples (C++, C-+) was a phenolic effect. However, in the pH not adjusted samples (C+-, C--) the increase in activity because of low pH dominates the decrease due to phenolic effect.

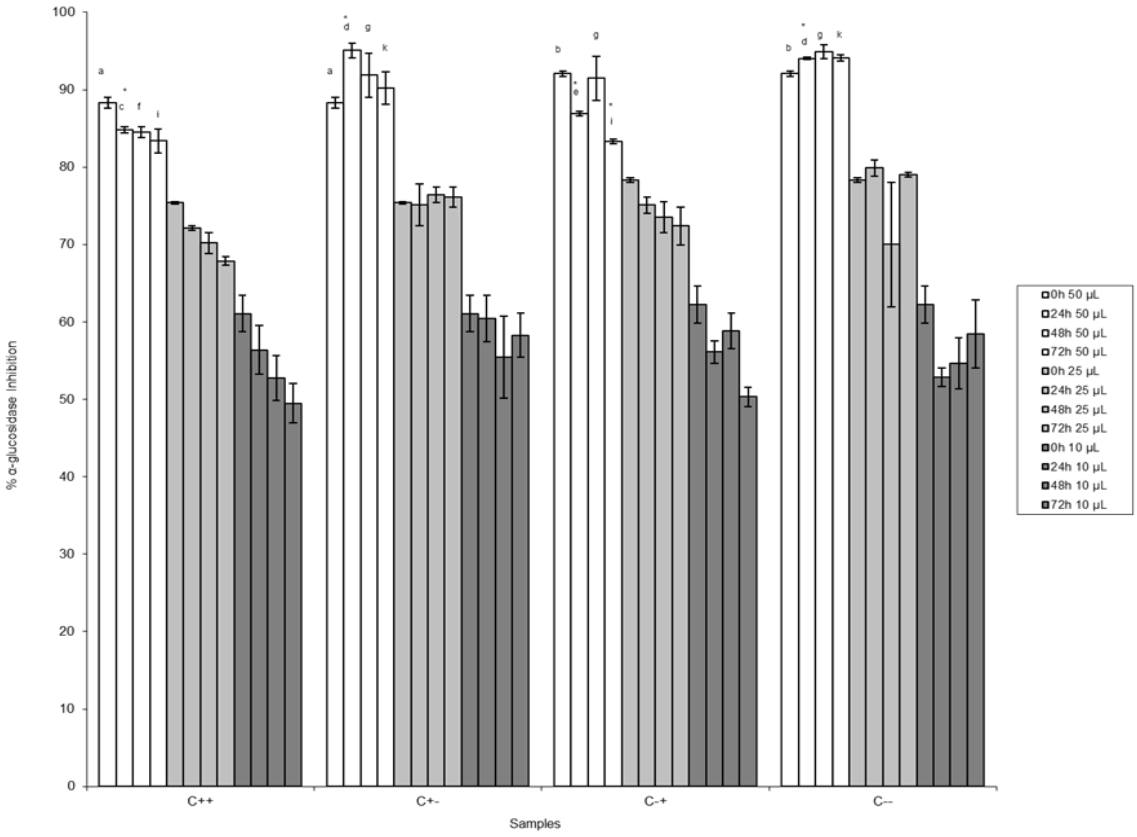

Figure 33:Dose-dependent changes in percentage α-glucosidase inhibitory activity of different pH treated extracts of fermented cherry juice using *Lactobacillus helveticus* at 0, 24, 48 and 72 h of fermentation.

The decrease in α-glucosidase inhibitory activity could be because of degradation of simple phenolics which have α-glucosidase inhibitory activity (Apostolidis *et al.,* 2006b; Kwon *et al.,* 2006b) or it could be because the compounds formed because of polymerization of simple phenolics may not be equally effective in inhibiting α-glucosidase. It could also be a combined effect.

For samples where final pH was not adjusted, good correlation (C+- = 0.6, C-- = 0.98) between lactic acid and α-glucosidase inhibition was observed. Although the phenolic effect may decrease the inhibitory activity, the pH effect may counteract to enhance inhibitory activity. In a similar study Apostolidis *et al.,* (2007), also reported an increase in inhibitory activity during fermentation of milk and soymilk where they had not adjusted the pH of the extracts.

5.2.4.5 α-Amylase Inhibition Assay

α-Amylase catalyzes hydrolysis of starch and mild inhibition of α-amylase is important in the management of hyperglycemia linked to type 2 diabetes. Because of high sugar content, the samples were diluted before the assay by 1:20 so as to get the final readings within the range of the spectrophotometer. No α-amylase inhibition was observed with the diluted fermented extracts.

5.2.4.6 ACE Inhibition Assay

Inhibition of ACE, an important enzyme in maintaining vascular tension, is considered a useful therapeutic approach in the treatment of high blood pressure (McCue *et al.,* 2005b). Polyphenols not only help normalizing blood pressure but they also help retard the development of hypertension (Appel *et al.,* 1997; Taubert *et al.,* 2003; Negishi *et al.,* 2004;). The 0 h values for ACE inhibition were moderate (31%) for both initial pH adjusted (C++, C+-) and pH not adjusted samples (C-+, C--; 40%)

indicating it has a reasonable potential in treating high blood pressure (Fig 34). At 24 h, only C++ had inhibition.

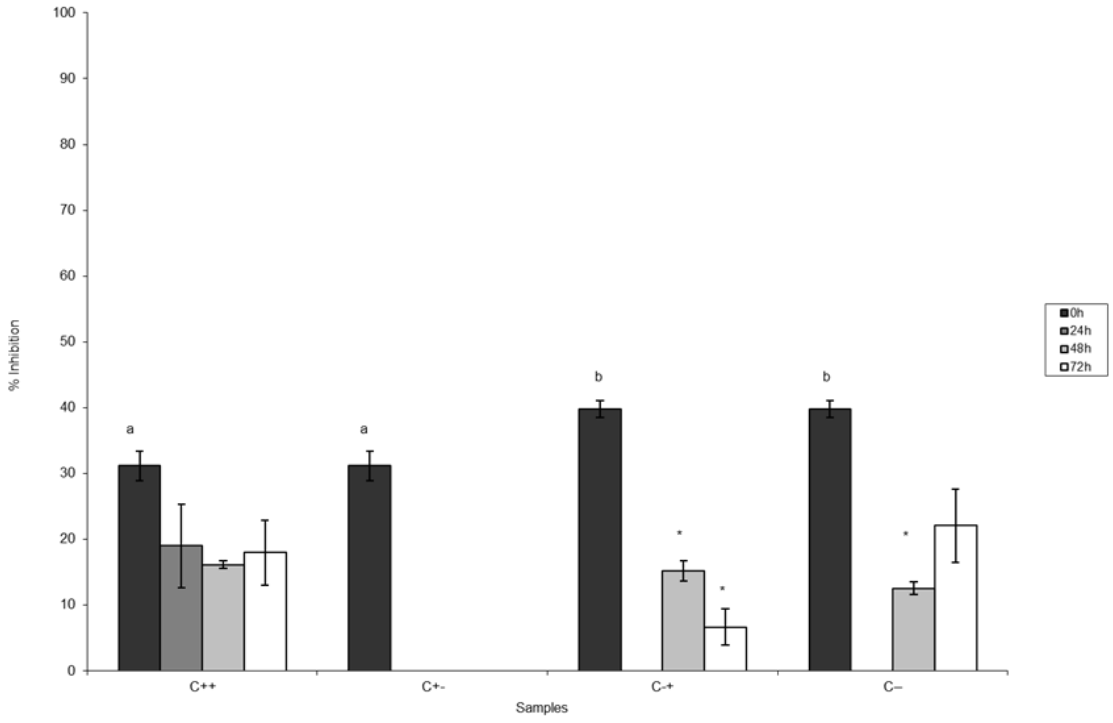

Figure 34: Changes in percentage ACE inhibitory activity of cherry juice with different pH treatments fermented using *Lactobacillus helveticus*

At 48 h, inhibitory activity did not change significantly ($P<0.05$) for C++ whereas initial pH not adjusted (C-+, C--) samples showed some inhibition. At 72 h, inhibition increased for C-- and C++ whereas it decreased for C-+. However, in none of the samples the changes were significant. Although lactic acid, simple phenolics and

small peptides have been suggested to be important for ACE inhibition (Frossard *et al.*, 2000; Apostolidis *et al.,* 2007), the trends obtained in this study for ACE inhibition cannot be explained at this time.

5.2.4.7 Protein Assay

Protein content was measured using the Bradford assay (Bradford, 1976). The protein content decreased for all samples as fermentation proceeded (Fig 35). Final pH adjusted samples were significantly higher ($P>0.05$) in protein content than samples where analysis was carried out at fermented acidic pH. This could be because adjusting the pH would lead to refolding of some proteins that is better reflected in the Bradford assay. The decrease in protein content at specific time points could be explained by the fact that lactic acid bacteria depend strongly on exogenous sources of nitrogen (Yamamoto *et al.*, 1993), indicating the action of nonspecific proteinases secreted during fermentation which might cause liberation of peptides. The decrease in protein content could also be because of protein denaturation due to lowering of pH during fermentation. The increase in protein content could be due to increase in enzyme activity of the fermenting bacteria.

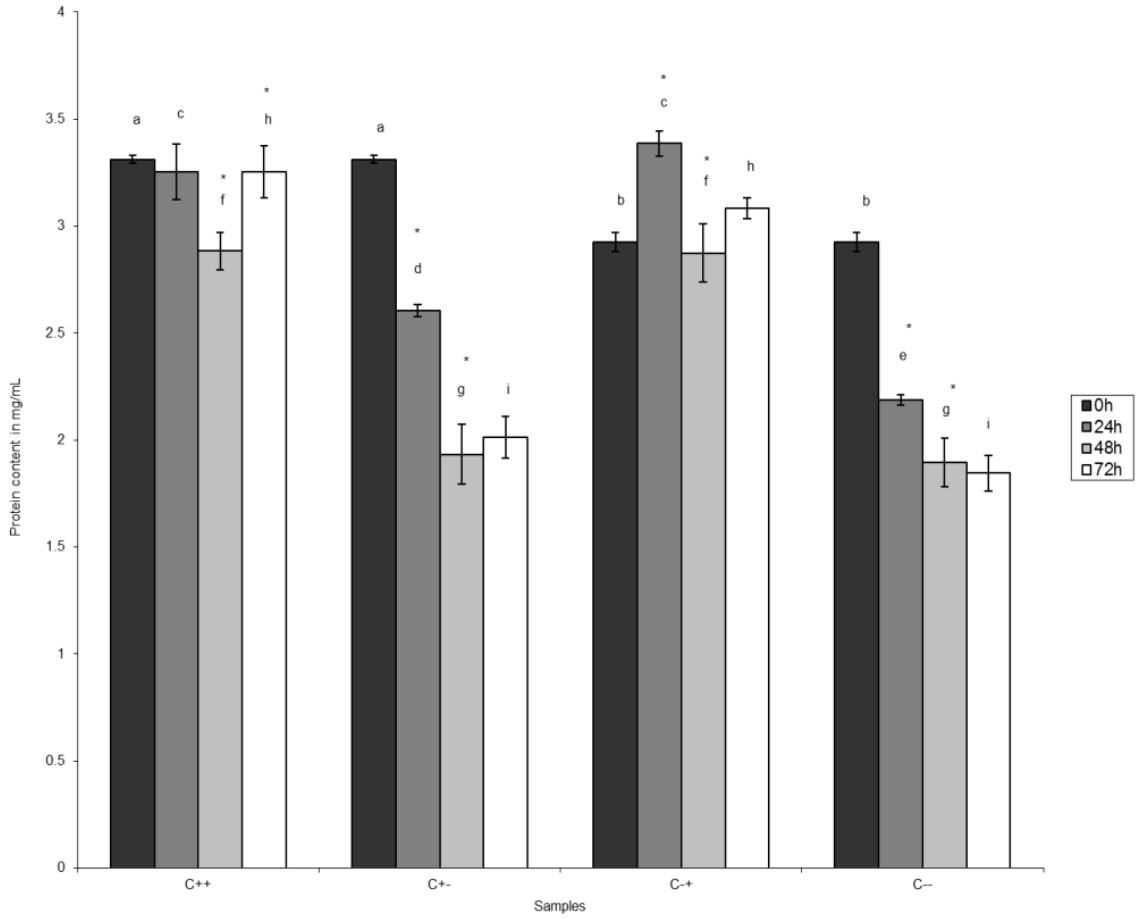

Figure 35: Effect of fermentation by *Lactobacillus helveticus* on the protein content (mg/mL) of cherry juice at 0, 24, 48 and 72 h of fermentation

5.2.4.8 Lactic Acid Content Measured by HPLC

Lactic acid in the sample was measured by using HPLC analysis. At 0 h some amount of lactic acid was present in the sample (Fig 36). At 24 h, the lactic acid content increased for all samples significantly ($P<0.05$) as expected. Lactic acid content increased significantly ($P<0.05$) at 48 h for all samples except C+-. At 72 h, lactic acid content decreased for samples where final pH was not adjusted whereas for final pH adjusted samples lactic acid content increased or remained constant. However this decrease or increase was not significant.

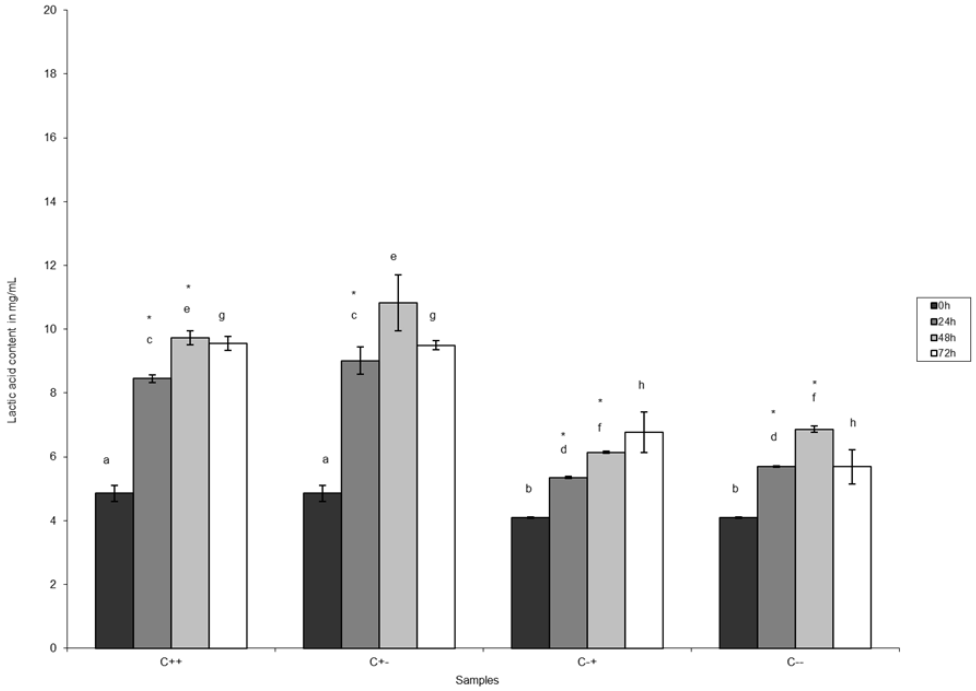

Figure 36:Lactic acid content (mg/mL) as measured by HPLC at 0, 24, 48 and 72 h of fermented cherry juice extracts using *Lactobacillus helveticus*.

5.2.4.9 High Performance Liquid Chromatography (HPLC) Analysis of Phenolic Profiles

The amount of a phenolic compound present is a result of the flux between degradation/formation of large polymeric phenolic compound and complete degradation of simple phenolics as a result of bacterial detoxification mechanism (McCue and Shetty, 2005).

Epicatechin, caffeic acid, and *p*-coumaric acid derivatives were the major phenolic compounds were found in fresh samples as well as the fermented extracts (Table 7 and 8). Resveratrol was also found in final pH adjusted samples (C++, C-+) at 24 and 48 h. The peaks were identified taking into account the retention time and the UV absorption spectra of the corresponding standards.

At 0 h, epicatechin and *p*-coumaric acid derivatives were found in pH adjusted and pH not adjusted samples. Caffeic acid was detected only in pH not adjusted samples. This could be because adjusting the pH may cause some changes to the structure of simple mono phenolics (Friedman and Jurgens, 2000) which might make it difficult to be detected by HPLC especially when it is present in such small amounts. At 0 h, the amounts of epicatechin detected were significantly different, the reason for this is not known.

At 24 h, epicatechin content decreased for initial pH adjusted samples (C++, C+-) whereas it increased for initial pH not adjusted samples (C-+, C--). Resveratrol was detected only in final pH adjusted samples (C++, C-+) whereas caffeic acid was detected only in final pH not adjusted samples (C+-, C--). *p*-Coumaric acid derivatives content decreased for all samples.

Table 7: HPLC Analysis of Individual Phenolic compounds of fermented extracts of pH adjusted Cherry juice (µg/mL)

Phenolic compound	C++				C-+			
	0 h	24 h	48 h	72 h	0 h	24 h	48 h	72 h
Epicatechin	98.1 ± 2.3	86.5 ± 3.4	78.2 ± 1.1	82.3 ± 4.0	59.8 ± 4.7	75.6 ± 7.8	90.5 ± 0.6	48.8 ± 1.2
Caffeic acid					1.0 ± 0.1			
Resveratrol		0.11 ± 0.01	0.1 ± 0.01			0.16 ± 0.004	0.14 ± 0.003	
p-Coumaric acid	1.29 ± 0.02	1.14 ± 0.02	1.09 ± 0.01	0.63 ± 0.01	1.29 ± 0.12	0.91 ± 0.04	1.22 ± 0.002	0.92 ± 0.06

Table 8: HPLC Analysis of Individual Phenolic compounds of fermented extracts of pH not adjusted extracts of Cherry juice (µg/mL)

Phenolic compound	C+-				C--			
	0 h	24 h	48 h	72 h	0 h	24 h	48 h	72 h
Epicatechin	98.1 ± 2.3	73.1 ± 5.1	66.2 ± 0.64	75.4 ± 5.2	59.8 ± 4.7	79.8 ± 2.3	74.5 ± 0.2	76.0 ± 0.7
Caffeic acid		0.82 ± 0.005	0.78 ± 0.01	0.89 ± 0.06	1.0 ± 0.1	0.97 ± 0.03	1.02 ± 0.02	1.0 ± 0.002
p-Coumaric acid	1.29 ± 0.02	1.14 ± 0.01	1.1 ± 0.01	1.38 ± 0.16	1.29 ± 0.12	1.1 ± 0.03	1.18 ± 0.003	1.4 ± 0.01

At 48 h, epicatechin content decreased for all samples except C-+. Resveratrol and caffeic acid content overall remained the same. Similar trend was observed for *p*-coumaric acid derivatives except in case C-+ where a significant increase was observed.

At 72 h, epicatechin content increased for all samples except C-+. Caffeic acid content increased for C+-, whereas it remained the same for C--. Resveratrol was not detected at 72 h, in the final pH adjusted samples (C++, C-+). *p*-coumaric acid

derivatives content decreased significantly for pH adjusted samples whereas a reverse trend was observed with final pH not adjusted samples.

Epicatechin is an antioxidant flavanol which has potent α-glucosidase inhibitory activity and mild α-amylase inhibitory potential (Tadera *et al.,* 2006; Matsui *et al.,* 2007). Epicatechin has also shown inhibitory potential against *S. aureus* at high concentrations (Akiyama *et al.,* 2001). Some of the functional properties of the fermented juice may be attributed to the presence of epicatechin in combination with other phenolics.

5.2.4.10 *Helicobacter pylori* Inhibition

Helicobacter pylori is a microaerophilic gram-negative pathogen that has been linked to stomach related diseases (Mitchell, 1999). Final pH adjusted samples (C++, C-+) had no inhibition at any time point. For the final pH not adjusted samples (C+-, C--) 24, 48 and 72 h fermented samples had inhibitory activity (Fig 37). Inhibition was observed only with 100 µL of the samples, 50 and 75 µL had no inhibition. At 0 h, no inhibition was observed.

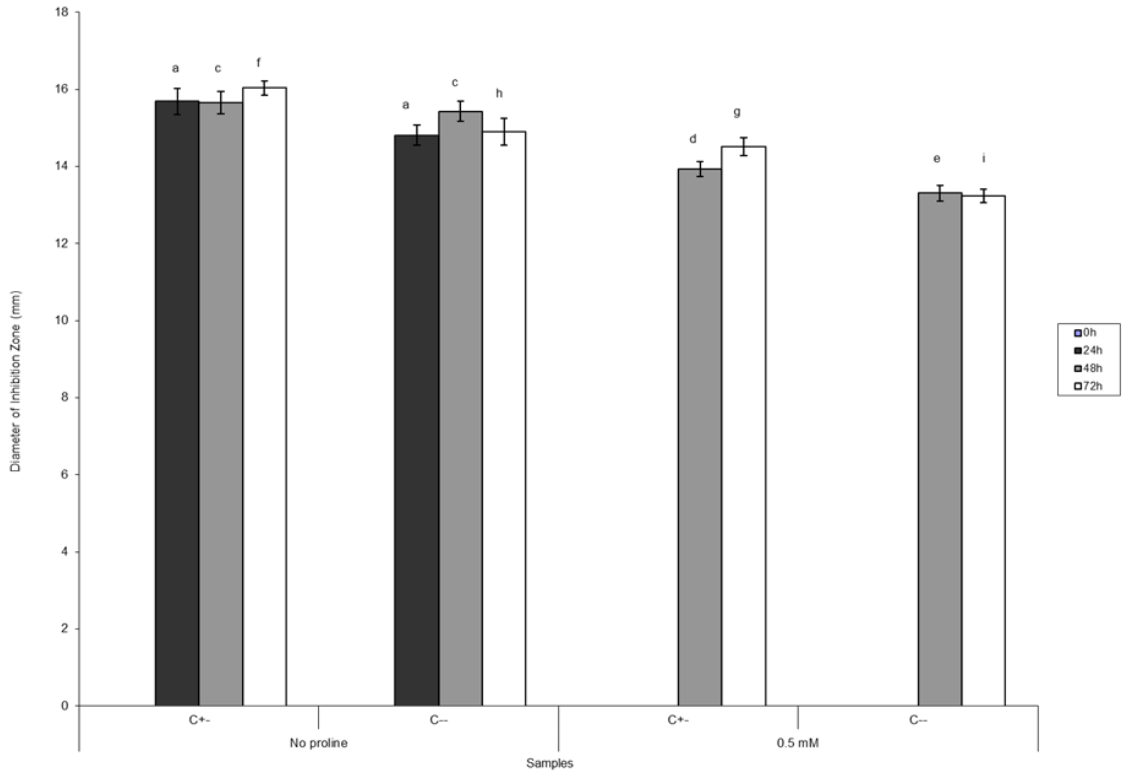

Figure 37: *Helicobacter pylori* inhibition by fermented cherry juice extracts at 0, 24, 48 and 72 h of fermentation and effect of addition of 0.5 mM proline on anti-*Helicobacter pylori* activity

As fermentation proceeded, inhibition was observed with fermented acidic extracts. At 24 h, C+- had higher inhibition (15.7 mm) than C-- (14.8 mm), however, the difference was not significant ($P>0.05$). For C+-, inhibition remained constant at 48 h, and increased slightly at 72 h, but none of the changes were significant. For C--, at 48 h, the inhibition potential increased and then at 72 h, it decreased without any significant difference ($P>0.05$). At 72 h, C+-, had significantly higher inhibition ($P<0.05$) than C--.

Midolo *et al.,* (1995), and Alakomi *et al.,* (2000), reported that sodium and potassium salts of lactic acid were less effective than their corresponding acid in inhibiting gram-negative bacteria. A much higher concentration of the salt was required to produce a comparable zone of inhibition as lactic acid (Midolo *et al.,* 1995). This explains the results obtained in this study where final pH adjusted samples had no inhibition. Adjusting the pH caused lactic acid to lose its inhibition properties. Similar results were obtained in another study with pH adjusted samples of fermented apple juice (data not published) where no inhibition was observed.

Inhibition of *H.* pylori was a combined lactic acid and phenolic effect. It has been reported that lactic acid can disrupt the outer membrane of gram-negative bacteria and act as a permeabilizer. This effect of lactic acid may increase the susceptibility to inhibition of the gram-negative bacteria by allowing other compounds like antibiotics and bacteriocins to penetrate (Alakomi *et al.,* 2000). Phenolic compounds have been shown to be inhibitory to gram-negative bacteria (Vattem *et al.,* 2005a). It is possible that phenolic compounds work in combination with lactic acid in inhibiting the bacteria. This is supported by the fact that although phenolic in C+- and C--, decrease or remain the same at 24 h, inhibition increases because of formation of lactic acid in the sample. After that although lactic acid content in the sample increases, inhibition does not change because the phenolic content in the fermented acidic sample do not change significantly. This leads us to the conclusion that phenolics cannot bring about inhibition on their own, unless there is certain amount of lactic acid present in the sample and after that inhibition of the bacteria is dictated by the changes in the phenolics present in the sample.

Previous work in our lab has provided some insights that in animal cell and bacterial model systems cellular redox response through proline- linked pentose phosphate pathway could be regulated through phenolics (Shetty and Wahlqvist, 2004; Lin *et al.,* 2005). The proline growth response assay is based on the rationale that oxidative phosphorylation could be disrupted in prokaryotic cells by proline mimicking small phenolics which can inhibit proline oxidation via proline dehydrogenase (PDH) at the plasma membrane level. If so, then addition of proline could overcome inhibition of the bacterium by proline mimicking phenolics present in the fermented extract. The results obtained in this study indicate that the antimicrobial effect of pH not adjusted fermented cherry juice was significantly ($P< 0.05$) reduced when proline was added at 0.5 mM in *H. pylori* agar plates. The results obtained in this study provide clues that the proline dehydrogenase inhibition caused by phenolics is one of the likely mechanisms contributing to the inhibition of *H. pylori*.

Fermented samples with caffeic acid content at 24, 48 and 72 h had *H. pylori* inhibition. Caffeic acid has been shown to have antimicrobial properties against *S. aureus* (Kwon *et al.,* 2007b). Kwon *et al.,* (2007b), reported that the inhibitory activity of caffeic acid against *S. aureus* was reversed with the addition of 0.5 mM to the agar plate which indicates that the site of action could be at proline dehyrogenase level.

In addition to the proline dehydrogenase inhibition by phenolics there could be other mechanisms contributing. Our findings here along with previous reports published by other authors (Midolo *et al.,* 1995; Alakomi *et al.,* 2000) suggests that lactic acid might aid the antimicrobial activity of other polyphenolic compounds by releasing lipopolysaccharide or other components, which can abolish the integrity of the outer

membrane. The antioxidant properties of phenolic compounds may allow them to act as antimicrobials by disrupting oxidative phosphorylation by quenching electrons from electron transport chain (ETC) along the bacterial membrane (Lin *et al.,* 2005). In addition they may also inhibit dehydrogenases linked proton efflux by interfering with the flow of electrons at the level of cytochromes (Vattem *et al.,* 2005a). It is also possible that simple phenolics, by hyper-acidification via proton donation at the plasma membrane can disrupt the H^+-ATPase required for ATP synthesis and cause intra-cellular cytosolic acidification (Shetty and Labbe, 1998; Shetty and Wahlqvist, 2004; Kwon *et al.,* 2007b). Another explanation is that partially hydrophobic polyphenols can stack themselves by attaching to the cell wall and cause destabilization of the membrane leading to membrane disruption or transport inhibition (Shetty and Wahlqvist, 2004). Phenolic phytochemicals may also inhibit *H. pylori* by restricting the supply of essential metal ions like Fe^{3+} and Ni^{2+} which are required for growth by the bacteria (Kumamoto *et al.,* 2001; Montecucco and Rappuoli, 2001) and by inhibiting ureases, which helps *H. pylori* survive in extremely acidic environment of the stomach by releasing ammonia (Matsubara *et al.,* 2003; Lin *et al.,* 2005).

5.2.4.11 Lactic Acid Bacterial Proliferation Assay

Probiotics are a class of living organisms which confer health benefits when ingested in certain numbers (Ljungh and Wadström, 2006). Extracts of cherry juice at fermented acidic pH (C+-, C--) which showed *H. pylori* inhibition were further tested for their effects on probiotic lactic acid bacteria. The effects were evaluated by determining the plate counts of probiotic bacteria at 0, 6, 9, 12 and 24 h of growth in

MRS broth medium. The concentrations of extracts used in this study were similar to those used in *H. pylori* inhibition studies.

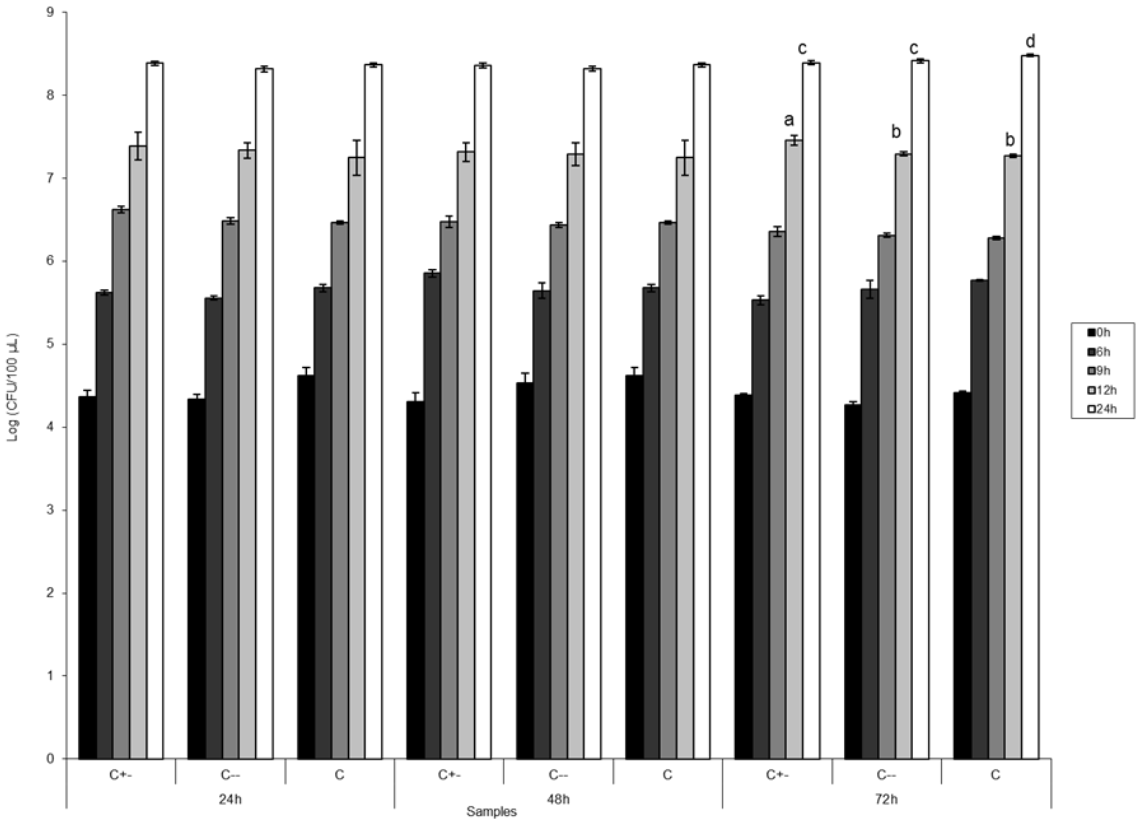

Figure 38: Effect of 24, 48, and 72 h fermented cherry juice extracts on *Bifidobacterium longum* proliferation (CFU/mL).

No inhibitory effects were seen on probiotic strain of *Bifidobacterium longum* as compared to control except in 72 h fermented extracts at 24 h of bacterial growth (Fig 38). Some stimulatory effects were seen with 72 h extracts at 12 h of bacterial growth. Overall with the extracts the growth rate was similar to that of the control. This suggests

that the fermented extracts might be able to inhibit *H. pylori* without affecting the intestinal probiotic bacteria. It has been reported that lipophilic phenolics were unable to inhibit lactic acid bacteria whereas more hydrophilic phenolics were completely inhibitory to lactic acid bacteria of human gastrointestinal tract origin (Puupponen-Pimia *et al.*, 2001). Another reason for the lack of inhibition could be because lactic acid bacteria use substrate level phosphorylation for energy generation whereas microaerobic bacteria such as *H. pylori* may depend on partial oxidative phosphorylation.

5.2.5 Conclusion

In this study, cherry juice was fermented over a period of 72 h and changes in functionality related to management of type 2 diabetes were investigated using *in vitro* assays. Effects of the fermented juice on the inhibition of pathogenic *H. pylori* and proliferation of probiotic *B. longum* were also evaluated. The results obtained in this study indicate that the total phenolics, DPPH linked antioxidant activity and α-glucosidase inhibition increase or decrease depending on the variations in the pH. Fermentation enhanced the functionality towards inhibition of gastric cancer linked pathogen *H. pylori* without affecting the beneficial lactic acid bacteria in the intestine. This provides further biochemical rationale for further animal and clinical studies to provide potentially novel and low-Cost approaches to manage hyperglycemia and *Helicobacter pylori* infections. Investigations into the mechanism of action against *H. pylori* revealed that one of the likely mechanisms of action was by inhibition of proline oxidation via proline dehyrogenase (PDH).

5.3 Evaluation of Antihyperglycemia and Antihypertension Potential of Pear juice Fermented with *Lactobacillus helveticus* Using *in Vitro* Assay Models

5.3.1 Abstract

Four cultivars of pear were homogenized to extract their juice, fermented using *Lactobacillus helveticus* for 0, 24, 48 and 72 h and their potential in management of hyperglycemia and hypertension were evaluated. The pH of the juices was adjusted to 6.0-7.0 before fermentation and assays at each time point were carried out at fermented acidic pH and by adjusting the pH to 6.0-7.0. Overall there was a decrease in total soluble phenolics and DPPH-linked free radical scavenging activity with fermentation over a period of 72 h. α-Glucosidase inhibition increased or remained constant for both pH adjusted and pH not adjusted samples. ACE inhibition decreased for all samples with fermentation. Epicatechin and quercetin derivatives were the major phenolic compounds detected in the fermented samples.

5.3.2 Introduction

Type 2 diabetes, cardiovascular diseases and chronic respiratory conditions are reaching epidemic proportions worldwide and the major reasons implicated in these chronic diseases are aging, refined high calorie diet lacking in fruits and vegetables, physical inactivity and stress (Wild *et al.*, 2004; Daar *et al.*, 2007) . It is projected that India and China will loose around half a trillion dollars and a quarter trillion dollars, respectively, as a result of these chronic diseases in the next decade (Daar *et al.*, 2007).

Intestinal α-glucosidase aides the subsequent absorption of carbohydrates hydrolyzed by pancreatic α-amylase (Elsenhans and Caspary, 1987). Therapeutic drugs for managing type 2 diabetes, such as acarbose, target the inhibition of these starch breakdown enzyme (Cheng and Fantus, 2005). These therapeutical approaches are associated with side effects such as abdominal distention, flatulence and possible diarrhea (Bischoff, 1994). Fruits and vegetables can provide naturally occurring α-glucosidase and α-amylase inhibitors without the associated side effects (Matsui *et al.*, 2001). Angiotensin-I converting enzyme (ACE) inhibition is considered important in controlling blood pressure by maintaining vascular tension in both diabetic and non-diabetic patients (Johnston and Franz, 1992). Phenolic compounds from plants and fermented foods such as yogurt may mimic synthetic ACE inhibitors and help in management of hypertension without side effects (Actis-Goreta *et al.*, 2003; Apostolidis *et al.*, 2006a; Apostolidis *et al.*, 2007). It was thought that such beneficial compounds would be mobilized by fermentation of fruit juice which could be targeted against the key enzymes linked to hyperglycemia and hypertension.

Oxidative stress can cause damage to DNA, proteins and lipids, resulting in degenerative diseases such as cancer, CVD and brain dysfunction (Ames *et al.,* 1993b). High intake of fruits and vegetables can provide the antioxidants, trace minerals and other bioactive compounds to counter this oxidative stress and maintain normal cell function. Although pear is classified in the group of fruits with low antioxidant capacity (Prior and Cao, 2000), it is suggested that the pear peel is an excellent source of antioxidant (Sanchez *et al.,* 2003). Phenolic compounds such as chlorogenic acid, caffeic acid, *p*-coumaroyl quinic and *p*-coumaric acids along with a number of other procyanidin and quercetin glycosides have been reported in pear (Schieber *et al.,* 2001; Lin and Harnly, 2008). Pear production in the USA is mostly concentrated in the northwest around the region of Washington and per capita consumption of all pear products was reported to be around 5.4 pounds (Agricultural marketing resource centre, www. agmrc.org).

In this study, we fermented juice extracted from four pear cultivars using *Lactobacillus helveticus* and investigated the changes in total phenolics, antioxidant activity, enzymes related to type 2 diabetes at 0, 24, 48 and 72 h of fermentation. Before fermentation, pH of the juice was adjusted to 6.0-7.0. After fermentation analysis at each time point was carried out by adjusting the pH and at fermented acidic pH to help us asses the contribution of both pH and phenolic effect at each time point.

5.3.3 Materials and Methods

5.3.3.1 Sample Extraction

Four cultivars of Pear: Anjou, Red Anjou, Bosc and Comice obtained from Washington. Whole Pears were washed with water and then cut into small pieces. The edible pieces were homogenized using a Waring blender for 5 min. The thick pulp was then centrifuged for 15 min at 15,000g, supernatants were collected and kept at -20^{0}C during the period of the study.

5.3.3.2 Bacterial Strain

The lactic acid bacteria strains used in this study, *Lactobacillus helveticus* was provided by Rosell Institute Inc. Montreal, Canada (Lot#: XA 0145, Seq#:00014160).

5.3.3.3 Fermentation

Initially 100 µL of frozen *Lactobacillus helveticus* stock were inoculated into 10 mL MRS broth (Difco) and incubated for 24 h at 37 °C. Then, 100 µL of the 24 h grown strain was re-inoculated into 10 mL MRS broth for 24 h at 37 ^{o}C. Ninety mL of the juice was put into a 125 mL Erlenmeyer flask; pH adjusted to 6 using NaOH and inoculated using 10 mL of the broth culture. Fermentation was carried out at 37 ^{o}C and 12.5 mL samples were taken out at 0, 24, 48 and 72 h. At every time point except for 0 h, two 12.5 mL samples were taken out, for one sample pH was adjusted to 6 by adding drops of 0.1M NaOH. For the non-pH adjusted sample; same number of drops of distilled water was added to keep the volume same. The samples were centrifuged at 15,000 g for 15 min and then used for the assays.

5.3.3.4 Absorbance of Sample and Colony Counts

Growth of bacteria in the sample was estimated using absorbance at 600 nm. More accurate estimations of the bacterial numbers were made by plate count technique. At 0, 24, 48 and 72 h, CFU/mL was determined by pipetting 100 µL of the sample, serially diluting and plating on MRS medium. The plates were incubated anaerobically at 37 ^{o}C for 24 h and individual colonies were counted. The pH of the samples was also measured at 24, 48 and 72 h.

5.3.3.5 Total Phenolics Assay

The total phenolics in all samples were determined by using a method modified by Shetty *et al.*, (1995). In brief, 0.5 mL of sample extract was added to a test tube and mixed with 0.5 mL of water and 5 mL of distilled water. To each sample, 0.5 mL of 50% (vol/vol) Folin-Ciocalteu reagent was added and mixed. The absorbance was read at 725 nm using a spectrophotometer (Genesys UV/Visible, Milton Roy, Inc., Rochester, NY). Different concentrations of gallic acid were used to develop a standard curve. Results were expressed as mg of gallic acid/mL of sample.

5.3.3.6 Antioxidant Activity by DPPH Radical Inhibition Assay

The antioxidant activity was determined by the DPPH radical scavenging method modified from Kwon *et al.*, (2006b). A 250-µL aliquot of the sample extract was mixed with 1,250 µL of DPPH (60 µM in ethanol). Absorbance was measured at 517 nm using the Genesys UV/Visible spectrophotometer. The readings were compared with the controls, containing 95% ethanol instead of sample extract. The percentage inhibition was calculated by:

$$\% \text{ inhibition} = \frac{(Absorbance_{control} - Absorbance_{extract})}{Absorbance_{control}} \times 100$$

5.3.3.7 α-Glucosidase Inhibition Assay

The α-glucosidase inhibitory activity was determined by an assay modified from McCue *et al.,* (2005a). α-Glucosidase was assayed by using 50 µL of sample extracts and 100 µL of 0.1 M phosphate buffer (pH 6.9) containing α-glucosidase solution (1 U/mL) and was incubated in 96-well plates at 25 °C for 10 min. After preincubation, 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 incubation, absorbance readings were recorded at 405 nm by a microplate reader (Thermomax, Molecular Devices Co., Sunnyvale, CA) and compared to a control that had 50 µL of buffer solution in place of the extract. The α-glucosidase inhibitory activity was expressed as percentage inhibition and was calculated with the same equation as for percentage inhibition in the DPPH radical inhibition assay. Dose dependency was tested using 25 µL and 10 µL of the sample, the volume made up to 50 µL using 0.1 M phosphate buffer (pH 6.9) and same protocol was followed.

5.3.3.8 α-Amylase Inhibition Assay

The α-amylase inhibitory activity was determined by an assay modified from McCue *et al.,* (2005a). A total of 500 µL of 1:10 dilution (due to the high sugar content) of sample extract and 500 µL of 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M

NaCl) containing α-amylase solution (0.5 mg/mL) were incubated at 25 °C for 10 min. After preincubation, 500 μL of a 1% starch solution in 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M NaCl) was added to each tube at timed intervals. The reaction was stopped with 1.0 mL of dinitrosalicylic acid color reagent. The test tubes were incubated in a boiling water bath for 5 min and cooled to room temperature. The reaction mixture was then diluted after adding 15 mL of distilled water, and the absorbance was measured at 540 nm using the Genesys UV/Visible spectrophotometer. The readings were compared with the controls, containing buffer instead of sample extract. The percentage α-amylase inhibitory activity was calculated with the same equation as for percentage inhibition in the DPPH radical inhibition assay.

5.3.3.9 ACE Inhibition Assay

ACE inhibition was assayed by a method modified by Kwon *et al.,* (2006b). The substrate hippuryl-histidyl-leucine (HHL) and the enzyme ACE-I from rabbit lung (EC 3.4.15.1) were used. Fifty μL of sample extracts were incubated with 100 μL of 1 M NaCl-borate buffer (pH 8.3) containing 2 mU of ACE-I solution at 37 °C for 10 min. After preincubation, 100 μL of a 5 mU substrate (HHL) solution was added to the reaction mixture. Test solutions were incubated at 37 °C for 1 h. The reaction was stopped with 150 μL of 0.5 N HCl. Five μL of the sample was injected in a high-performance liquid chromatography (HPLC) apparatus (Agilent 1100 series equipped with autosampler and DAD 1100 diode array detector, Agilent Technologies, Palo Alto, CA). The solvents used for gradient were (1) 10 mM phosphoric acid (pH 2.5) and (2)

100% methanol. The methanol concentration was increased to 60% for the first 8 min and to 100% for 5 min and then was decreased to 0% for the next 5 min (total run time, 18 min). The analytical column used was an Agilent Nucleosil 100-5C18, 250 mm × 4.6 mm inside diameter, with packing material of 5 μm particle size at a flow rate of 1 mL/minute at ambient temperature. During each run, the absorbance was recorded at 228 nm, and the chromatogram was integrated using the Agilent Chemstation (Agilent Technologies) enhanced integrator for detection of liberated hippuric acid (A). Hippuric acid standard was used to calibrate the standard curve and retention time. The percentage inhibition was calculated by:

$$\%\ \text{inhibition} = \frac{(A_{\text{control}} - A_{\text{extract}})}{(A_{\text{control}} - A_{\text{blank}})} \times 100$$

5.3.3.10 Protein Assay

Protein content was measured by the method of Bradford assay (Bradford, 1976). The dye reagent concentrate (Bio-Rad protein assay kit II, Bio-Rad Laboratory, Hercules, Calif., USA) was diluted 1:4 with distilled water. Five mL of diluted dye reagent was added to 100 μL of fermentation supernatant. After vortexing and incubating for 5 min, the absorbance was measured at 595 nm against a 5 mL reagent blank and 100 μL buffer using a UV-VIS Genesys spectrophotometer (Milton Roy, Inc., Rochester, N.Y., USA).

5.3.3.11 HPLC Determination of Lactic Acid Content

Five μl of sample was injected using Agilent ALS 1100 autosampler into Agilent 1100 series HPLC (Agilent Technologies) 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, 25 min). The analytical column used was Agilent Zorbax SB-C18, 250 × 4.6 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 210 nm and integrated using Agilent Chemstation enhanced integrator. Pure standards of lactic acid (purchased from Sigma Chemical Co. in 100% methanol were used to calibrate the standard curve and retention times.

5.3.3.12 High Performance Liquid Chromatography (HPLC) Analysis of Phenolic Profiles

The extracts (2 mL) were filtered through a 0.2 μm filter. A volume of 5 μL of extract was injected using an Agilent ALS 1100 auto sampler into an Agilent 1100 series HPLC (Agilent Technologies, Palo Alto, CA) equipped with a 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, 25 min). The analytical column used was Agilent Zorbax SB-C_{18}, 250 x 4.6 mm i.d., with packing material of 5 μm particle size at a flow rate of 1 mL/min at room temperature. During each run the absorbance was recorded at 226 and 306 nm and the chromatogram was integrated using Agilent Chemstation enhanced integrator. Pure standards of gallic acid, catechin,

epicatechin, quercetin, p-coumaric acid, caffeic acid and resveratrol in 100% methanol were used to calibrate the standard curves and retention times.

5.3.3.13 Statistical Analysis

All experiments were performed at least in duplicate or triplicate. Analysis at every time point from each experiment was carried out in duplicate or triplicate. Means, standard errors and standard deviations were calculated from replicates within the experiments and analyzed using Microsoft Excel XP. Significant difference between the treatment was determined using the student t-test at the 0.05 probability level.

5.3.4 Results and Discussion

5.3.4.1 pH, Absorbance and Colony Counts

pH of the juice was adjusted to 6.0 before fermentation. pH decreased for all samples at 24 h to around 3.8 as expected. Thereafter pH remained constant for all samples (Fig 39). Fermentation started at 0 h with CFU/mL around 7 log. At 24 h it decreased for all the samples. After 24 h, the numbers steadily decreased for all samples representing the death phase of the bacteria growth curve. Absorbance for all the samples followed a bacterial growth curve; it increased at 24 h, then it increased or remained the same and then at 72 h, it decreased. The decrease in pH and increase in absorbance suggest that pear juice is a good substrate for *Lactobacillus helveticus* fermentation. The slight decrease in colony counts at 24 h could be because the bacteria have already reached the death phase at 24 h.

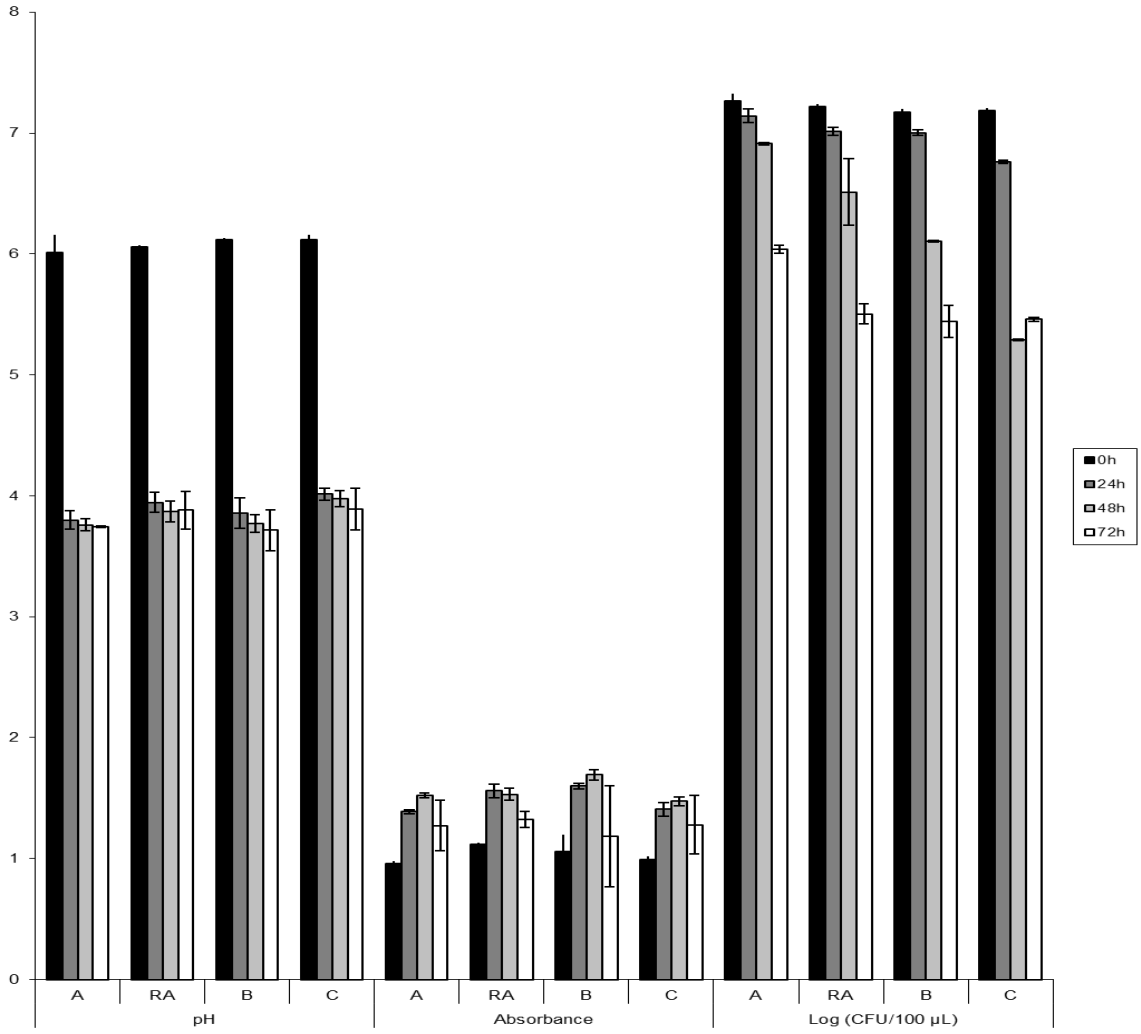

Figure 39: pH, absorbance and colony counts of fermented pear juice extracts at various time points.

5.3.4.2 Total Soluble Phenolics

Total phenolic content was determined using Folin-Ciocalteu method which involves measuring the intensity of a blue complex formed by the reduction of the reagent by phenolic compounds. The intensity of the blue complex is linearly proportional to the concentration of phenols present in the sample (Singleton *et al.*, 1999). At 0 h all the pear cultivar juices had total phenolic content of around 0.4 mg/mL. Bosc had the highest (0.42 mg/mL) whereas Anjou had the lowest (0.38 mg/mL) (Fig 40). For both pH adjusted and not adjusted samples total phenolics decreased as fermentation proceeded.

For pH adjusted samples, at 24 and 48 h total phenolic content decreased for all samples. This decrease was not significant ($P>0.05$) for Anjou at 24 h and 48 h, whereas it was not significant for Red Anjou at 48 h. At 72 h, there was no significant change in total phenolics for any pH adjusted sample.

For samples where pH was not adjusted a similar overall effect was seen. Total phenolics decreased significantly ($P<0.05$) for all samples at 24 h. At 48 h, total phenolics decreased for all samples however this was not significant ($P>0.05$) for Comice. At 72 h, only the increase in Red Anjou was significant ($P<0.05$) whereas, the changes in remaining cultivars were not significant.

Total phenolics present in a fermented substrate is suggested to be a results of phenolic mobilization linked to a flux between the formation/degradation of large polymeric phenolics and the degradation of small phenolics (McCue and Shetty, 2005). The degradation of small phenolics could be a possible detoxification mechanism for bacteria and yeast (McCue and Shetty, 2005).

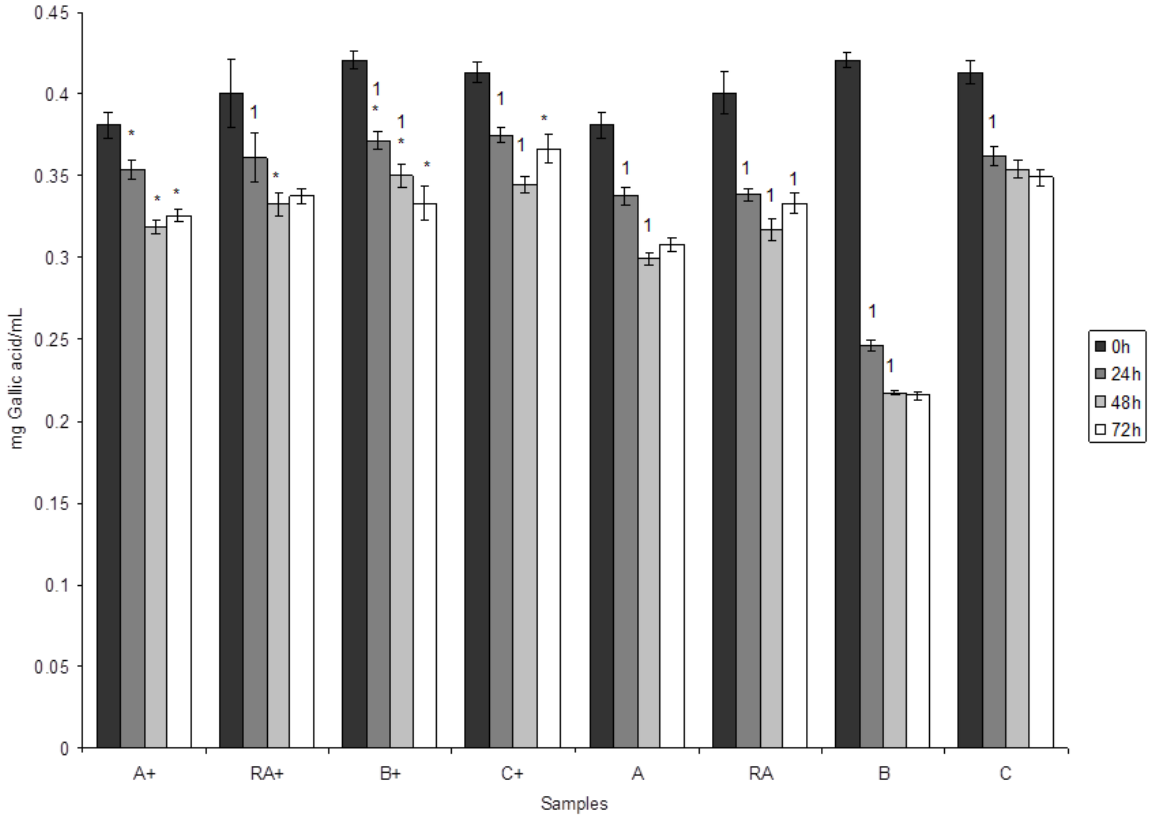

Figure 40: Total phenolic content changes in fermented pear juice at 0, 24, 48 and 72 h of fermentation.

Apostolidis *et al.*, (2007), fermented milk and soymilk with *Lactobacillus helveticus* and *Lactobacillus bulgaricus* and found a decrease in total phenolics with fermentation at 24 h. Our results are also supported by the research of Kwon, *et al.*, (2006b), where a decrease in total phenolics was observed in Kefir culture mediated soymilk fermentation. The significant increase in Red Anjou at 72 h for acidified (pH not adjusted) samples could be because of degradation of large polymeric phenolic

structures present in the sample. Further investigation of the enzymes related to these biochemical changes would be the focus of our future studies.

At all time points the pH adjusted samples had higher amount of total phenolics than the pH not adjusted samples however it was not significant ($P>0.05$) at all time points. It has been suggested that a pH of greater than 7 can cause spectral changes to the phenolic compounds and multi-ring aromatic structures are more resistant to such changes than monocyclic phenolic compounds (Friedman and Jurgens, 2000). Although the pH range tested in their research was high, these results might explain the difference in total phenolics in pH adjusted and not adjusted samples. Also this difference in pH adjusted samples and pH not adjusted samples was observed more at 48 and 72 h than at 24 h. This possibly suggests that the smaller monocyclic phenolics formed during fermentation are less resistant to the pH changes which agrees with the findings of Friedman and Jurgens, (2000). We have observed a similar decrease in total phenolic content in fermentation of apple and cherry juice (data not shown).

5.3.4.3 Antioxidant Activity by DPPH Radical Inhibition Assay

Although pear has been suggested to be classified in the group of low antioxidant fruits (Prior and Cao, 2000), in this study, DPPH-linked free radical scavenging activity was high (73-80%) for all samples at 0 h indicating whole pear juice has a good potential for free radical inhibition (Fig 41).

For pH adjusted samples, at 24 h, antioxidant activity decreased for Anjou and Comice, it remained the same for Red Anjou and it increased for Bosc, however none of these changes were significant ($P>0.05$). At 48 h, antioxidant activity increased for all

samples, however this increase was significant ($P<0.05$) only in case of Anjou. At 72 h, the antioxidant activity decreased for all samples significantly ($P<0.05$).

For samples where analysis was carried out at fermented acidic pH, the antioxidant activity increased at 24 and 48 h for all samples however this was significant ($P<0.05$) only in case of Anjou at 48 h. At 72 h, like in pH adjusted samples, the antioxidant potential decreased for all samples significantly ($P<0.05$) except in case of Comice where it was not significant ($P>0.05$).

Only in case of Anjou at 24 and 48 h, a statistically significant ($P<0.05$) difference was observed between pH adjusted and pH not adjusted samples. At 72 h, only Red Anjou had significant difference between pH adjusted and pH not adjusted samples. The structural changes caused by adjusting the pH might be responsible for the change in potential antioxidant activity.

No correlation was found between total phenolics and DPPH linked antioxidant activity for the fermented samples except in case of pH not adjusted samples of Anjou where a high inverse correlation ($r = -0.81$) was observed. An inverse relationship has been reported during soymilk fermentation with lactic acid bacteria. It could be due to mobilization and likely polymerization of phenolics (McCue and Shetty, 2005; Apostolidis *et al.,* 2007). An increase in the antioxidant activity during soymilk fermentation due to liberation of aglycone of isoflavones through catalytic action of β-glucosidase activity and also intracellular antioxidants of starter organisms has been suggested (Wang *et al.,* 2006).

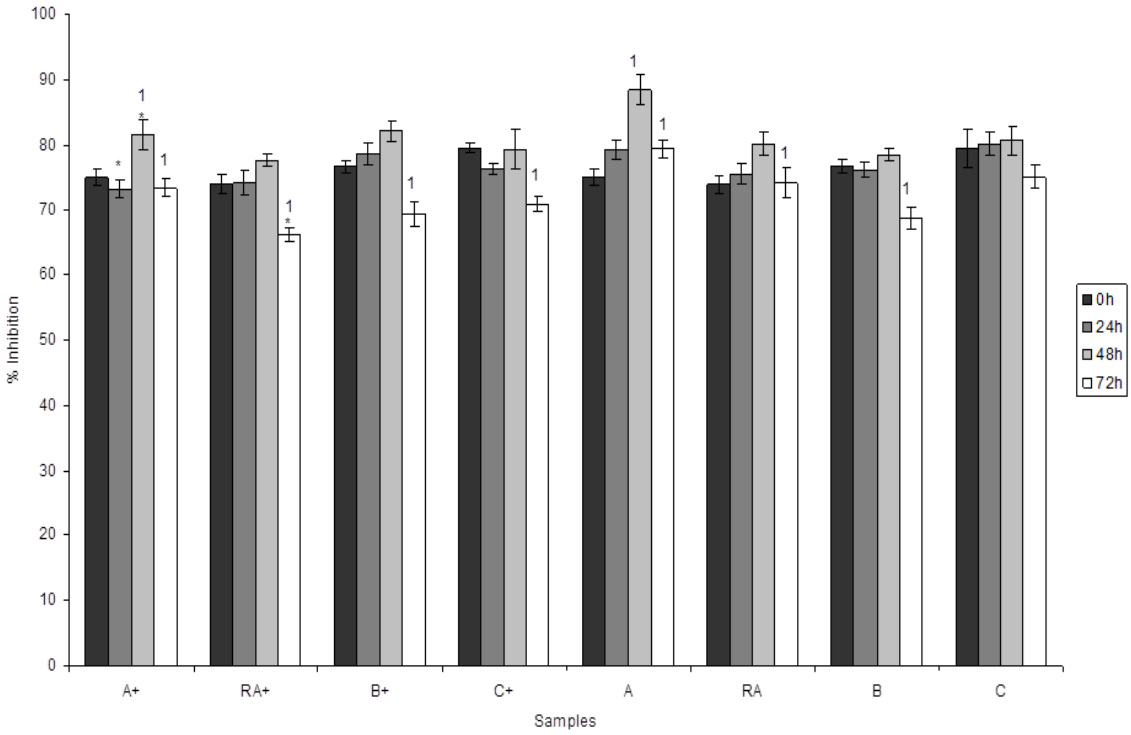

Figure 41: Changes in the DPPH scavenging activity (% Inhibition) of extracts of fermented pear juice using *Lactobacillus helveticus* at 0, 24, 48 and 72 h of fermentation.

5.3.4.4 α-Glucosidase Inhibition Assay

Moderate α-glucosidase inhibitory activity (40-60%) was observed at 0 h which indicates whole pear juice has reasonable potential to inhibit intestinal starch breakdown enzyme and reduce post-prandial rise in blood glucose.

For pH adjusted samples, at 24 h α-glucosidase inhibitory activity decreased or remained constant for all samples (Fig 42). The decrease was significant ($P<0.05$) only in case of Red Anjou. At 48 h, inhibitory activity increased for all samples significantly except in case of Anjou and Comice where the increase was not significant. At 72 h,

there was no significant change in α-glucosidase inhibitory activity of any sample. Dose dependency studies using 25 µL and 10 µL indicated a dose dependent response.

For pH not adjusted samples, after 0 h the inhibitory activity increased for all samples at 24 h (Fig 43). At 48 h, the inhibitory potential increased significantly ($P>0.05$) in case of Bosc and Comice whereas it remained constant for Anjou and Red Anjou. At 72 h, inhibition increased in case of Anjou and Red Anjou whereas it decreased slightly in case of Bosc and Comice. The changes were significant ($P<0.05$) only in case of Red Anjou. Dose dependency studies using 10 µL and 25 µL indicated a dose dependent response. Overall a similar trend was observed with different doses of the sample.

The inhibitory activity when the analysis was carried out at a fermented acidic pH was significantly higher ($P<0.05$) than the corresponding pH adjusted samples at all time points. The only exception to this was Anjou at 24 and 48 h. This suggests that fermenting whole pear juice with *Lactobacillus helveticus* can enrich the juice with compounds that can potentially help counter hyperglycemia linked to type 2 diabetes.

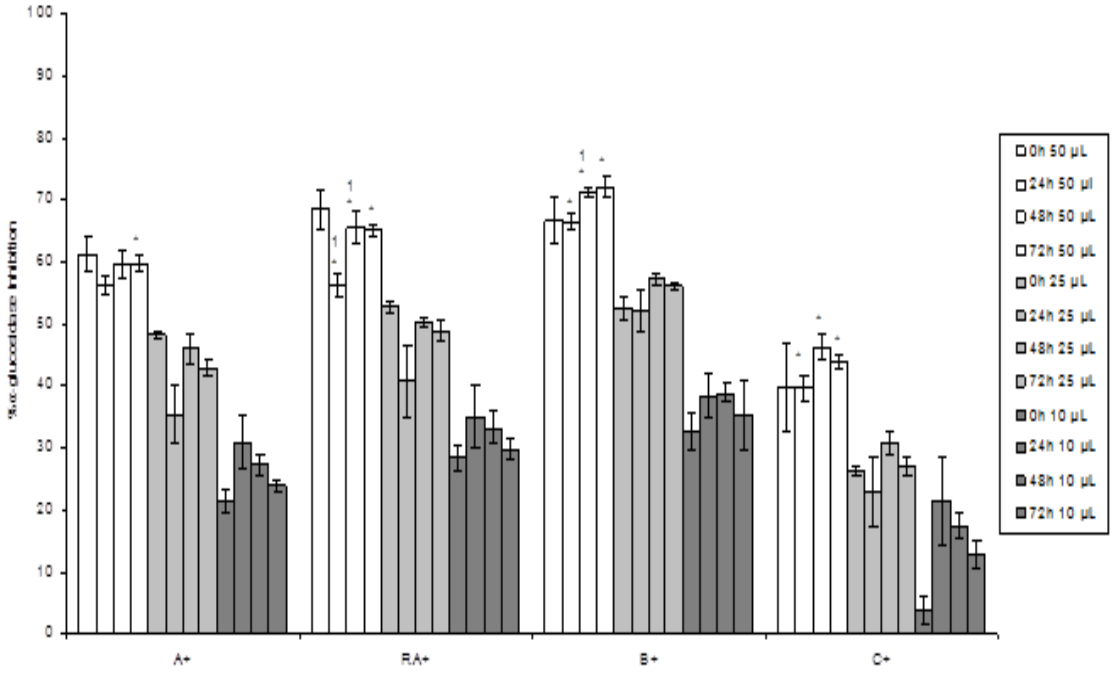

Figure 42: Dose-dependent changes in percentage α-glucosidase inhibitory activity of pH adjusted extracts of fermented pear juice at 0, 24, 48 and 72 h of fermentation.

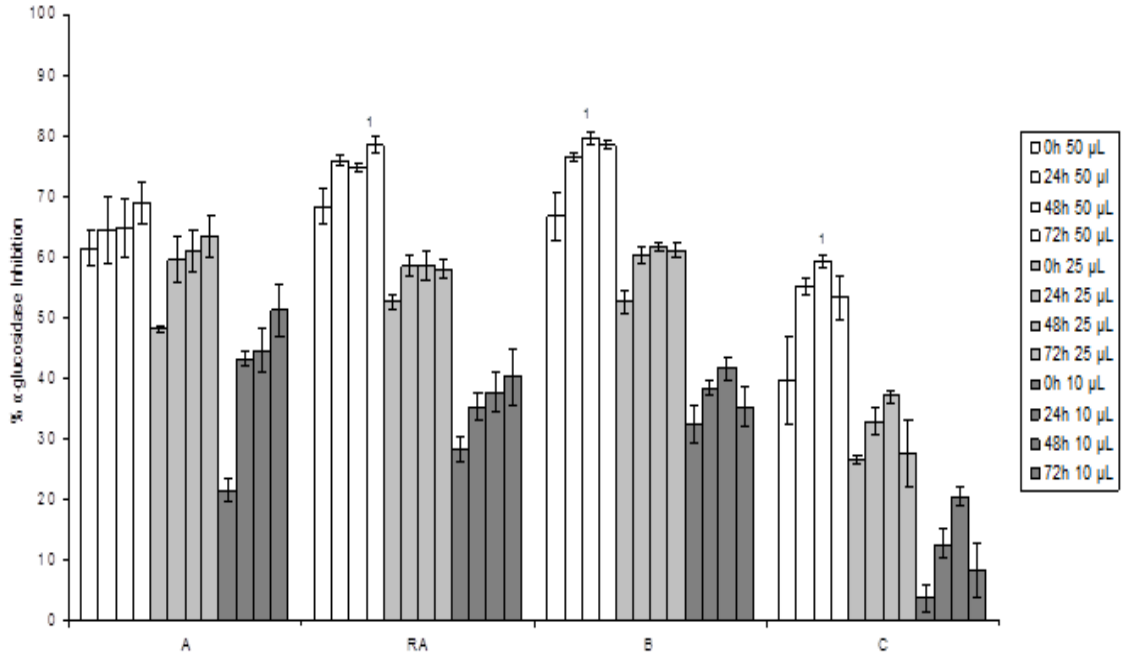

Figure 43: Dose-dependent changes in percentage α-glucosidase inhibitory activity of *Lactobacillus helveticus* fermented pear juice at fermented acidic pH at 0, 24, 48 and 72 h of fermentation.

α-Glucosidase inhibitory activity in the fermented samples seems to be a combined phenolic and pH effect. In pH adjusted samples the changes in α-glucosidase inhibitory activity at a given time point was dependent on degradation/formation of simple phenolics which have been suggested to have inhibitory activity (Apostolidis *et al.*, 2006b; Kwon *et al.*, 2006b). The degradation of simple phenolics could be a detoxification mechanism for the bacteria whereas formation could be a result of breakdown of large polymeric phenolics (McCue and Shetty, 2005). For pH adjusted

samples, a weak inverse correlation in case of Anjou and Red Anjou (-0.61, -0.75) was observed between α-glucosidase inhibitory activity and lactic acid content whereas little or no correlation was observed for Bosc and Comice (0.21, 0.61) which indicate that adjusting the pH may dramatically reduce the effect of lactic acid present in the sample on α-glucosidase inhibition. A strong inverse correlation was observed between Bosc and Comice (-0.81, -0.82) with total phenolics whereas no correlation was observed with Anjou and Red Anjou (0.16, 0.21).

For the pH not adjusted samples, the increase in the α-glucosidase inhibitory activity was more related to the pH of the sample. For all cultivars, a high correlation (r = 0.79-0.98) between lactic acid and α-glucosidase inhibition was observed. An inverse correlation was observed between total phenolics and the α-glucosidase for all cultivars (r = -0.79 - -0.99). Although the phenolic effect may decrease the inhibitory activity, the pH effect may counteract to enhance the inhibitory activity. Apostolidis *et al.*, (2007), reported an increase in α-glucosidase inhibitory activity during fermentation of milk and soymilk where pH of the extracts was not adjusted at the time of analysis.

5.3.4.5 α-Amylase Inhibition Assay

One of the means by which commercial drugs help reduce blood glucose is by inhibition of pancreatic α-amylase which catalyzes hydrolysis of starch thereby reducing the post-prandial rise in blood glucose. Because of high sugar content, the samples were diluted before the assay by 1:10 so as to get the final readings within the

range of the spectrophotometer. No α-amylase inhibition was observed with the diluted fermented extracts.

5.3.4.6 ACE Inhibition Assay

Hypertension is one of the long term complications of diabetes. Inhibition of ACE, an important enzyme in maintaining vascular tension, is considered a useful therapeutic approach in the treatment of high blood pressure (Johnston and Franz, 1992). ACE inhibition at 0 h for pear was low indicating pear juice has a mild ACE inhibition potential (Fig 44). For both pH adjusted and not adjusted samples ACE inhibitory potential decreased with fermentation. At 48 h, the potential further decreased for all samples and for Red Anjou no inhibition was observed. At 72 h, none of the samples had ACE inhibition.

For pH not adjusted samples, the inhibition potential decreased for all samples at 24 h. At 48 h, the potential further decreased for all samples except Anjou where it did not change. ACE inhibition potential further decreased at 72 h, for all samples and was very close to 0.

Lactic acid has been suggested to have significant effect on ACE inhibition. Apostolidis *et al.,* (2007), reported that lactic acid may have a synergistic effect on peptide mobilization which may have significance in ACE inhibition. This effect was more pronounced when lactic acid was present at 0.5% or 1%, whereas no effect was observed when lactic acid was present at 1.5% and 3%.

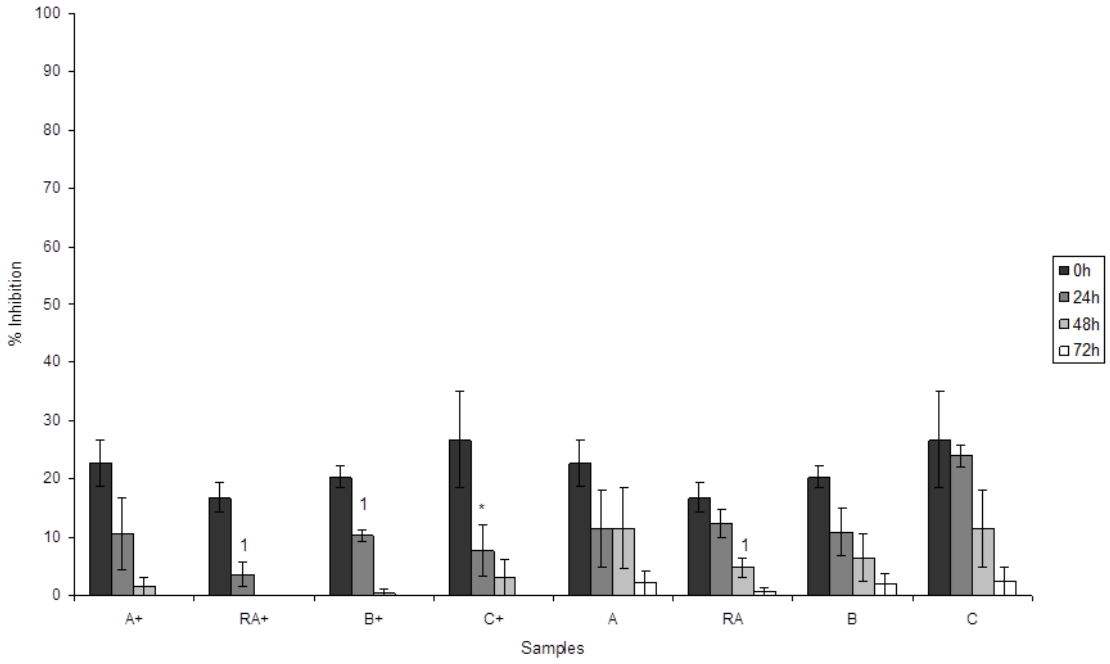

Figure 44: Changes in percentage ACE inhibitory activity (% Inhibition) of pear juice fermented using *Lactobacillus helveticus.*

Frossard *et al.,* (2000), have observed *in vivo* lactate production during administration of ACE inhibitors to humans. Total phenolics and ACE inhibition had good correlation for both pH adjusted and pH not adjusted samples (0.75-0.98). Lactic acid and ACE had a high inverse correlation (-0.62 - -0.99). The decrease in inhibition in this study seems to be more of a phenolic effect and it may be because of degradation of small phenolics during fermentation which had ACE inhibitory potential. A similar decrease in ACE inhibition was observed by Kwon *et al.,* (2006a) during Kefir

mediated soymilk fermentation. By contrast, Apostolidis *et al.*, (2007), found an increase in ACE inhibition when milk was fermented with lactobacillus.

5.3.4.7 Protein Assay

Protein content was measured using the Bradford assay (Bradford, 1976). Protein content in the samples was highest for Bosc (7.8 mg/mL) and lowest for Anjou (3.8 mg/mL) (Fig 45). For pH adjusted samples, changes in protein content during fermentation were not significant ($P>0.05$) except for Comice where it increased significantly at 72 h.

For pH not adjusted samples, protein content decreased for all samples till 48 h. at 72 h, protein content increased for all samples. Significant changes were observed in Bosc at 24 and 48 h and Red Anjou at 72 h. For all samples the pH adjusted samples were higher in protein content than the pH not adjusted ones. However, this difference was not significant ($P<0.05$) only for Red Anjou at 24 and 72 h.

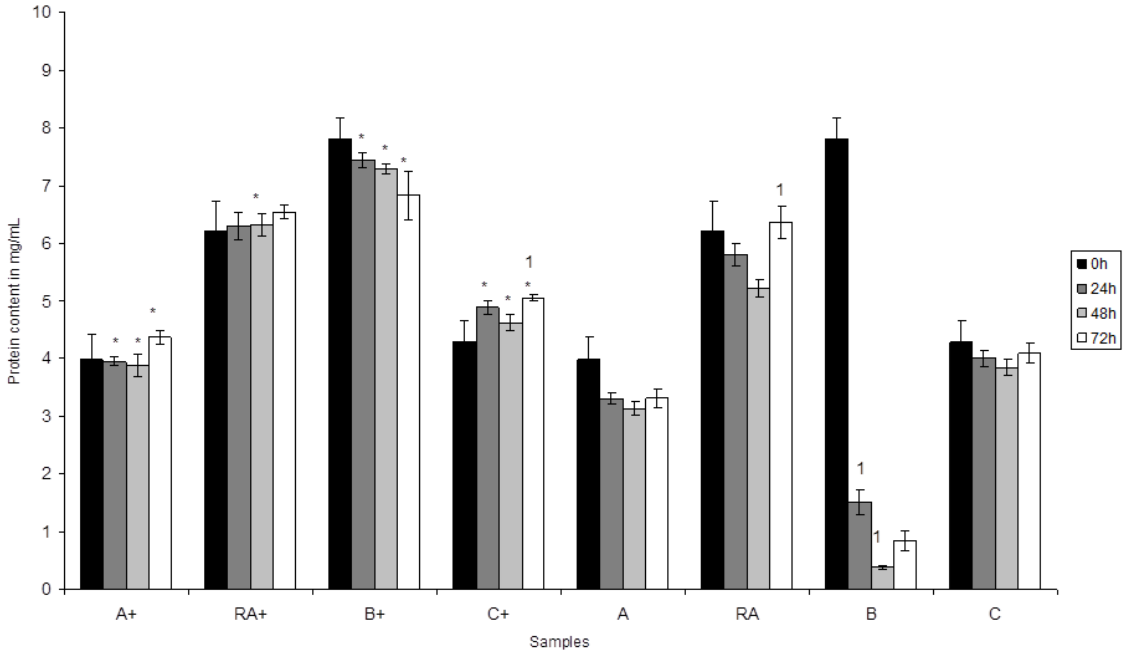

Figure 45: Effect of fermentation by *Lactobacillus helveticus* on the protein content (mg/mL) of pear juice at 0, 24, 48 and 72 h of fermentation.

The decrease in protein content could be because lactic acid bacteria depend strongly on exogenous sources of nitrogen (Yamamoto *et al.*, 1993). The secretion of nonspecific proteinases for obtaining this exogenous source of nitrogen might cause liberation of peptides causing a decrease in protein content. Also the low pH during fermentation may cause protein denaturation. This might explain the higher protein content in pH adjusted samples because of refolding of some proteins due to pH adjustment which may be better reflected in the Bradford assay.

5.3.4.8 Lactic Acid Content Measured by HPLC

Lactic acid in the sample was measured by using HPLC analysis. At 0 h some amount of lactic acid was present in the sample (Fig 46). Lactic acid content increased for both pH adjusted and pH not adjusted samples as fermentation proceeded. At 48 h, lactic acid content increased for all samples except pH adjusted sample of Red Anjou where a slight decrease was observed. At 72 h, lactic acid content increased for all samples except for pH adjusted and not adjusted samples of Bosc where a slight decrease was observed however this decrease was not significant (P>0.05).

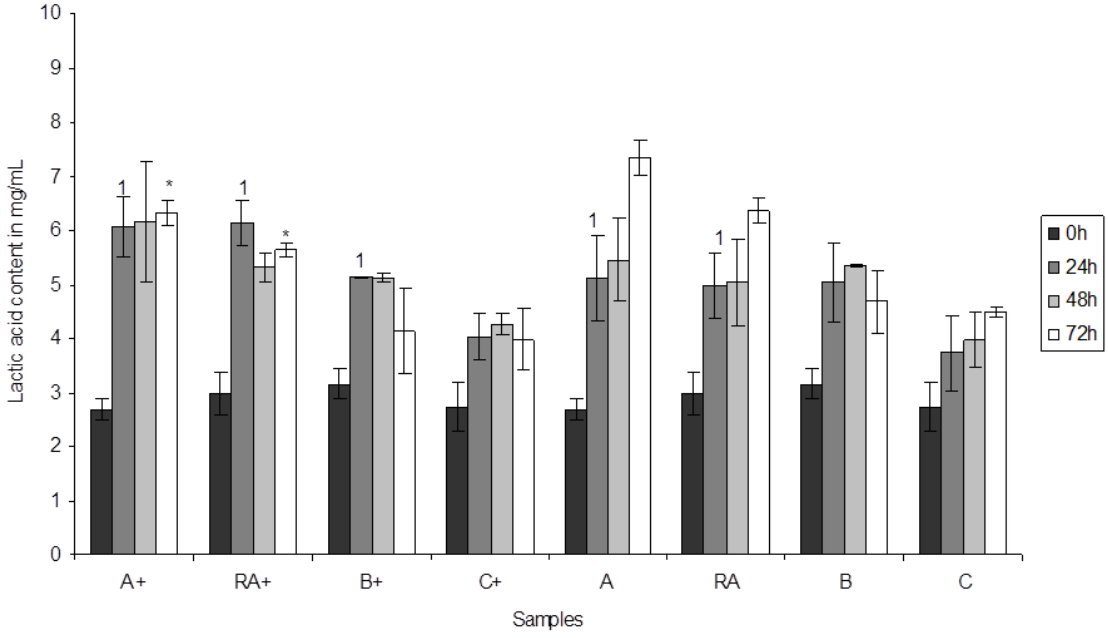

Figure 46: Lactic acid content (mg/mL) as measured by HPLC at 0, 24, 48 and 72 h of fermented pear juice extracts using *Lactobacillus helveticus*.

5.3.4.9 High Performance Liquid Chromatography (HPLC) Analysis of Phenolic Profiles

Epicatechin and quercetin derivatives were the major phenolic compounds found in fresh samples as well as the fermented extracts although the amounts changed as fermentation proceeded (Table 9, 10, 11 and 12). *p*-Coumaric acid, caffeic acid, and resveratrol were also found in some samples at certain time points. The peaks were identified taking into account the retention time and the UV absorption spectra of the corresponding standards.

Epicatechin was found in all samples at all-time points except pH not adjusted samples of Anjou at 48 and 72 h. Caffeic acid was found in pH adjusted and not adjusted samples of Anjou at 48 and 72 h. *p*-Coumaric acid was found in Anjou and Red Anjou at 0 h. Following that it was not detected in the pH not adjusted samples of Red Anjou. Similarly resveratrol was found in both pH adjusted and not adjusted samples of Anjou and Red Anjou at 0 h. At 24 h, it was found in pH adjusted samples of the same however after that it was only found in samples of Red Anjou. Quercetin derivatives were found initially at 0 h in all cultivars except Bosc. At 24 h, it was found in pH adjusted samples of Anjou, Red Anjou and Comice. For the pH not adjusted samples at 24 h, it was found in Red Anjou and Comice but not Anjou. At 48 and 72 h, it was found only in samples of Red Anjou and Comice.

Epicatechin is an antioxidant flavanol which has potent α-glucosidase inhibitory activity and mild α-amylase inhibitory potential (Tadera *et al.*, 2006; Matsui *et al.*, 2007). Some of the functional properties of the fermented juice may be attributed to the presence of epicatechin in combination with other phenolics. The increase or decrease

in a phenolic compound detected by the HPLC method used at a specific time point is determined by the flux between formation of that phenolic compound by degradation of large polymeric phenolics and complete degradation of that compound. It could also be because adjusting the pH may cause some changes to the structure of small phenolics (Friedman and Jurgens, 2000) which might make it difficult to be detected by HPLC especially when it is present in such small amounts.

Table 9: HPLC Analysis of Individual Phenolic compounds of fermented extracts of Anjou (µg/mL)

Phenolic compound	pH adjusted				pH not adjusted			
	0 h	24 h	48 h	72 h	0 h	24 h	48 h	72 h
Epicatechin	35.5 ± 6.1	17.6 ± 1.6	18.6 ± 6.7	26.1 ± 4.2	35.5 ± 6.1	17.4 ± 1.4		
Caffeic acid			0.094 ± 0.012	0.055 ± 0.001			0.075 ± 0.012	0.11 ± 0.001
p-Coumaric acid	0.037 ± 0.006	0.038 ± 0.002	0.06 ± 0.00	0.023 ± 0.006	0.037 ± 0.006	0.06 ± 0.001	0.052 ± 0.008	0.04 ± 0.004
Resveratrol	0.017 ± 0.0	0.018 ± 0.002			0.017 ± 0.0			
Quercetin derivatives	0.0706 ± 0.012	0.0332 ± 0.01			0.0706 ± 0.012			

Table 10: HPLC Analysis of Individual Phenolic compounds of fermented extracts of Red Anjou (µg/mL)

Phenolic compound	pH adjusted				pH not adjusted			
	0 h	24 h	48 h	72 h	0 h	24 h	48 h	72 h
Epicatechin	25.5 ± 2.4	21.3 ± 3.5	14.9 ± 2.7	18.1 ± 0.09	25.5 ± 2.4	19.5 ± 1.5	23.7 ± 1.6	15.2 ± 2.1
p-Coumaric acid	0.036 ± 0.006	0.022 ± 0.001	0.042 ± 0.006	0.044 ± 0.00	0.036 ± 0.006			
Resveratrol	0.01 ± 0.001	0.008 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.01 ± 0.001		0.011 ± 0.00	0.014 ± 0.00
Quercetin derivatives	0.012 ± 0.00	0.011 ± 0.002	0.011 ± 0.00	0.012 ± 0.00	0.012 ± 0.00	0.012 ± 0.004	0.01 ± 0.00	0.014 ± 0.001

Table 11: HPLC Analysis of Individual Phenolic compounds of fermented extracts of Bosc (µg/mL)

Phenolic compound	pH adjusted				pH not adjusted			
	0 h	24 h	48 h	72 h	0 h	24 h	48 h	72 h
Epicatechin	31.7 ± 8.6	19.2 ± 1.4	24.7 ± 4.4	15.9 ± 1.8	31.7 ± 8.6	21.5 ± 4.5	27.5 ± 1.2	46.6 ± 12.5

Table 12: HPLC Analysis of Individual Phenolic compounds of fermented extracts of Comice (µg/mL)

Phenolic compound	Comice pH adjusted				pH not adjusted			
	0 h	24 h	48 h	72 h	0 h	24 h	48 h	72 h
Epicatechin	31.6 ± 8.6	26.2 ± 2.1	23.9 ± 2.5	25.3 ± 2.9	31.6 ± 8.6	21.3 ± 3.9	20.1 ± 3.1	31.2 ± 5.1
Quercetin derivatives	0.078 ± 0.008	0.053 ± 0.003	0.05 ± 0.003	0.03 ± 0.00	0.078 ± 0.008	0.075 ± 0.009	0.087 ± 0.019	0.05 ± 0.002

5.3.4.10 Conclusion

Diabetes and other chronic oxidation linked diseases are an emerging challenge globally and fruits have been shown to have good potential in managing oxidation linked diseases. In this study, we fermented pear juice and changes in functionality related to total phenolics, antioxidant potential, key enzymes in carbohydrate metabolism and hypertension linked ACE were evaluated. Total phenolics decreased with fermentation and DPPH linked antioxidant activity increased till 48 h and then it decreased for most samples at 72 h. α-Glucosidase inhibition did not change significantly for pH adjusted samples whereas for pH not adjusted samples there was a significant increase in inhibition for most samples. ACE inhibition potential decreased with fermentation for all samples. This suggests that fermentation of pear is a good strategy to enhance antioxidant potential and inhibition against α-glucosidase to reduce post-prandial rise in blood glucose.

CHAPTER 6

STIMULATION OF BIOACTIVES IN RESPONSE TO ELICITORS

6.1 Stimulation of Total Phenolics and Antioxidant Enzymes in Fava Bean (*Vicia faba*) in Response to Phytochemical Elicitors

6.1.1 Abstract

Improvement in the health relevant functionality of fava bean (*Vicia faba)* was investigated by priming seeds with phytochemical elicitors. Activities of the key enzymes of the pentose phosphate pathway, tricarboxylic acid cycle, antioxidant enzymes as well as metabolic regulation by proline oxidation via Proline dehydrogenase were evaluated. Although phytochemical elicitation resulted in higher phenolic (day 13, 17) and L-DOPA (day 13, 20) content in seedlings when compared to control, the difference was not significant. Glucose-6-phosphate dehydrgenase and succinate dehydrogenase activity were higher in elicitor treatments (day 13 and 20) and the activity was significantly higher on day 20 when compared to the control. Elicitor treatments stimulated superoxide dismutase and catalse response however mixed trends were observed with phenolic polymerizing guiacol peroxidase activity. Proline content correlated with glucose-6-phosphate dehydrgenase activity in all except one treatment indicating proline synthesis may be coupled to pentose phosphate pathway stimulation via NADPH/$NADP^+$ coupled redox balance. Induction of a stress like state by phytochemical elicitors may be responsible for stimulating phenolic stimulation and antioxidant enzyme response indicating a better defense response for countering stress from the elicitor treatments.

6.1.2 Introduction

Recent research has highlighted the potential of phenolic phytochemicals, identified by a hydroxyl group attached to at least one aromatic ring, as an important source of antioxidants in the diet. The health beneficial effects of phenolic compounds are attributed, but not limited to its antioxidant potential. They can act as substrates for chemical reactions, they inhibit or promote certain chemical reactions, can inhibit pathogens or influence proliferation of beneficial gut bacteria and scavenge toxic compounds in the intestine (Dillard and German, 2000). Phenolic biosynthesis is dependent on a network of routes based on closely regulated steps of the anabolic pathways providing carbon precursors and the biosynthetic pathways involved in formation of the aromatic ring and modifications thereof to produce a range of phenolic compounds (Crozier, et al., 2009). Anabolic pathways include the glycolytic pathway providing phosphenolpyruvate and the pentose phosphate pathway providing carbon precursor in the form of erythrose-4-phosphate whereas the biosynthetic pathway includes shikimate pathway involved in formation of phenylalanine which is the starting compound for all phenolic phytochemicals. Phenylalanine is then modified through the phenylpropanoid and the flavonoid pathway to produce different health relevant phenolic compounds commonly consumed in the diet (Schijlen et al., 2004; Crozier et al., 2009; Randhir et al., 2009).

Parkinson's disease (PD), a motor neurodegenerative disorder, is characterized by deficits of dopamine and acetylcholine caused by breakdown of the dopaminergic and cholinergic system (Calabresi et al., 2006). Of the few dietary sources available,

fava bean also known as the broad bean, is one of the richest sources of L-DOPA (Levo dihydroxy phenylalanine). L-DOPA is converted to dopamine in the cytosol by an aromatic amino acid decarboxylase (Elsworth and Roth, 1997). Fava bean due to its high L-DOPA content and antioxidant potential to counter ROS, also implicated in the dopaminergic and cholinergic system breakdown (Jenner, 2003), presents a low Cost, diet based solution to manage PD. Further increase in intra-renal concentration of dopamine has shown an increase in urinary sodium excretion which may have implications in treating hypertension, heart failure, renal failure and liver cirrhosis (Vered et al., 1997). Animal studies have shown the potential of fava beans in preventing experimentally induced convulsions by blocking glycine receptors in the brain (Salih and Mustafa, 2008).

Priming is a process where plants are sensitized for a stronger activation of defense responses (Conrath et al, 2006). Seed priming with phenolic elicitors mimics a stress like state and induces phenolic secondary metabolite synthesis that have diverse physiological functions in stress response (Dixon and Paiva, 1995; Winkel-Shirley, 2002; Randhir, et al., 2009; Shetty and Wahlqvist, 2004). Copanogulu (2010), presents an excellent review discussing the potential of priming in improving the quality of food crops.

Oxidative damage to DNA, protein and other macromolecules are associated with degenerative diseases of aging that include cancer, CVD, immune system breakdown, and brain dysfunction (Ames et al., 1993a). Aerobic respiration can constantly produce reactive oxygen species (ROS) such as superoxide radical, hydrogen peroxide and hydroxyl radicals (Shigenaga et al., 1994). As the balance between

oxidant and antioxidant changes, cellular antioxidants such as tocopherols, ascorbate, glutathione and antioxidant enzymes such as superoxide dismutase, catalase and glutathione peroxidase are induced to maintain the cellular antioxidant homeostasis (Foyer and Noctor, 2005). Reducing equivalents in the form of NADH and $FADH_2$ is needed to regenerate these antioxidant defense systems in order to maintain efficiency (Mates and Sanchez-Jimenez, 1999; Mates, Perez-Gomez and De Castro, 1999; Nordberg and Arner, 2001). Proline, a nitrogen containing imino acid, acts as an osmolyte and a ROS scavenger, and is produced under different kinds of stresses (Matysik, 2002). Proline synthesis under stress takes place by reducing pyroline-5-carboxylate (P5C) that requires NADPH leading to the accumulation of $NADP^+$ (Verbruggen and Hermans, 2008). $NADP^+$ is a co-factor for the enzyme glucose-6-phosphate dehydrogenase which catalyzes the rate limiting step of the pentose phosphate pathway (PPP) (Kruger and von Schaewen, 2003). Using this rationale, an alternate model for proline metabolism was proposed where proline biosynthesis is linked to stimulation of Pentose Phosphate Pathway (PPP) through $NADP^+$/NADPH redox balance (Shetty and Wahlqvist, 2004). Precursors can then be provided through this stimulated PPP for driving anabolic reactions including phenolic and nucleotide synthesis and proline synthesized can be transported to the mitochondria to provide reducing equivalents for oxidative phosphorylation, serving as an alternate non NADH route to ATP synthesis for rapid recovery from stress (Hare and Cress, 1997; Shetty and Wahlqvit, 2004).

Use of natural peptide and phytochemical elicitors has been described in stimulating phenolic biosynthesis and antioxidant enzyme response in different bean

sprouts (Randhir and Shetty, 2005; Randhir et al., 2004). Using this rationale, we investigated the effects of different natural phenolic based phyto-elicitors in metabolic regulation during initial growth phase of germinating fava bean and its influence on stimulating phenolic biosynthesis and antioxidant enzyme response.

6.1.3 Materials and Methods

6.1.3.1 Elicitor, Treatment and Germination

Five elicitors and control were used in this study. Elicitors, their source, composition and dose used are described in Table 13. Dry seeds of fava bean (*Vicia faba*) were obtained from Casablanca Halal Market, Hadley, MA. For each treatment, 20 dry seeds were soaked in 200 mL distilled water in a 250 mL Erlenmeyer flask for 24 h. The pre-soaked seeds were then germinated in sterilized potting soil at 20 ^{o}C for 21 days.

Table 13: Elicitor Treament used in priming Fava bean

Elicitor	Source	Description	Concentration
Chitosan Oligosachharide (COS)	Kong Poong Bio (Jeju, South Korea)	Soluble Chitin from crab shell	1000 ppm
Nutricran	Decas Cranberry Products Inc (Carver, MA)	Soluble juice parts from cranberry	1000 ppm
Gropro	Icelandic Bioenhancer (Harrison, NY)	Marine peptide extracts from seaweed and fish byproducts with minimum of 1% glutamic acid and proline and at least 0.1% total phenolics	1 ml/L
Origanox	Barrington Nutritionals (Harrison , NY)	Dried oregano powder	1000 ppm

6.1.3.2 Leaf Sample Extraction

Two hundred mg of leaf sample was taken on day 13, 17, and 20 for analysis. Two hundred mg leaf samples were collected from at least 3 different germinating seeds. A cold pestle and mortar was used to thoroughly grind 200 mg of the leaf tissue in cold enzyme extraction buffer (0.5 % polyvinylpyrrolidone (PVP), 3 mM EDTA. 0.1 M potassium phosphate buffer of pH 7.5). The sample was centrifuged at 12000 x g for 15 min at 2-5 ºC and stored on ice. The supernatant was used for further analysis.

For total phenolic and ABTS assay 50 mg of the leaf tissue was immersed in 2.5 mL of 95% ethanol and kept in the freezer for 48-72 h. Samples were homogenized using a tissue tearor (Biospec Products, Bartleville, OK) and centrifuged at 12000 x g for 10 min. Supernatant was used for further analysis.

6.1.3.3 Total Protein Assay

Protein content was measured by the method of Bradford assay (Bradford, 1976). The dye reagent concentrate (Bio-Rad protein assay kit II, Bio-Rad Laboratory, Hercules, CA) was diluted 1:4 with distilled water. A volume of 5 mL of diluted dye reagent was added to 50 µL of the leaf tissue extract. After vortexing and incubating for 3 min, the absorbance was measured at 595 nm against a 5 mL reagent blank and 50 µL buffer solutions using a UV-VIS Genesys spectrophotometer (Milton Roy, Inc., Rochester, NY).

6.1.3.4 Glucose-6-Phosphate Dehydrogenase (G6PDH) Assay

A modified version of the assay described by Deutsch (1983) was followed. The enzyme reaction mixture containing 5.88 µmol ß-NADP, 88.5 µmol $MgCl_2$, 53.7 µmol glucose-6-phosphate, and 0.77 mmol maleimide was prepared. This mixture was used to obtain baseline (zero) of the spectrophotometer reading at 340 nm wavelength. To 1 mL of this mixture, 100 µL of the enzyme sample was added. The rate of change in absorbance per minute was used to quantify the enzyme in the mixture using the extinction co-efficient of NADPH (6.22 $mM^{-1}cm^{-1}$).

6.1.3.5 Succinate Dehydrogenase (SDH) Assay

Modified method described by Bregman (1987) was used to assay the activity of succinate dehydrogenase. The assay mixture consisted of the following: 1.01 mL of 0.4 M potassium phosphate buffer (pH 7.2); 40 µL of 0.15 M sodium succinate (pH 7.0); 40 µL of 0.2 M sodium azide; and 10 µL of 6.0 mg/mL DCPIP. This mixture was used to obtain baseline (zero) of the spectrophotometer reading at 600 nm wavelength. To 1.0 mL of this mixture, 200 µL of the enzyme sample was added. The rate of change in absorbance per minute was used to quantify the enzyme in the mixture using the extinction co-efficient of DCPIP (19.1 $mM^{-1}cm^{-1}$).

6.1.3.6 Superoxide Dismutase (SOD) Assay

A competitive inhibition assay was performed that used xanthine-xanthine oxidase-generated superoxide to reduce nitroblue tetrazolium (NBT) to blue formazan. Spectrophotometric assay of SOD activity was carried out by monitoring the reduction of NBT at 560 nm (Oberley and Spitz, 1985). The reaction mixture contained 13.8 mL of 50 mM potassium phosphate buffer (pH 7.8) containing 1.33 mM DETAPAC; 0.5 mL of 2.45 mM NBT; 1.7 mL of 1.8 mM xanthine and 40 IU/mL catalase. To 0.8 mL of reagent mixture 100 μL of phosphate buffer and 100 μL of xanthine oxidase was added. The change in absorbance at 560 nm was measured every 20 sec for 2 min and the concentration of Xanthine oxidase was adjusted to obtain a linear curve with a slope of 0.025 absorbance per min. The phosphate buffer was then replaced by the enzyme sample and the change in absorbance was monitored every 20 sec for 2 min. One unit of SOD was defined as the amount of protein that inhibits NBT reduction to 50% of the maximum.

6.1.3.7 Catalase (CAT) Assay

A method originally described by Beers and Sizer, (1952), was used to assay the activity of catalase. To 1.9 mL of distilled water 1 mL of 0.059 M hydrogen peroxide (Merck's Superoxol or equivalent grade) in 0.05 M potassium phosphate, pH 7.0 was added. This mixture was incubated in a spectrophotometer for 4-5 min to achieve temperature equilibration and to establish blank rate. To this mixture 0.1 mL of diluted enzyme sample was added and the disappearance of peroxide was followed

spectrophotometrically by recording the decrease in absorbance at 240 nm for 2-3 min. The change in absorbance ΔA_{240}/min from the initial (45 sec) linear portion of the curve was calculated. One unit of catalase activity was defined as amount that decomposes one micromole of H_2O_2

$$\text{Units /mg} = \frac{\Delta A_{240}/\text{min} \times 1000}{43.6 \times mg\ enzyme/mL\ of\ reaction\ mixture}$$

6.1.3.8 Guaiacol Peroxidase (GPX) Assay

Modified version of assay developed by Laloue et al., (1997), was used. Briefly, the enzyme reaction mixture contained 0.1 M potassium phosphate buffer (pH 6.8), 56 mM guaiacol solution and 0.2 mM hydrogen peroxide. To 1 mL of this reaction mixture, 50 µL of enzyme sample was added. The absorbance was noted at zero time and then after 5 min. The rate of change in absorbance per minute was used to quantify the enzyme in the mixture using the extinction co-efficient of the oxidized product tetraguaiacol (26.6 $mM^{-1}cm^{-1}$).

6.1.3.9 Total Soluble Phenolics Assay

The total phenolic content in grass leaves was analyzed by the Folin-Ciocalteu method (Shetty et al., 1995). One milliliter of supernatant was transferred into a test tube and mixed with 1 mL of 95 % ethanol and 5 mL of distilled water. To each sample 0.5 mL of 50 % (v/v) Folin-Ciocalteu reagent was added and mixed. After 5 min, 1 mL of 5 % Na_2CO_3 was added to the reaction mixture and allowed to stand for 60 min. The

absorbance was read at 725 nm. The absorbance values were converted to total phenolics and were expressed in milligrams equivalents of gallic acid per grams Fresh weight (FW) of the sample. Standard curves were established using various concentrations of gallic acid in 95 % ethanol.

6.1.3.10 ABTS Cation Radical and Antioxidant Activity Assay

The total antioxidant activity of fava bean leaf extract was measured by the $ABTS^+$ radical cation-decolorization assay involving preformed $ABTS^+$ radical cation (Re et al., 1999). ABTS [2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)] (Sigma Chemical Co.St. Louis, MO) was dissolved in water to a 7 mM concentration. $ABTS^+$ radical cation was prepared by reacting 5 mL of 7 mM ABTS stock solution with 88 µL of 140 mM potassium persulphate, and mixture was allowed to stand in the dark at room temperature for 12-16 h before use. Prior to assay $ABTS^+$ stock solution was diluted with 95% ethanol (ratio 1:88) to give an absorbance at 734 nm of 0.70 ± 0.02, and was equilibrated to 30 °C . One milliliter ABTS was added to glass test tubes containing 50 µL of each tissue extract, and mixed by vortex mixer for 30 s. After 2.5 min incubation in the dark, mixtures were read at 734 nm. The readings were compared with controls, which contained 50 µL of 95% ethanol instead of the extract. The Trolox reference standard for relative antioxidant activities was prepared with 5 mM stock solution of Trolox in ethanol for introduction into the assay system at concentrations within the activity range of the assay (0-20 µM final concentration) for preparing a standard curve to which all data were referenced. The percent inhibition was calculated by:

$$\% \text{ inhibition} = \left(\frac{[A_{734}^{control} - A_{734}^{extract}]}{[A_{734}^{control}]}\right) \times 100$$

6.1.3.11 HPLC Analysis of Proline

High performance liquid chromatography (HPLC) analysis was performed using an Agilent 1100 liquid chromatograph equipped with a diode array detector (DAD 1100). The analytical column was reverse phase Nucleosil C18, 250 nm x 4.6 mm with a packing material of 5 µm particle size. The extract samples were eluted out in an isocratic manner with a mobile phase consisting of 20 mM potassium phosphate (pH 2.5 by phosphoric acid) at a flow rate of 1 mL min^{-1} and detected at 210 nm. L-Proline (Sigma chemicals, St. Louis, MO) dissolved in the 20 mM potassium phosphate solution was used to calibrate the standard curve. The amount of proline in the sample was calculated as mg of proline per milliliter and converted and reported as mg g^{-1} FW.

6.1.3.12 HPLC Analysis of L-DOPA

High performance liquid chromatography (HPLC) analysis was performed using an Agilent 1100 liquid chromatograph equipped with a diode array detector (DAD 1100). The analytical column was a reverse phase Supleco Discovery C18, 250 mm × 4.6 mm with a packing material of 5 µm particle size. The extract samples were eluted out in an isocratic manner with a mobile phase composition of 18% methanol and 82% buffer consisting of 0.01M ammonium acetate at pH 5.4, at a flow rate of 1 mL/min.

The retention time and spectrum of sample was compared with that of standard L-DOPA (Sigma). The amount of L-DOPA was determined from the area obtained at 280 nm as compared to the pure standard of known concentration. The amount of L-DOPA in the sample was calculated as mg of L-DOPA per milliliter and converted and reported as mg g^{-1} FW.

6.1.3.13 Statistical Analysis

All experiments were performed with six replications with a minimum of four replications for each treatment. Samples were collected from at least three different germinating seeds. The effect of each treatment was determined by the analysis of variance (ANOVA) of SAS (version 8.2; SAS Institute, Cary, NC). Differences among different treatments were determined by the Fishers least significant difference (LSD) test at the 0.05 probability level. Standard error was calculated using Microsoft Excel 2010.

6.1.4 Results and Discussion

6.1.4.1 Changes in Total Soluble Phenolics, Free Radical-linked Antioxidant Activity and L-DOPA content of Fava Bean Leaf

Total soluble phenolics was assayed using the Folin-ciocalteau method and was measured as gallic acid equivalents (Fig 47). On day 13, phenolic content were similar for all samples (13.5-15.2 mg/g). On day 17, phenolic content increased in Gropro (17.6%), decreased in Origanox (17.7%) and COS (6.9%), and remained the same for the Nutricran, Craninsulin and water. On day 20, there was a slight increase in phenolic content for COS (2.7%) and Origanox (3.4%) treatments whereas it decreased for

Nutricran (21%), GroPro (23.5%), Craninsulin (16.7%) and water (3.6%) treatments. Highest phenolic content was found in GroPro treatment on day 17 (17.3 mg/g) and lowest was found in response to Origanox (11.5 mg/g) treatment on the same day.

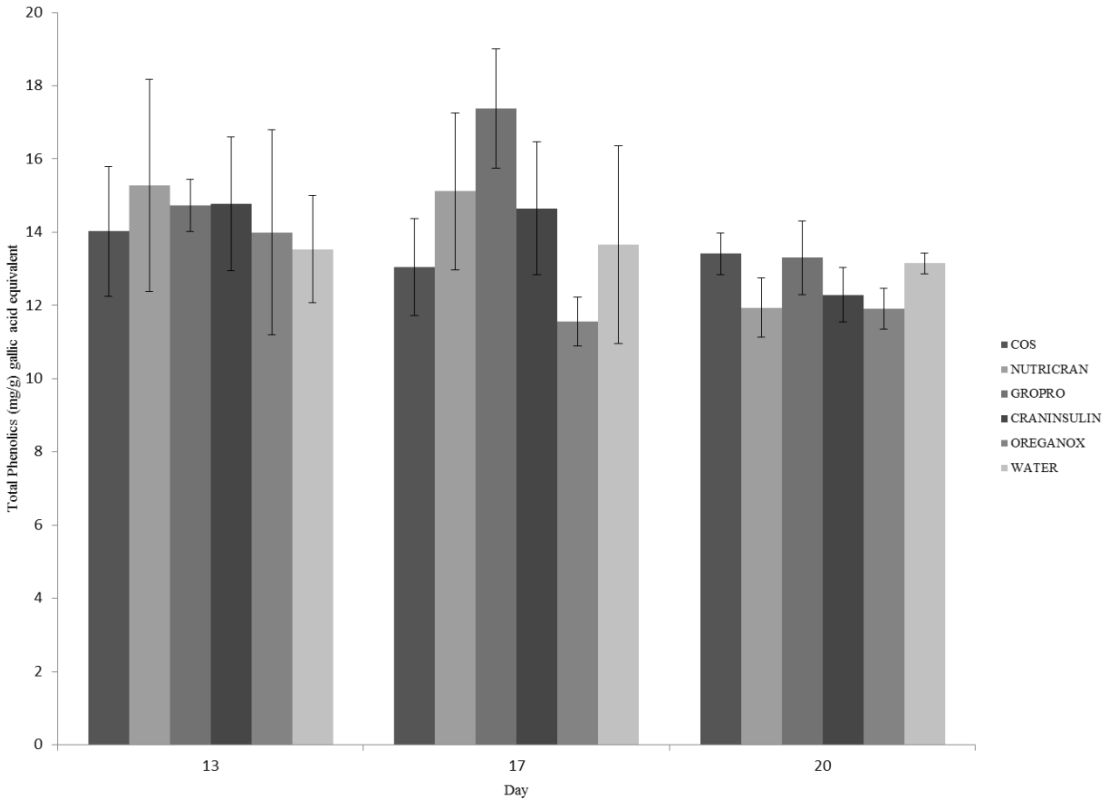

Figure 47: Effect of priming on Total soluble phenolic content (mg g^{-1} FW) of fava bean leaf tissue (13, 17, and 20 d).

For all treatments the baseline value for total antioxidant activity (ABTS assay) was high (96-98% inhibition) in fava bean leaves over the growth phase (day 13, 17, 20) (Fig 48). Total antioxidant activity linked to free radical scavenging for elicitor treatments were not significantly different ($P>0.05$) from control treatment. Antioxidant

activity in fava bean leaves did not change for treatment or control during the growth phase (day 13, 17, 20).

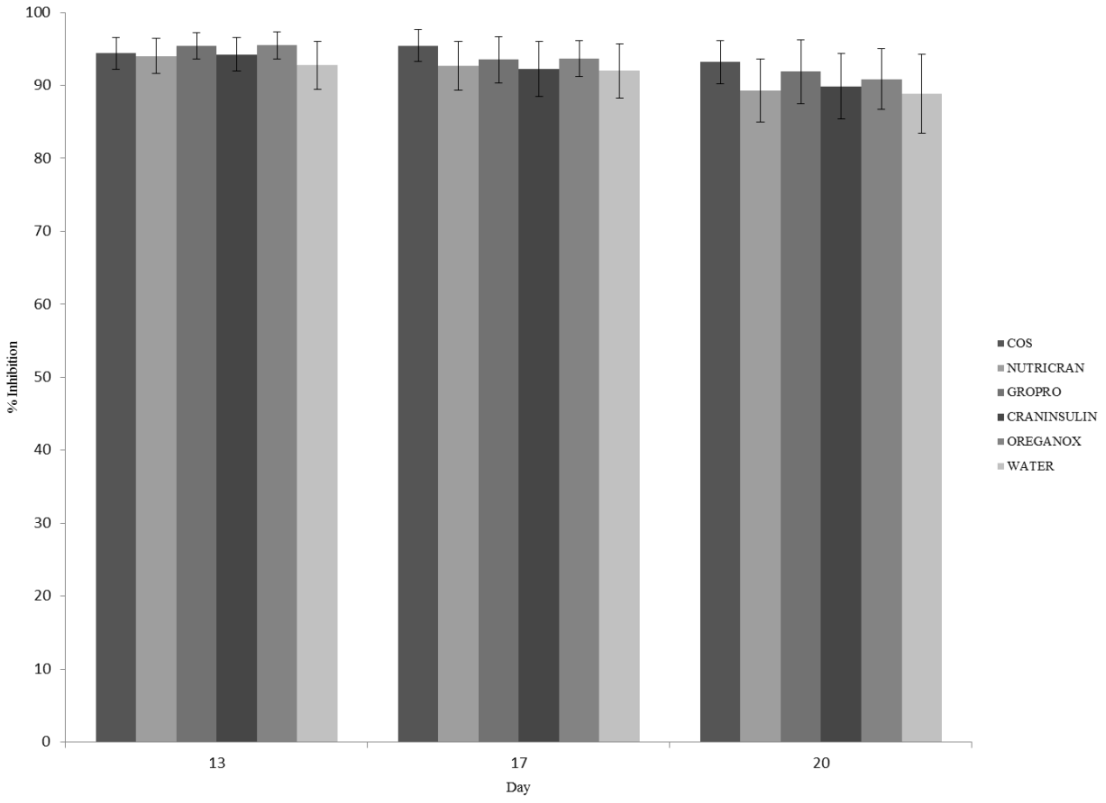

Figure 48: Effect of priming on Total antioxidant activity (ABTS%) of fava bean leaf tissue (13, 17, and 20 d).

L-DOPA content was determined using High Performance Liquid Chromatography (Fig 49). On day 13, highest L-DOPA content was found in Nutricran (12.3 mg/g) treatment whereas lowest was found in Origanox (4.7 mg/g) treatment. On day 17, L-DOPA content decreased sharply for Nutricran (31.7%) treatment, GroPro (37%) treatment, Craninsulin (47.4%) treatment , decreased for COS (17.3%) treatment and water (10.3%) whereas it increased sharply for Origanox (53.1%). On day 20, L-

DOPA content decreased for all treatments (27-63.8%). Highest L-DOPA content was found in Nutricran (12.3 mg/g) treatment on day 13 whereas lowest was found in Origanox (2.6 mg/g) on day 20.

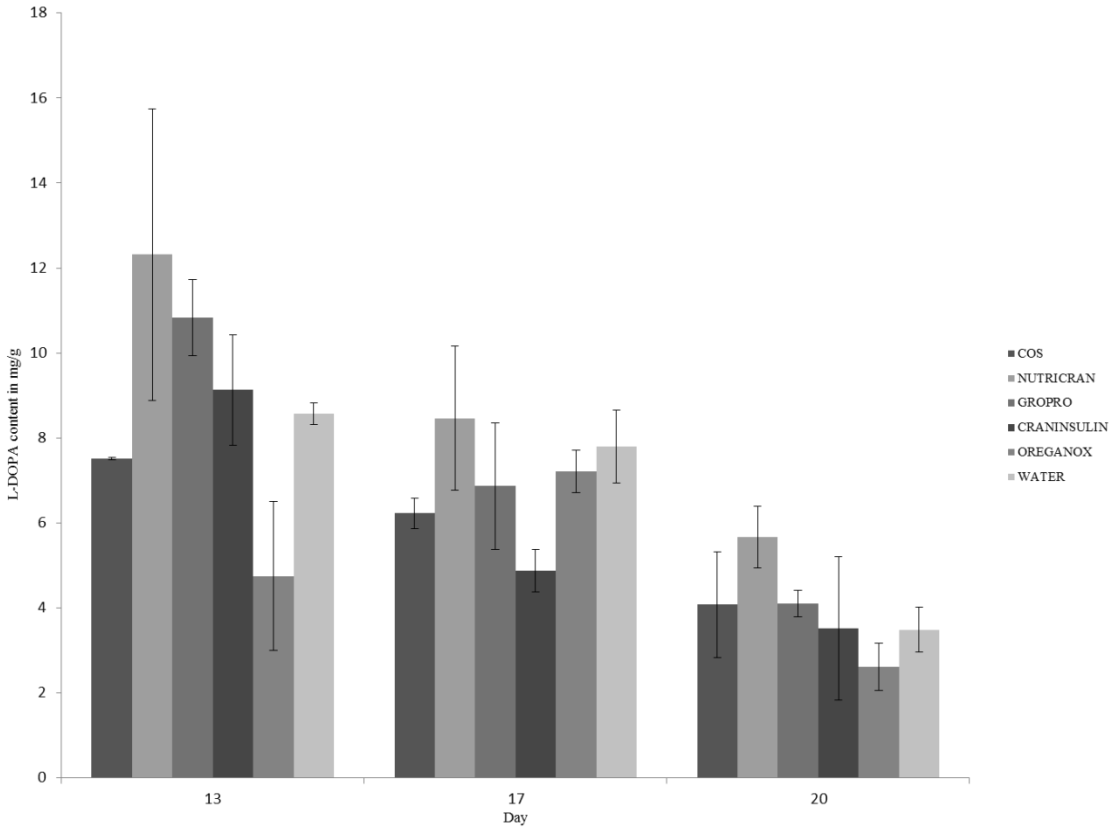

Figure 49: Effect of priming on L-DOPA content (mg g^{-1} FW) of fava bean leaf tissue (13, 17, and 20 d).

6.1.4.2 Glucose-6-Phosphate Dehydrogenase (G6PDH) and Succinate Dehydrogenase (SDH) Activity in Fava Bean Leaves

G6PDH activity decreased over the initial growth phase (day 13, 17, 20) for all treatments (Fig. 50). On day 13, Origanox (136.1 nmol/min/mg protein) treatment had

the highest G6PDH activity whereas Craninsulin (110.7 nmol/min/mg protein) treatment had the lowest activity. However there was no significant difference in G6PDH activity among different treatments. On day 17, the G6PDH activity decreased for all treatments and all the elicitor treatments had slightly lower activity (96.7-104.1 nmol/min/mg protein) as compared to control (108.6 nmol/min/mg protein). On day 20, G6PDH activity slightly decreased for all elicitor treatments (3.6-11.9%) whereas it decreased sharply for the control (34.8%).

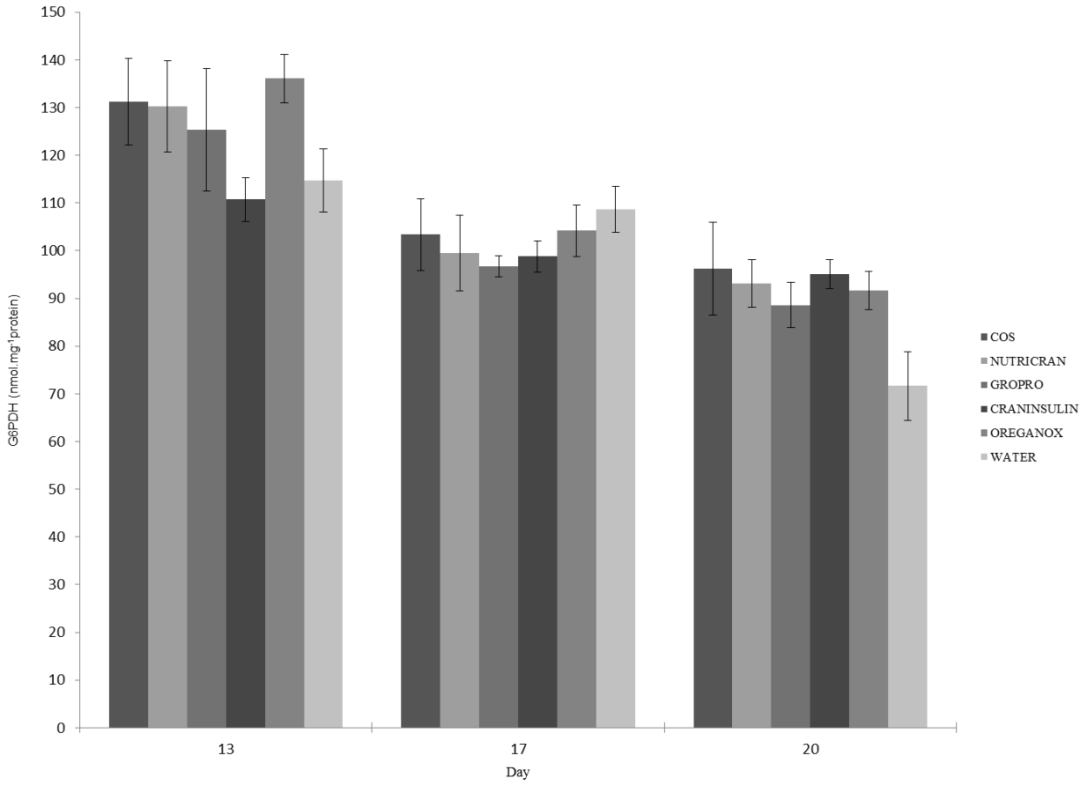

Figure 50: Effect of priming on Glucose-6-phosphate dehydrogenase activity (nmol mg^{-1} protein) of fava beans leaf tissue (13, 17, and 20 d).

In order to investigate the effect of the elicitor treatment in modulating the Tricarboxylic Acid Cycle (TCA), the activity of the key enzyme SDH was assayed (Fig

51). On day 13, higher SDH activity was found in COS (3.4 nmol/min/mg protein), Nutricran (3.8 nmol/min/mg protein) and Origanox (3.4 nmol/min/mg protein) treatments as compared to GroPro (2.9 nmol/min/mg protein), Craninsulin (2.6 nmol/min/mg protein) and control (2.6 nmol/min/mg protein) treatments. On day 17, SDH activity decreased in COS (22.6%), Nutricran (26.35), GroPro (10.3%) and Origanox (14.7%) treatments whereas it increased slightly in Craninsulin (8.9%) and water (1.8%) treatments. On day 20, SDH activity decreased for COS (8.1%), Nutricran (7.1%), GroPro (3.8%) and water (27%) treatments whereas it increased for Cranisnulin (6.5%) and Origanox (13.1%) treatments. On day 20, Origanox treatment had significantly higher ($P<0.05$) SDH activity than COS, GroPro and water treatments. Highest SDH activity was found in Nutricran treatment on day 13 (3.8 nmol/min/mg protein) whereas lowest was found in water on day 20 (1.9 3.8 nmol/min/mg protein) treatment.

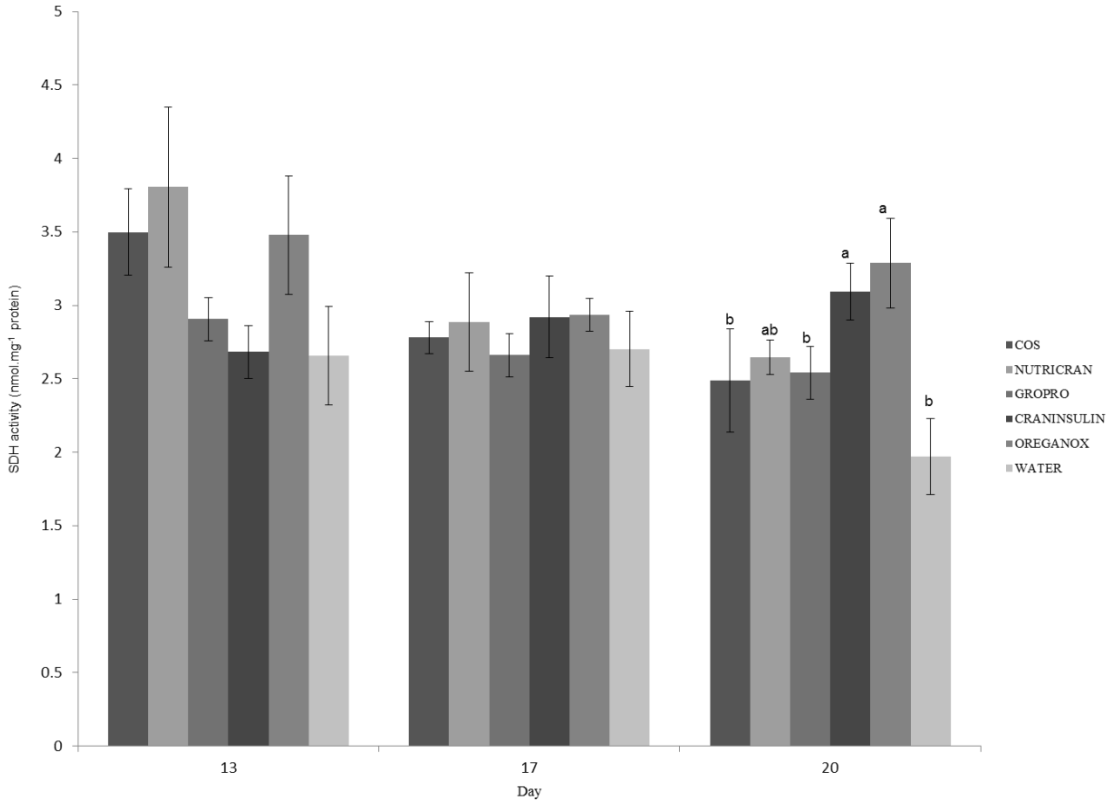

Figure 51: Effect of priming on Succinate Dehydrogenase activity (nmol mg-1 protein) of fava beans leaf tissue (13, 17, and 20 d).

6.1.4.3 Proline Dehydrogenase (PDH) Activity and Proline Content in Fava Bean Leaves

PDH can mediate proline oxidation as a potential alternate energy source via oxidative phosphorylation in the mitochondria. On day 13, Craninsulin (12.1 nmol/min/mg protein) treatment had lower PDH activity as compared to COS (14.1 nmol/min/mg protein), Nutricran (14.1 nmol/min/mg protein), Gropro (13.9

nmol/min/mg protein), Origanox (13.1 nmol/min/mg protein) and water (14.1 nmol/min/mg protein) treatments (Fig 52). On day 17, PDH activity increased slightly for all treatments (2.2-11.1%). On day 20, PDH activity decreased for COS (14.1%), Nutricran (8.7%), GroPro (7.8%) and water (11.1%) treatments whereas it increased for Craninuslin (7.5%) and oregano (23.7%) treatments. Highest PDH activity was found in Origanox treatment on day 20 (16.6 nmol/min/mg protein) whereas lowest was found in Craninsulin treatment on day 13 (12.1 nmol/min/mg protein).

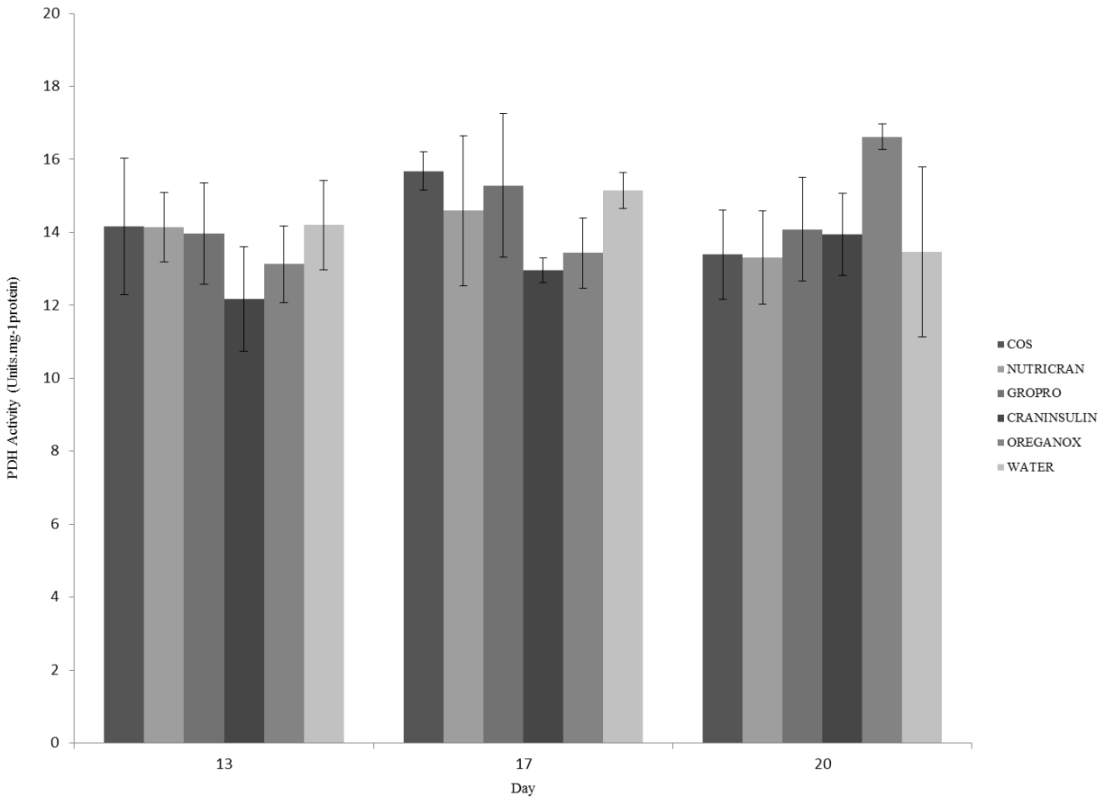

Figure 52: Proline dehydrogenase activity (Units mg^{-1} protein) of fava beans leaf tissue (13, 17, and 20 d).

Proline accumulation was high in Nutricran treated fava bean on day 13 (9.8 mg/g) as compared to COS (6.87 mg/g), GroPro (7.7 mg/g), Craninsulin (7.4 mg/g), Origanox (7.2 mg/g) and water (7.9 mg/g) treatments (Fig 53). Proline content decreased on day 17 for all treatments (6.2-23.4%). On day 20, proline content increased for Nutricran (32%) and Origanox (28.2%) treatments whereas it decreased for COS (15.5%), GroPro (19.7%), Craninsulin (15.8%) and water (22.6%) treatments. Highest proline accumulation was found in Nutricran (9.8 mg/g) treatment on day 13 whereas lowest was found in Craninsulin treatment on day 20 (4.8 mg/g).

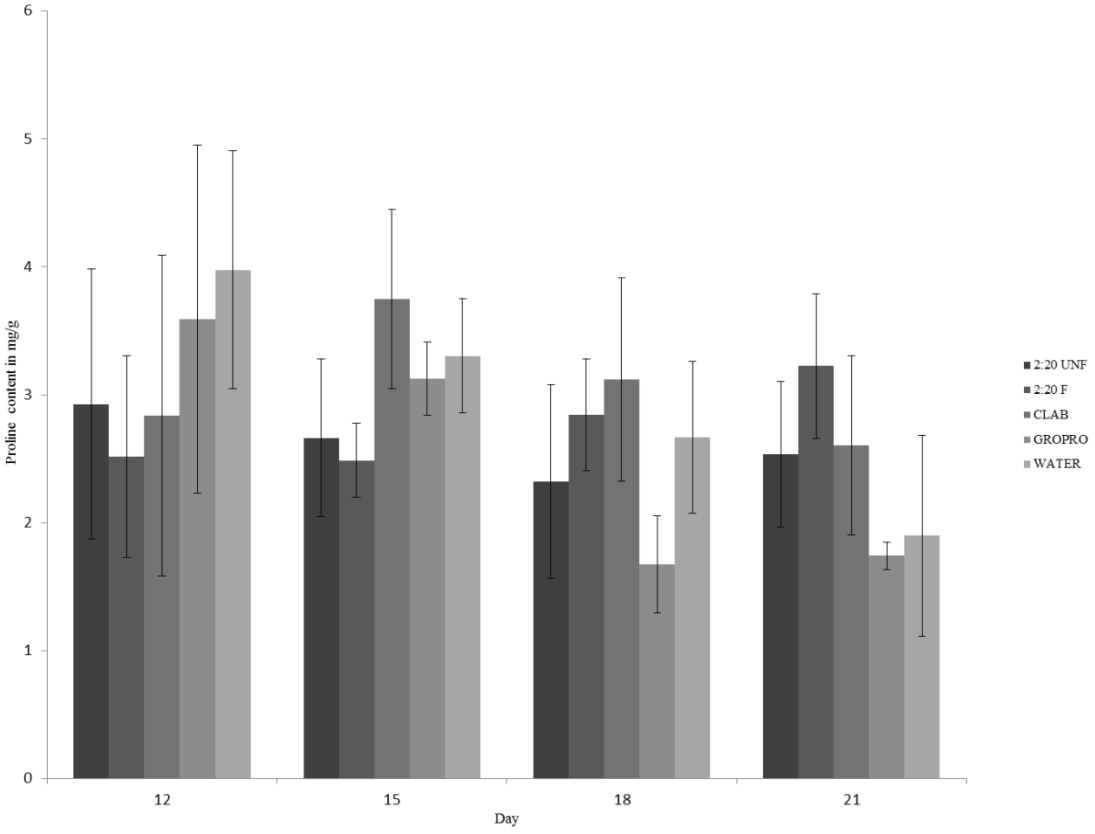

Figure 53: Total proline content (mg g^{-1} FW) of fava beans leaf tissue (13, 17, and 20 d).

6.1.4.4 Changes in Superoxide Dismutase (SOD), Catalase (CAT), and Guaicol peroxidase (GPX) Activity of Fava Bean Leaves

To investigate the effects of elicitor treatments on inducing antioxidant enzymes, the activity of three key antioxidant enzymes; SOD, CAT and GPX were assayed. On day 13, SOD activity was lower in Craninsulin treatment (0.74 units/mg protein) as compared to COS (0.89 units/mg protein), Nutricran (0.98 units/mg protein), GroPro (0.85 units/mg protein), Origanox (1.01 units/mg protein), and water (0.90 units/mg protein) treatments (Fig 54). On day 17, SOD activity decreased for Nutricran (9.5%), GroPro (2.4%) and Origanox treatments (4.0%) whereas it increased for COS (3.1%), Craninsulin (22.3%), and water (2.4%) treatments. The increase or decrease for each treatment on day 17, was reversed on day 20 except for nutricran treatment where a decrease was observed for day 17 and day 20. Higher SOD activity was found in Origanox (1.04 units/mg protein) treatment as compared to COS (0.85 units/mg protein), Nutricran (0.89 units/mg protein), GroPro (0.93 units/mg protein), Craninsulin (0.87 units/mg protein) and water (0.86 units/mg protein) treatments. Highest SOD activity was found in Origanox (1.04 units/mg protein) treatment on day 20, whereas lowest was found in craninsulin (0.74 units/mg protein) treatment on day 13.

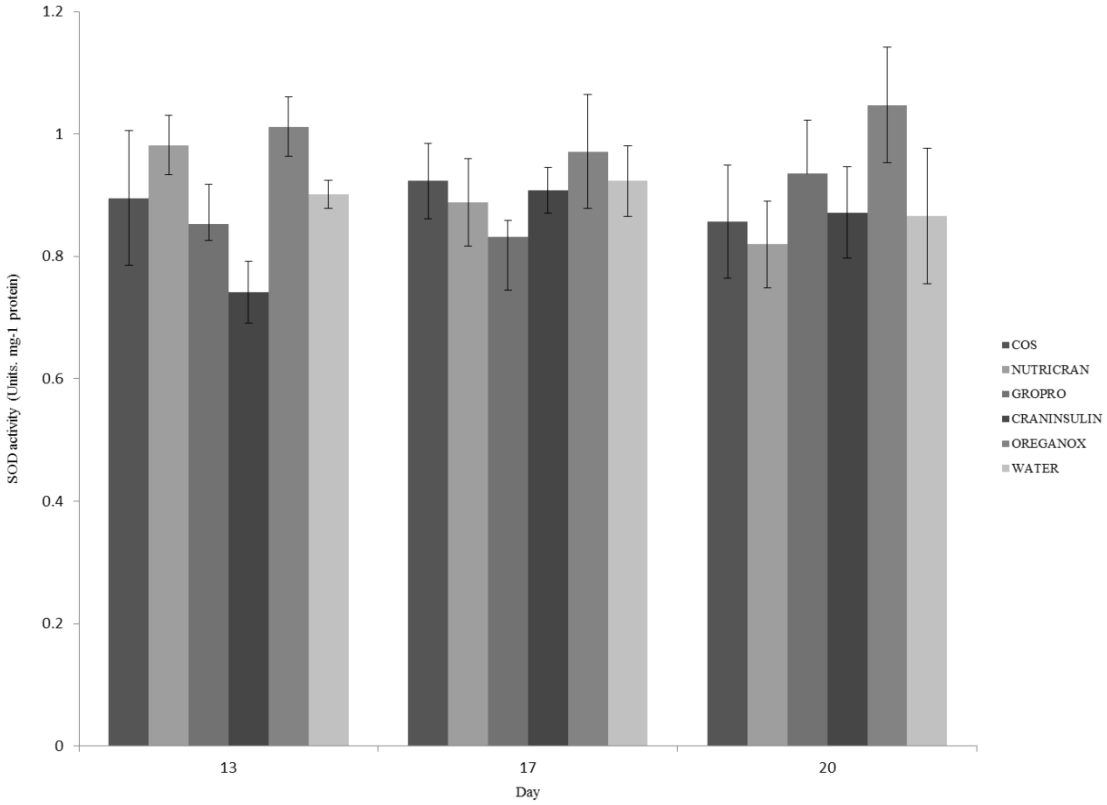

Figure 54: Superoxide dismutase activity (Units mg-1 protein) of fava beans leaf tissue (13, 17, and 20 d).

On day 13, Nutricran (45 units/min/mg protein) and Craninsulin (47.6 units/min/mg protein) treatments had higher CAT activity than COS (31.6 units/min/mg protein), GroPro (37.2 units/min/mg protein), Origanox (41.2 units/min/mg protein) and water (35.6 units/min/mg protein) treatments (Fig 55).

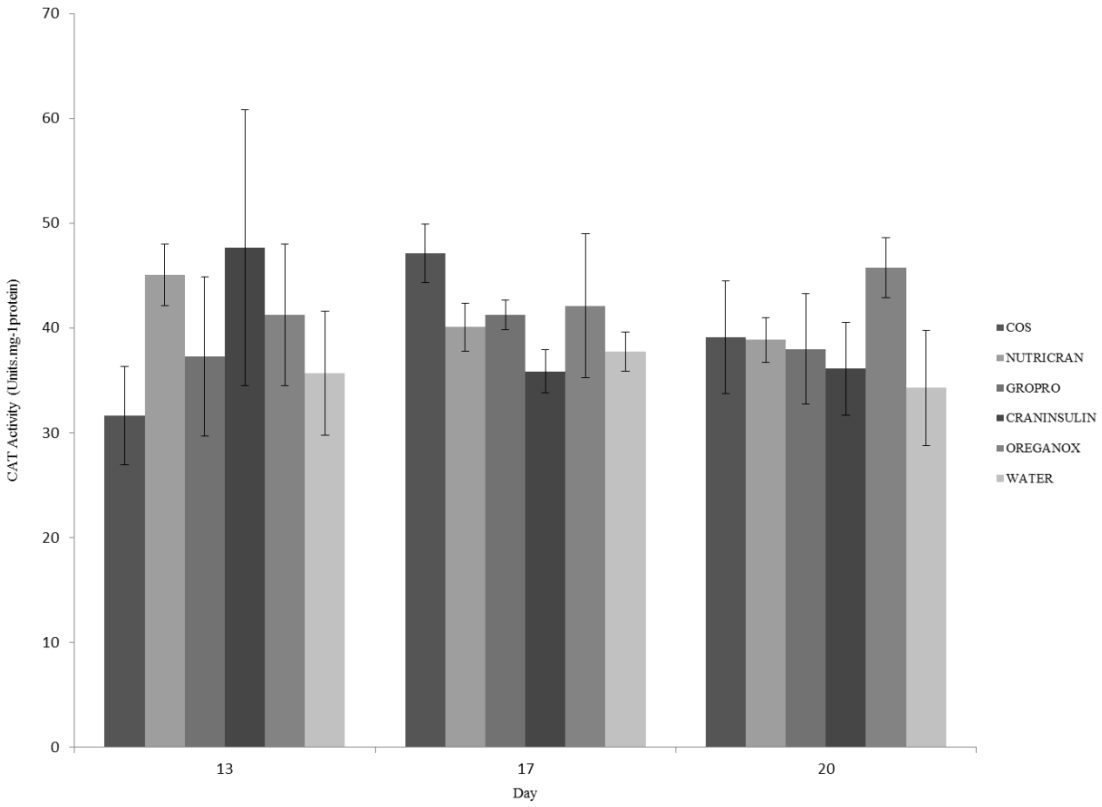

Figure 55: Catalase activity (Units mg^{-1} protein) of fava beans leaf tissue (13, 17, and 20 d).

CAT activity decreased on day 17 in Nutricran (11%) and Craninsulin (24.7%) treatments whereas it increased in COS (48.8%), GroPro (10.6%), Origanox (2.1%), and water (5.7%) treatments. On day 20, the increase (0.7-8.6%) or decrease (2.9-9.1%) in CAT activity was small in all treatments except COS (17.6%) treatment where the decrease was moderate-sharp. Highest CAT activity was found in Craninsulin treatment on day 13 whereas lowest was found in water on day 20.

GPX converts phenolics to lignin which is required for the structural development of the plant during its growth phase. On day 13, Origanox (91.6

nmol/min/mg protein) treatment had higher activity as compared to COS (67.7 nmol/min/mg protein), Nutricran (66.1 nmol/min/mg protein), GroPro (82.6 nmol/min/mg protein), Craninsulin (76 nmol/min/mg protein) and water (84.3 nmol/min/mg protein) treatments however, the difference was not significant (P>0.05) amongst treatments (Fig 56). On day 17, GPX activity decreased for all treatments (1.2-43.8%). On day 20, the activity decreased further for COS (3.5%), Nutricran (17.7%), Craninsulin (8.3%) and Origanox (25.1%) treatments whereas it increased for GroPro (27.9%) and Water (30.2%) treatments.

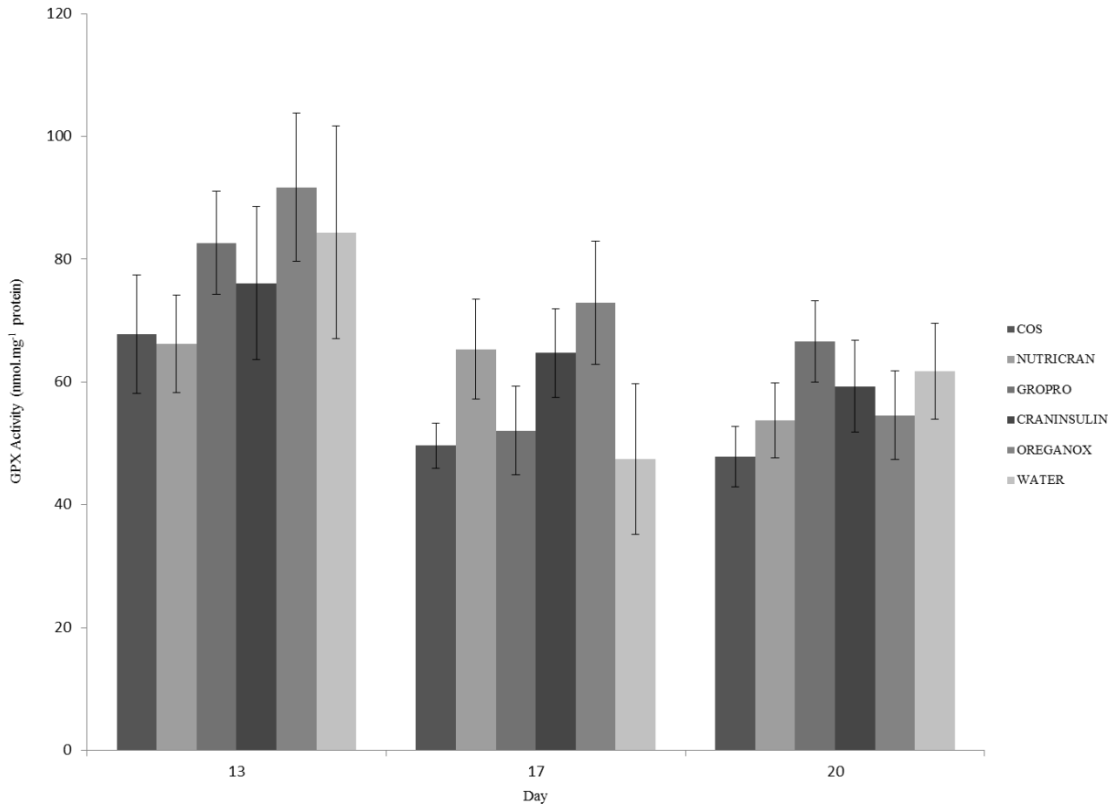

Figure 56: Guaiacol peroxidase activity (µmol mg^{-1} protein) of fava beans leaf tissue (13, 17, and 20 d).

6.1.5 Discussion

G6PDH catalyzes the rate limiting step of the pentose phosphate pathway which provides reducing power and provides intermediates for biosynthetic pathways (Kruger and von Schaewen, 2003) (Fig. 57). G6PDH activity decreased over the initial growth phase (day 13, 17, 20). Elicitor treatments, except Craninsulin, had higher G6PDH activity on day 13 as compared to control. G6PDH activity decreased on day 17 and control had slightly higher activity than elicitor treatments. On day 20, all elicitor treatments had higher activity than control. The cyclical rise and fall in G6PDH activity may be due to an allosteric feedback inhibition mechanism due to sugar phosphates being synthesized by the enzyme (Randhir et al., 2009). A decrease in G6PDH activity during the growth phase in response to peptide and phenolic phytochemical elicitors during germination has been previously reported (Randhir et al., 2002; Randhir and Shetty, 2003; Andarwulan and Shetty, 1999). Higher initial activity of this enzyme may be because during seed germination oxidative pentose phosphate pathway up-regulation is needed (Hare et al., 2003).

Respiration which drives carbon flux towards two essential needs of oxidation of substrates to provide energy and provide intermediates required for biosynthesis consists of a range of metabolic pathways converging into TCA cycle (Botha et al., 1992). SDH, a key enzyme of the TCA cycle, generates NADH for oxidative phosphorylation and was assayed to investigate the energy demands of the germinating seed. Overall, SDH activity remained constant for all elicitor treatments over the initial growth phase, after a slight increase on day 17 followed by a slight decrease on day 20. In control treatment activity decreased sharply on day 20. Elicitor treatments had higher

SDH activity than control on day 13 and day 20 suggesting stimulation of the TCA cycle for meeting a higher cellular energy demand of the germinating seed required for growth and development (Noguchi et al., 1996; Sarkar et al., 2009a). A combination of higher G6PDH and SDH activity on day 13 and day 20 in the elicitor treatments may provide a balance between the anabolic and catabolic needs and subsequently drive anabolic precursors towards phenolic synthesis and antioxidant enzyme response in fava bean leaves.

The decrease in phenolic content in elicitor treatments on day 20 may represent phenolics being portioned for lignification which are required for the structural development of the plant (Vattem et al., 2005c). Phenolic content correlated well with the G6PDH activity for all treatments (r =0.67-0.93) except GroPro (r = 0.04). This suggests that GroPro treatment may be driving majority of the carbon flux towards phenolic synthesis whereas in other treatments a part of it may be portioned towards nucleotide, amino acid and plant growth regulator synthesis. This is further supported by the fact that in GroPro treatment antioxidant enzyme (SOD and CAT) response was lower as compared to the control. Phenolic biosynthesis and antioxidant enzyme response may be stimulated by elicitors by inducing a signaling cascade or by stress like perception due to involvement of ROS, changes in ion fluxes and reversible phosphorylation/dephosphorylation (Randhir et al., 2009; Dixon and Paiva, 1995; Shetty and Wahlqvist, 2004). It is possible that proline or glutamate present in GroPro or small proline mimicking phenolics present in cranberry or oregano based elicitors (Craninsulin, Nutricran and Origanox) may stimulate phenolic biosynthesis (Kwok et al., 1998; Apostolidis et al., 2008). Stimulation of phenolic synthesis in response to

various elicitors by biosynthetic pathway up-regulation has been reported (Conceicao et al., 2006; Daayf et al., 2003; Vattem et al., 2005c; Lozoya et al., 1991; Zahringer et al., 1978).

Elicitor treatments did not have a significant effect on the antioxidant potential of fava bean leaf. High antioxidant capacity of fava bean has been previously reported (Amarowicz et al., 1996; Berghofer et al., 1998).

L-DOPA content decreased over the initial growth phase. L-DOPA content correlated with G6PDH activity in all treatments (r = 0.88-0.99) except Origanox (r = 0.23) which suggests stimulation of the pentose phosphate pathway may stimulate L-DOPA synthesis.

PDH activity was high in Origanox treatment on day 20 which suggests proline oxidation in the mitochondria to support oxidative phosphorylation to provide a non NADH route to ATP synthesis (Shetty and Wahlqvist, 2004; Hare and Cress, 1997). Proline accumulation is regulated by proline synthesis from glutamate via TCA cycle and proline oxidation by PDH in the mitochondria. Proline content correlated with G6PDH activity in all treatments (r = 0.82-0.99) except Origanox (r = 0.31) indicating proline biosynthesis may regulate PPP via NADPH/$NADP^+$ redox balance (Shetty and Wahlqvist, 2004).

SOD acts on superoxide radicals producing hydrogen peroxide. Catalase acts on hydrogen peroxide converting it to water and molecular oxygen (Jaleel et al., 2009). It has been reported that mitochondrial metabolism can produce ROS (Ames et al., 1993a) and it is possible that this high amount of ROS may have triggered a higher SOD response. This is further confirmed by the fact that SDH activity correlated with SOD

activity in all treatments (r = 0.67-0.97) except GroPro (r = -0.61) and COS (r = 0.37) treatments. CAT activity was higher in elicitor treatments as compared to control. Elicitor treatments may induce antioxidant enzyme response either by inducing a stress like state during germination (Randhir et al., 2009) and/or by ROS produced due to up regulation of the TCA cycle. An increase in SOD and CAT activity during germination in sunflower seeds in order to protect the seedling from the external environment has been reported (Bailly et al., 2000; Bailly, 2004). SOD activity was reported to increase in dark germinated *Mucuna pruriens* sprouts by peptide and phytochemical elicitors (Burguieres et al., 2007; Randhir et al., 2009).

GPX is involved in catalyzing polymerization of substrates and it plays an important role in redox reactions in plasma membranes, cell wall modifications and in defense mechanism (Sarkar et al., 2009a). GPX activity decreased overall during the initial growth phase suggesting a decrease in the need for lignification. High phenolic content in GroPro on day 17 may induce a higher GPX activity as seen in the treatment on day 20. In other treatments phenolics available for lignification may be low and that may explain the decrease in GPX activity during the initial growth phase. GPX activity has been reported to increase in light and dark germinated fava beans, mung beans, corn and pea by various peptide and phytochemical elicitors (Randhir and Shetty, 2003; Andarwulan and Shetty, 1999; Randhir et al., 2002; Randhir and Shetty, 2005).

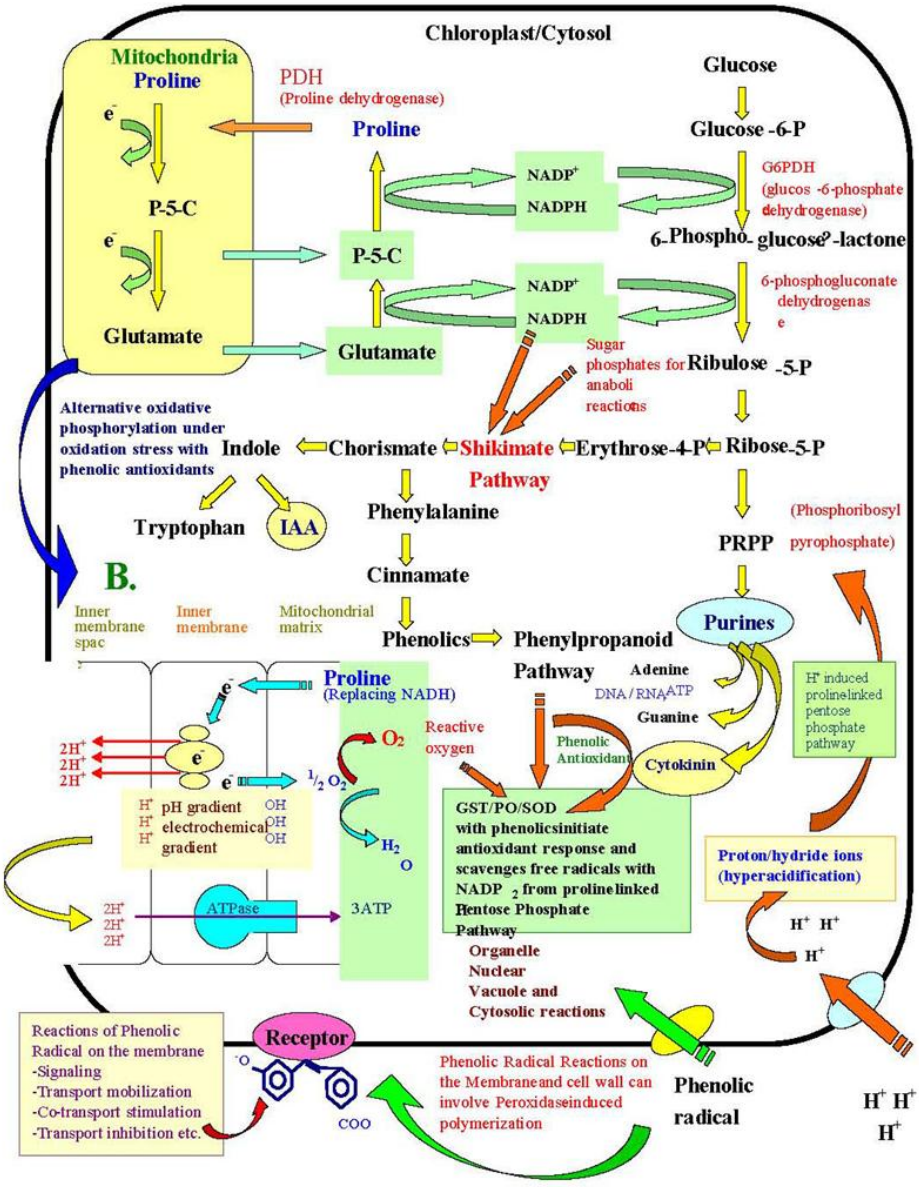

Figure 57: Proline linked Pentose Phosphate Pathway

6.1.6 Conclusion

This study provides a perspective into metabolic regulation by energy partitioning using elicitors to improve defense responses of the germinating seed during initial growth phase. Phenolic biosynthesis in response to elicitor treatment was likely stimulated by using proline as an alternate energy source and by a stimulated carbon flux through the TCA cycle. This novel approach and innovative strategy provides basis for practical applications of using such elicitors for improving nutritional and health relevant bioactive functional value of legumes. This provides a rationale metabolic basis for improving legume sprouts with enhanced health benefits to counter oxidation-linked chronic diseases.

6.2 Stimulation of Total Phenolics and Antioxidant Enzymes in Fava Bean (*Vicia faba*) in Response to Fermented Apple Extracts

6.2.1 Abstract

Fava bean is an important legume with bioactive functional phenolics for managing Parkinson disease. Effect of elicitors in the form of a fermented apple juice with live probiotic bacteria and a marine bio-enhancer on the stimulation of bioactive phenolic biosynthesis and associated antioxidant enzyme response were evaluated in germinating fava bean on day 12, 15, 18 and 21 of its growth phase. Significant stimulation of phenolic biosynthesis and antioxidant enzymes was observed in the treatments. Glucose-6-phosphate dehydrogenase (G6PDH) activity was not significantly stimulated in the treatments. Succinate dehydrogenase (SDH) activity was higher in the treatments along with antioxidant enzymes: superoxide dismutase (SOD), catalase (CAT) and guaiacol peroxidase (GPX). Proline dehydrogenase (PDH) activity in response to fermented extracts was higher indicating proline oxidation is an important alternate metabolite for ATP generation. L-DOPA, an important phenolic for Parkinson's disease management was stimulated in the elicitor treatments. Overall elicitor treatments support metabolic regulation for energy efficient phenolic biosynthesis and antioxidant enzyme response.

6.2.2 Introduction

Epidemiological evidence related to the decrease in non-communicable chronic diseases due to consumption of fruits and vegetables has been in part, attributed to phenolic secondary metabolites (Crozier et al., 2009). Protective effect of phenolic compounds is due to their free radical scavenging and metal ion chelating ability

thereby alleviating increased oxidative damage, a causative process in the development of many age related diseases (Petti and Scully, 2009). Diet relevant phenolic secondary metabolite classes which include phenolic acids, hydroxycinnamates and flavonoids are synthesized by a complex, enzymatically regulated network of routes primarily based on the shikimate, phenylpropanoid and flavonoid pathways (Crozier et al., 2009). Significant progress has been made in directing phenolic bioactive synthesis in plants by genetic engineering and such new developments seem promising. However, challenges from isolation and cloning of structural genes and complexity of pathways to accumulation of unknown compounds and regulatory hurdles still persist (Crozier et al., 2009; Schijlen et al., 2004) and therefore new approaches in metabolic stimulation of bioactives for diverse benfits are essential.

Fava bean (*Vicia faba*) first incorporated in the eastern Mediterranean diet and commonly called as the broad bean is known for its protein content, complex carbohydrates and secondary metabolites including phenolics and L-DOPA (levo dihydroxy phenylalanine) (Kones, 2010; Randhir et al., 2002). Increase in intra-renal concentration of dopamine through high Fava bean content of dopamine precursor, L-DOPA, has shown an increase in urinary sodium excretion which may have implications in treating hypertension, heart failure, renal failure and liver cirrhosis (Vered et al., 1997). Fava bean is one of the few diet based treatments available for therapy of Parkinson disease, caused by the imbalance of dopamine and acetylcholine in the brain (Randhir et al., 2002). Fava bean extracts protected against picrotoxin induced convulsions and deaths in mice and may have potential in reducing rates of epilepsy (Salih and Mustafa, 2008).

Elicitor-linked priming is a process where plants are sensitized for faster and/or stronger defense responses leading to an increase in resistance to biotic and abiotic stress (Beckers and Conrath, 2007). Seed priming with phenolic phytochemical elicitors followed by germination can stimulate phenolic biosynthesis via up regulation of pentose phosphate pathway (Randhir et al., 2009). Potential of priming in enhancing health beneficial antioxidants to improve the quality of food crops has been reviewed (Capanoglu, 2010). Both biotic and abiotic stress can induce phenolic secondary metabolites such as phenylpropanoids and flavonoids which have diverse physiological functions in stress response (Dixon and Paiva, 1995; Winkel-Shirley, 2002). Flavonoids may modulate stress response by several different mechanisms including acting as an antioxidant, inducing antioxidant enzyme response, screening harmful radiation, binding phytotoxins and regulating auxin transport (Shetty and Wahlqvist, 2004; Winkel-Shirley, 2002).

Cellular antioxidant homeostasis is maintained by antioxidant enzymes such as superoxide dismutase (SOD), Catalase (CAT), Glutathione peroxidase (GPX), Glutathione S-transferase (GST) and molecular antioxidants such as tocopherols, ascorbate, glutathione by a series of redox reactions (Foyer and Noctor, 2005; Mates et al., 2008). Reactive oxygen species (ROS) may be produced as a result of normal metabolic processes such as respiration or may be induced by exogenous sources such as UV, toxins, and inflammation (Shao et al., 2008). Regeneration of these antioxidant defense systems, an energy intensive process, is essential to maintain efficiency and requires cellular reducing equivalents in the form of NADPH and $FADH_2$ (Mates and Sanchez-Jimenez, 1999; Mates et al., 1999; Nordberg and Arner, 2001). In plants,

imposition of biotic and abiotic stresses lead to accumulation of free proline by reduction of pyroline-5-carboxylate (P5C) using reducing equivalents in the form of NADPH which leads to formation of $NADP^+$ (Verbruggen and Hermans, 2008). $NADP^+$ is a co-factor of the Glucose-6-Phosphate Dehydrogenase (G6PDH), an enzyme which drives the rate limiting step of the pentose phosphate pathway. Using this rationale, an alternate model for proline metabolism was proposed where proline biosynthesis is linked to stimulation of Pentose Phosphate Pathway (PPP) through $NADP^+$/NADPH redox balance (Shetty and Wahlqvist, 2004). This up-regulated PPP provides excess metabolic intermediates for anabolic reactions including secondary metabolite synthesis whereas proline and P5C can act as a redox couple for oxidative phosphorylation providing a non-NADH route for ATP synthesis (Hare and Cress, 1997; Shetty, 2004). Further, phenolic antioxidants may mediate the antioxidant response of the cell, not only by acting as direct scavengers of ROS but also by stimulating proline biosynthesis which provides excess NADPH to aide cellular antioxidant defense response (Shetty and Wahlqvist, 2004).

Mobilization of phenolics using fermentation based processing using lactic acid bacteria has been shown in soymilk (McCue and Shetty, 2005; Wang et al., 2006). Liquid state /submerged fermentation involves reduced or lack of oxygen and hence represents a reductive state for maintaining redox protective bioactive compounds. Here we investigated the effect of mobilized phenolic from fermented apple juice extracts containing live probiotic bacteria to elicit endogenous phenolic biosynthesis and associated cellular antioxidant response in fava bean leaf tissue targeted as bioactive ingredient source for Parkinson's disease management.

6.2.3 Materials and Methods

6.2.3.1 Elicitor and Treatment

One cultivar of Apple: Red delicious; was obtained from Stop and Shop stores, Hadley, MA. Whole Apples were washed with water and then cut into small pieces. Edible pieces were homogenized using a Waring blender for 5 min. The thick pulp was then centrifuged for 15 min at 15,000 g, supernatants were collected and kept at -20^{0}C during the period of the study. Treatments used were as follows A) Apple juice was fermented using *Lactobacillus helveticus* (LA) provided by Rosell Institute Inc. Montreal, Canada (Lot#: XA 0145, Seq#:00014160). Five mL of the overnight grown culture of LA was added to 45 mL of apple juice and fermented for 24 h at 37 ^{o}C. Twenty mL of this fermented extract was made up to 200 mL by adding distilled water and this was used as one treatment. B) As a control to this treatment, 20 mL of unfermented apple juice was used instead of fermented juice. C) As a control for the presence of probiotic bacteria, twenty mL of fermented apple juice was centrifuged and the bacterial pellet was washed with distilled water twice and put in 180 mL of distilled water. D) GroPro, consisting of marine peptide extracts from extracts of fish and seaweed byproducts with a minimum of 1% glutamic acid and proline and 0.1% total phenolics (Icelandic Bioenhancer, Harrison, NY). Two mL of GroPro was added to 100 mL water and then 1 mL of this diluted elicitor was added to 199 mL of water and used for soaking seeds. E) As a control to all treatments 200 mL of distilled water was used to soak seeds.

6.2.3.2 Seed Treatment and Germination

Dry seeds of fava bean (*Vicia faba*) were obtained from Casablanca Halal Market, Hadley, MA. For each treatment, 20 dry seeds were soaked in 200 mL distilled water in a 250 mL Erlenmeyer flask for 24 h. Five treatments used were a) 10% fermented apple extracts (2:20 F) b) 10% unfermented apple extracts (2:20 UNF) c) probiotic bacteria (similar cfu/mL as fermented extracts) (CLAB) d) GroPro e) distilled water (W). The pre-soaked seeds were then germinated in sterilized potting soil at 20 ^{o}C for 21 days. 2:20 F and GroPro are referred to as elicitor treatments whereas 2:20 UNF, CLAB and Water are referred to as controls.

6.2.3.3 Leaf Sample Extraction

Two hundred mg of leaf sample was taken on day 12, 15, 18 and 21 for analysis. Two hundred mg leaf samples were collected from at least 3 different germinating seeds. A cold pestle and mortar was used to thoroughly grind 200 mg of the leaf tissue in cold enzyme extraction buffer (0.5 % polyvinylpyrrolidone (PVP), 3 mM EDTA. 0.1 M potassium phosphate buffer of pH 7.5). Grinding was carried out in ice. The sample was centrifuged at 12000 x g for 15 min at 2-5 ºC and stored on ice. The supernatant was used for further analysis.

For total phenolic and ABTS assay 50 mg of the leaf tissue was immersed in 2.5 mL of 95% ethanol and kept in the freezer for 48-72 h. Samples were homogenized using a tissue tearor (Biospec Products, Bartleville, OK) and centrifuged at 12000 x g for 10 min. Supernatant was used for further analysis.

6.2.3.4 Total Protein Assay

Protein content was measured by the method of Bradford assay (Bradford, 1976). The dye reagent concentrate (Bio-Rad protein assay kit II, Bio-Rad Laboratory, Hercules, CA) was diluted 1:4 with distilled water. A volume of 5 mL of diluted dye reagent was added to 50 µL of the leaf tissue extract. After vortexing and incubating for 3 min, the absorbance was measured at 595 nm against a 5 mL reagent blank and 50 µL buffer solutions using a UV-VIS Genesys spectrophotometer (Milton Roy, Inc., Rochester, NY).

6.2.3.5 Glucose-6-Phosphate Dehydrogenase (G6PDH) Assay

A modified version of the assay described by Deutsch (1983) was followed. The enzyme reaction mixture containing 5.88 µmol ß-NADP, 88.5 µmol $MgCl_2$, 53.7 µmol glucose-6-phosphate, and 0.77 mmol maleimide was prepared. This mixture was used to obtain baseline (zero) of the spectrophotometer reading at 340 nm wavelength. To 1 mL of this mixture, 100 µL of the enzyme sample was added. The rate of change in absorbance per minute was used to quantify the enzyme in the mixture using the extinction co-efficient of NADPH (6.22 $mM^{-1}cm^{-1}$).

6.2.3.6 Succinate Dehydrogenase (SDH) Assay

Modified method described by Bregman (1987) was used to assay the activity of succinate dehydrogenase. The assay mixture consisted of the following: 1.01 mL of 0.4 M potassium phosphate buffer (pH 7.2); 40 µL of 0.15 M sodium succinate (pH 7.0); 40 µL of 0.2 M sodium azide; and 10 µL of 6.0 mg/mL DCPIP. This mixture was used to obtain baseline (zero) of the spectrophotometer reading at 600 nm wavelength. To 1.0 mL of this mixture, 200 µL of the enzyme sample was added. The rate of change in absorbance per minute was used to quantify the enzyme in the mixture using the extinction co-efficient of DCPIP (19.1 $mM^{-1}cm^{-1}$).

6.2.3.7 Superoxide Dismutase (SOD) Assay

A competitive inhibition assay was performed that used xanthine-xanthine oxidase-generated superoxide to reduce nitroblue tetrazolium (NBT) to blue formazan. Spectrophotometric assay of SOD activity was carried out by monitoring the reduction of NBT at 560 nm (Oberley and Spitz, 1985). The reaction mixture contained 13.8 mL of 50 mM potassium phosphate buffer (pH 7.8) containing 1.33 mM DETAPAC; 0.5 mL of 2.45 mM NBT; 1.7 mL of 1.8 mM xanthine and 40 IU/mL catalase. To 0.8 mL of reagent mixture 100 µL of phosphate buffer and 100 µL of xanthine oxidase was added. The change in absorbance at 560 nm was measured every 20 sec for 2 min and the concentration of Xanthine oxidase was adjusted to obtain a linear curve with a slope of 0.025 absorbance per min. The phosphate buffer was then replaced by the enzyme sample and the change in absorbance was monitored every 20 sec for 2 min. One unit of

SOD was defined as the amount of protein that inhibits NBT reduction to 50% of the maximum.

6.2.3.8 Catalase (CAT) Assay

A method originally described by Beers and Sizer, (1952), was used to assay the activity of catalase. To 1.9 mL of distilled water 1 mL of 0.059 M hydrogen peroxide (Merck's Superoxol or equivalent grade) in 0.05 M potassium phosphate, pH 7.0 was added. This mixture was incubated in a spectrophotometer for 4-5 min to achieve temperature equilibration and to establish blank rate. To this mixture 0.1 mL of diluted enzyme sample was added and the disappearance of peroxide was followed spectrophotometrically by recording the decrease in absorbance at 240 nm for 2-3 min. The change in absorbance ΔA_{240}/min from the initial (45 sec) linear portion of the curve was calculated. One unit of catalase activity was defined as amount that decomposes one micromole of H_2O_2

$$\text{Units /mg} = \frac{\Delta A_{240}/\text{min} \times 1000}{43.6 \times mg\ enzyme/mL\ of\ reaction\ mixture}$$

6.2.3.9 Guaiacol Peroxidase (GPX) Assay

Modified version of assay developed by Laloue et al., (1997), was used. Briefly, the enzyme reaction mixture contained 0.1 M potassium phosphate buffer (pH 6.8), 56 mM guaiacol solution and 0.2 mM hydrogen peroxide. To 1 mL of this reaction mixture, 50 µL of enzyme sample was added. The absorbance was noted at zero time

and then after 5 min. The rate of change in absorbance per minute was used to quantify the enzyme in the mixture using the extinction co-efficient of the oxidized product tetraguaiacol (26.6 $mM^{-1}cm^{-1}$).

6.2.3.10 Total Soluble Phenolics Assay

The total phenolic content in grass leaves was analyzed by the modified Folin-Ciocalteu method (Shetty et al., 1995). One milliliter of supernatant was transferred into a test tube and mixed with 1 mL of 95 % ethanol and 5 mL of distilled water. To each sample 0.5 mL of 50 % (v/v) Folin-Ciocalteu reagent was added and mixed. After 5 min, 1 mL of 5 % Na_2CO_3 was added to the reaction mixture and allowed to stand for 60 min. The absorbance was read at 725 nm. The absorbance values were converted to total phenolics and were expressed in milligrams equivalents of gallic acid per grams Fresh weight (FW) of the sample. Standard curves were established using various concentrations of gallic acid in 95 % ethanol.

6.2.3.11 ABTS Cation Radical and Antioxidant Activity Assay

The total antioxidant activity of fava bean leaf extract was measured by the $ABTS^+$ radical cation-decolorization assay involving preformed $ABTS^+$ radical cation (Re et al., 1999). ABTS [2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)] (Sigma Chemical Co.St. Louis, MO) was dissolved in water to a 7 mM concentration. $ABTS^+$ radical cation was prepared by reacting 5 mL of 7 mM ABTS stock solution with 88 µL of 140 mM potassium persulphate, and mixture was allowed to stand in the

dark at room temperature for 12-16 h before use. Prior to assay $ABTS^{+}$ stock solution was diluted with 95% ethanol (ratio 1:88) to give an absorbance at 734 nm of 0.70 ± 0.02, and was equilibrated to 30 °C. One milliliter ABTS was added to glass test tubes containing 50 µL of each tissue extract, and mixed by vortex mixer for 30 s. After 2.5 min incubation in the dark, mixtures were read at 734 nm. The readings were compared with controls, which contained 50 µL of 95% ethanol instead of the extract. The Trolox reference standard for relative antioxidant activities was prepared with 5 mM stock solution of Trolox in ethanol for introduction into the assay system at concentrations within the activity range of the assay (0-20 µM final concentration) for preparing a standard curve to which all data were referenced. The percent inhibition was calculated by:

$$\% \text{ inhibition} = \left(\frac{[A_{734}^{\text{control}} - A_{734}^{\text{extract}}]}{[A_{734}^{\text{control}}]}\right) \times 100$$

6.2.3.12 HPLC Analysis of Proline

High performance liquid chromatography (HPLC) analysis was performed using an Agilent 1100 liquid chromatograph equipped with a diode array detector (DAD 1100). The analytical column was reverse phase Nucleosil C18, 250 nm x 4.6 mm with a packing material of 5 µm particle size. The extract samples were eluted out in an isocratic manner with a mobile phase consisting of 20 mM potassium phosphate (pH 2.5 by phosphoric acid) at a flow rate of 1 mL min^{-1} and detected at 210 nm. L-Proline (Sigma chemicals, St. Louis, MO) dissolved in the 20 mM potassium phosphate

solution was used to calibrate the standard curve. The amount of proline in the sample was calculated as mg of proline per milliliter and converted and reported as mg g^{-1} FW.

6.2.3.13 HPLC Analysis of L-DOPA

High performance liquid chromatography (HPLC) analysis was performed using an Agilent 1100 liquid chromatograph equipped with a diode array detector (DAD 1100). The analytical column was a reverse phase Supleco Discovery C18, 250 mm × 4.6 mm with a packing material of 5 µm particle size. The extract samples were eluted out in an isocratic manner with a mobile phase composition of 18% methanol and 82% buffer consisting of 0.01M ammonium acetate at pH 5.4, at a flow rate of 1 mL/min. The retention time and spectrum of sample was compared with that of standard L-DOPA (Sigma). The amount of L-DOPA was determined from the area obtained at 280 nm as compared to the pure standard of known concentration. The amount of L-DOPA in the sample was calculated as mg of L-DOPA per milliliter and converted and reported as mg g^{-1} FW.

6.2.3.14 Statistical Analysis

All experiments were performed with six replications with a minimum of four replications for each treatment. Samples were collected from at least three different germinating seeds. The effect of each treatment was determined by the analysis of variance (ANOVA) of SAS (version 8.2; SAS Institute, Cary, NC). Differences among different treatments were determined by the Fishers least significant difference (LSD) test at the 0.05 probability level. Standard error was calculated using Microsoft Excel 2010.

6.2.4 Results

6.2.4.1 Changes in Total Soluble Phenolics, Free Radical-linked Antioxidant Activity and L-DOPA content of Fava Bean Leaf

Total soluble phenolic content in fava bean leaves was assayed using the Folin-Ciocalteu method (Fig 58). On day 12, the phenolic content in fava bean leaves were higher in 2:20 F (10.4 mg/g), CLAB (9.7 mg/g) and GroPro (10.1 mg/g) treatments as compared to 2:20 UNF (8.3 mg/g) and water (8.4 mg/g) treatments. On day 15, 2:20 F (10.5 mg/g) treatment had significantly higher ($P<0.05$) phenolic content as compared to all other treatments (7.9-8.4 mg/g). On day 18, elicitor treatments 2:20 F (9.9 mg/g) and GroPro (9.0 mg/g) were significantly higher ($P<0.05$) than the other treatments (7.2-7.8 mg/g). On day 20, 2:20 F (9.9 mg/g) was significantly higher ($P<0.05$) than all the other treatments (7.3-8.1 mg/g) except the unfermented control (2:20 UNF) (8.8 mg/g) treatments. Highest phenolics were found in 2:20 F (10.5 mg/g) treatments on day 15 and lowest was found in CLAB (7.2 mg/g) treatments on day 18.

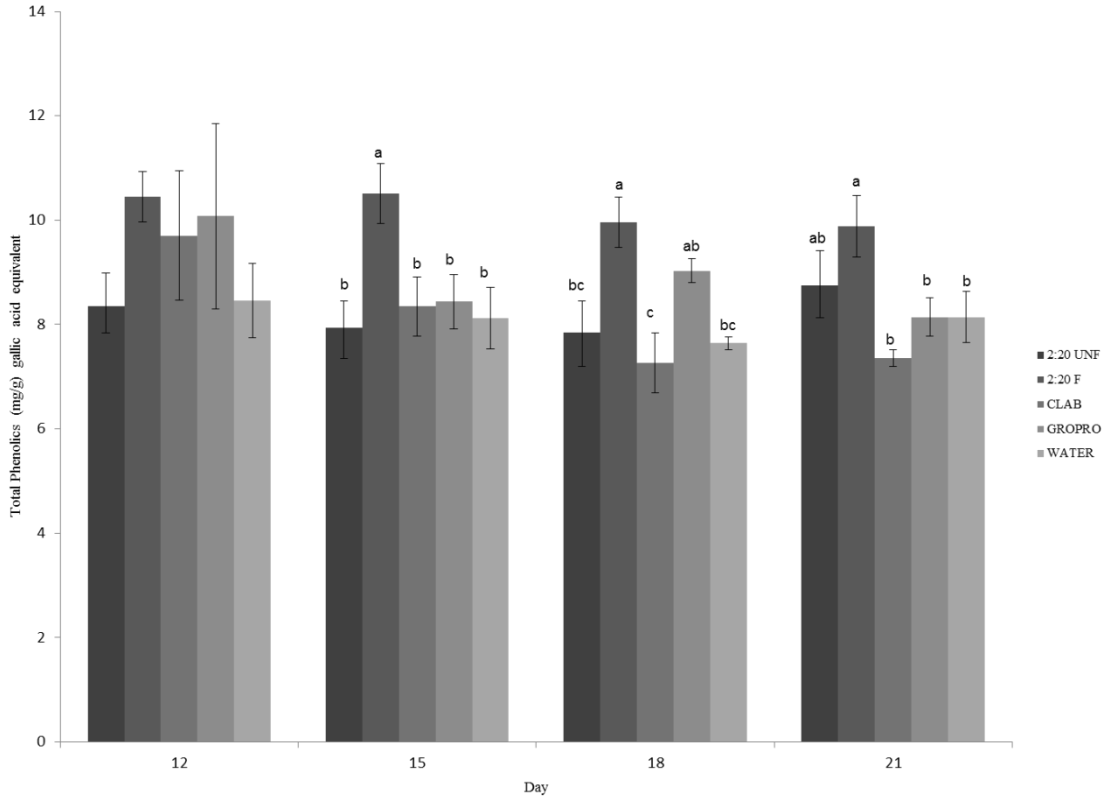

Figure 58: Effect of priming on Total soluble phenolic content (mg g-1 FW) of fava bean leaf tissue (12, 15, 18 and 21 d).

For all treatments the baseline value for total antioxidant activity (ABTS assay) was high (96-98% inhibition) in fava bean leaves over the growth phase (day 12, 15, 18, 21) (Fig 59). Total antioxidant activity linked to free radical scavenging for elicitor treatments were not significantly different ($P>0.05$) from control treatments. Antioxidant activity in fava bean leaves did not change for treatment or control during the growth phase (day 12, 15, 18, 21).

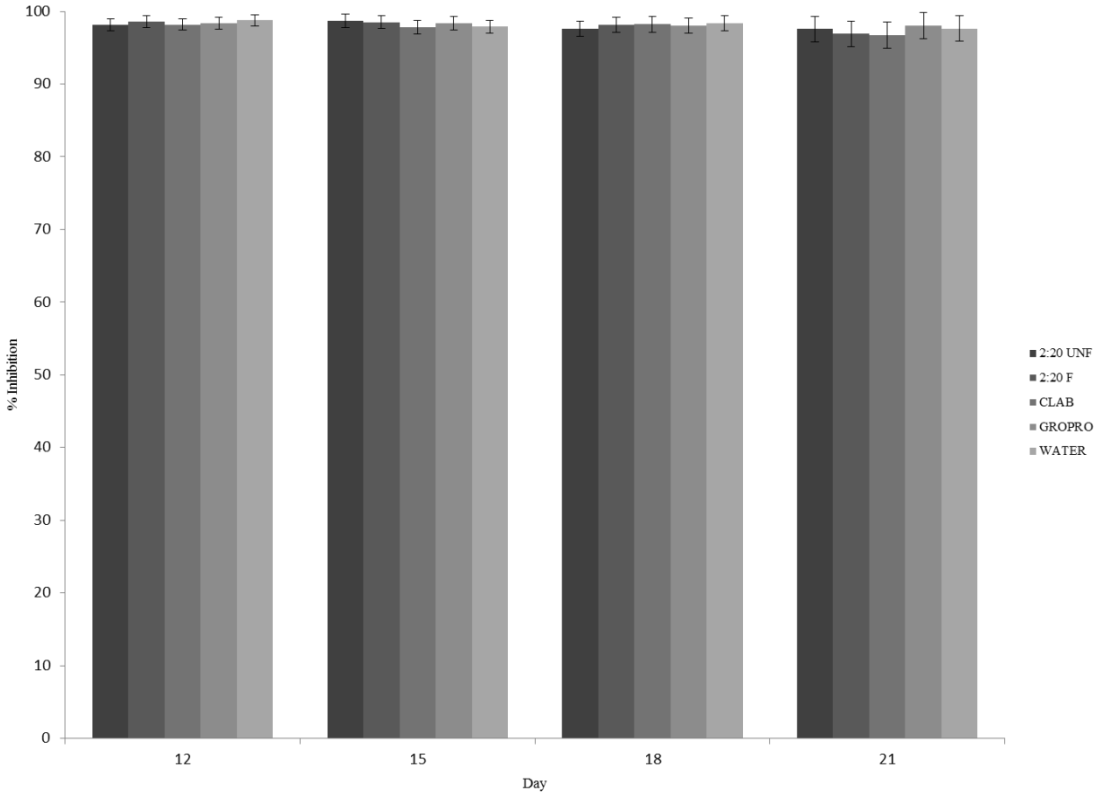

Figure 59: Effect of priming on Total antioxidant activity (ABTS%) of fava bean leaf tissue (12, 15, 18 and 21 d).

L-DOPA content was evaluated using HPLC. Elicitor treatments had higher baseline L-DOPA content (7.9-8.0 mg/g) as compared to the controls (3.7-6.1 mg/g) on day 12 (Fig 60). L-DOPA content decreased in individual treatments except for GroPro (7.9 mg/g) treatment on day 15. However a similar trend (elicitors higher than controls) was maintained. This trend was further maintained on day 18 and day 21 where elicitor treatments (2:20 F and GroPro) had higher L-DOPA content than controls (2:20 UNF, CLAB and water). Highest L-DOPA content was found in 2:20 F (8.0 mg/g) treatment on day 12 whereas lowest was found in CLAB (3.7 mg/g) treatment on the same day.

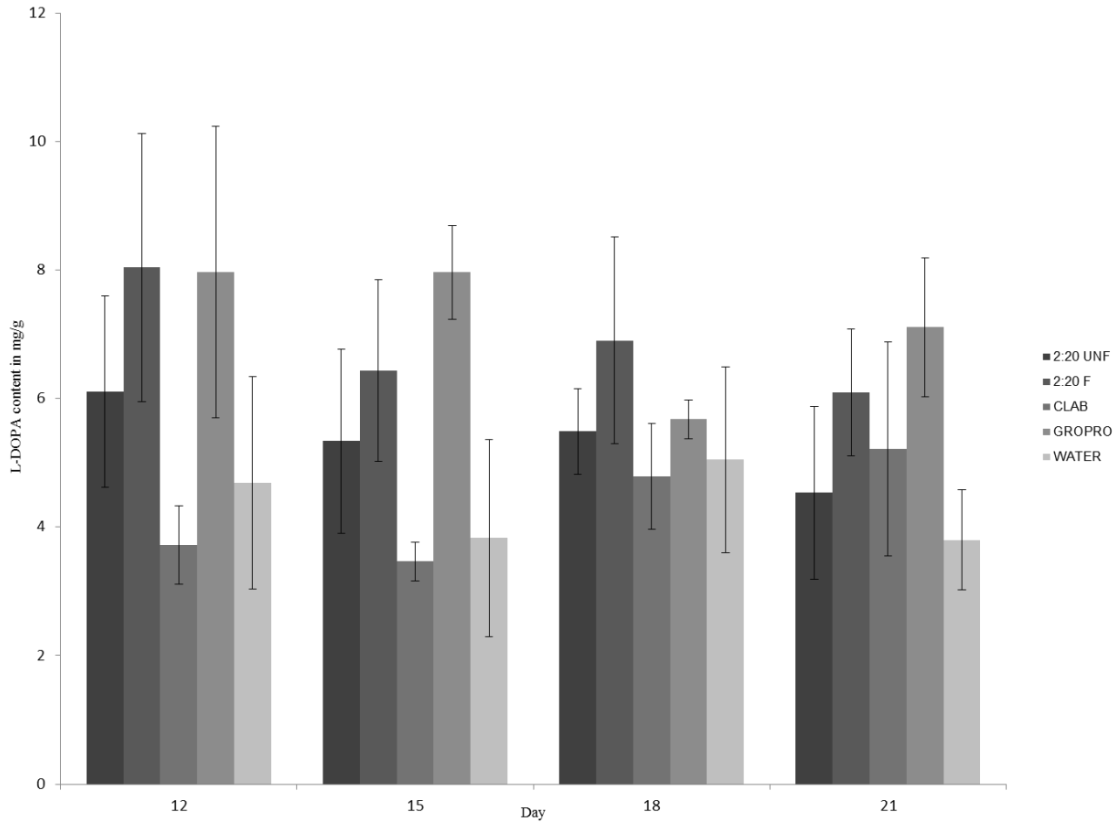

Figure 60: Effect of priming on L-DOPA content (mg g-1 FW) of fava bean leaf tissue (12, 15, 18 and 21 d).

6.2.4.2 Glucose-6-Phosphate Dehydrogenase (G6PDH) and Succinate Dehydrogenase (SDH) activity in fava bean Leaves

G6PDH catalyzes the rate limiting step of the Pentose Phosphate Pathway (PPP) and the activity of this enzyme modulates the stimulation/retardation of the PPP (Puskas et al., 2000). On day 12, GroPro (136.5 nmol/min/mg protein) and CLAB (136.5 nmol/min/mg protein) treatment had higher G6PDH activity than 2:20 UNF (110.6 nmol/min/mg protein), 2:20 F (125.2 nmol/min/mg protein), and water (120.8

nmol/min/mg protein) treatments (Fig 61). On day 15, activity remained same or slightly decreased for all treatments (3.6-12%) except in case of GroPro treatment where the activity decreased sharply (25%). On day 18, G6PDH activity decreased for all treatments (5.9-16.9%) however significant difference ($P>0.05$) was not found between treatments. On day 21, G6PDH activity increased for the fermented (130.9 nmol/min/mg protein) and the unfermented treatments (121.1 nmol/min/mg protein) whereas it remained the same for CLAB (113.8 nmol/min/mg protein), GroPro (115.9 nmol/min/mg protein) and water (104.9 nmol/min/mg protein) treatments. Overall the rise or fall in the activity of this enzyme in individual treatments was not significant over the growth phase.

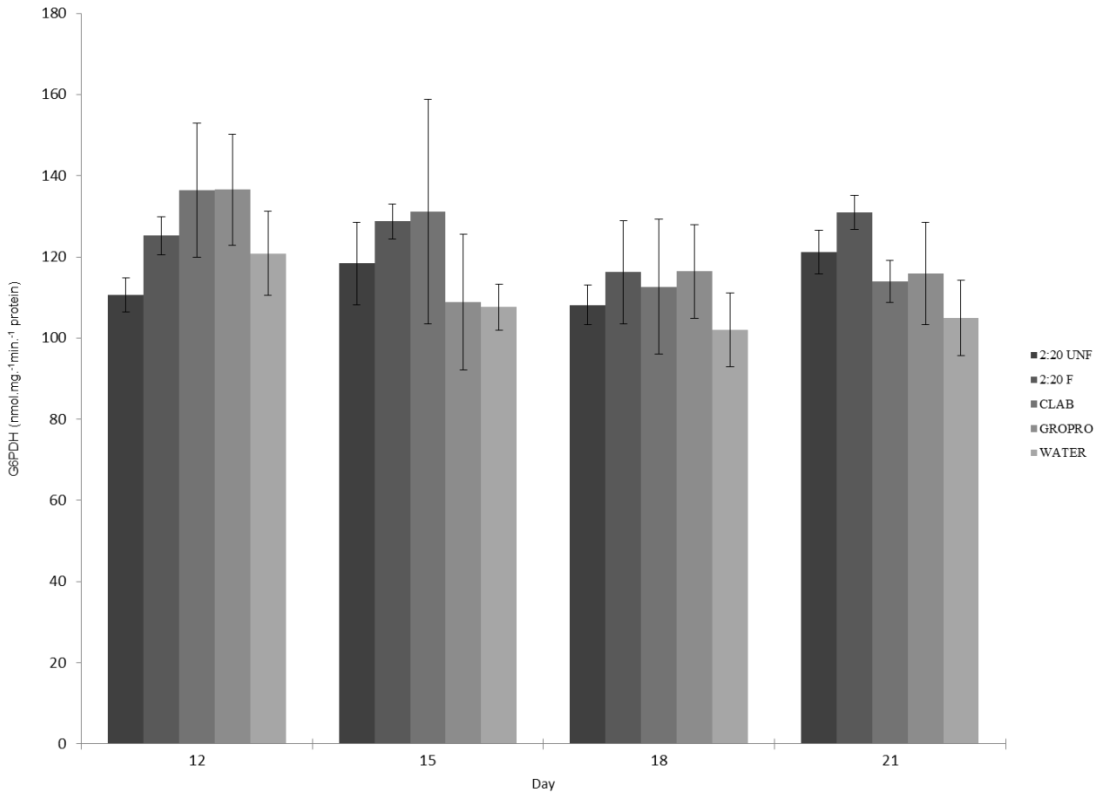

Figure 61: Effect of priming on Glucose-6-phosphate dehydrogenase activity (nmol mg-1 protein) of fava beans leaf tissue (12, 15, 18 and 21 d).

In order to investigate the effect of the elicitor treatment in modulating the Tricarboxylic Acid Cycle (TCA), the activity of the key enzyme SDH was assayed (Fig 62). On day 12, the fermented elicitor (3.3 nmol/min/mg protein) treatment had higher SDH activity as compared to all the other treatments (2.1-2.9 nmol/min/mg protein). On day 15, SDH activity sharply increased in case of 2:20 F (31%), CLAB (106%) and water (31%) treatments. On day 18, the activity of this key enzyme remained the same in 2:20 F (4.8 nmol/min/mg protein), increased in GroPro (4.3 nmol/min/mg protein), 2:20 UNF (3.4 nmol/min/mg protein) treatments whereas it decreased in CLAB (3.5 nmol/min/mg protein) and water (3.3 nmol/min/mg protein treatments. Although on day

21, the activity decreased in all the treatments (3-45%), SDH activity was higher in the fermented elicitor treatment as compared to all other treatments. Overall a general trend for all the treatments was that the activity was low on day 12, then it increased either on day 15 or day 18 and then decreased again on day 21. Highest SDH activity was found in 2:20 F (4.8 nmol/min/mg protein) treatment on day 15 and lowest was found in CLAB (2.2 nmol/min/mg protein) treatment on day 12.

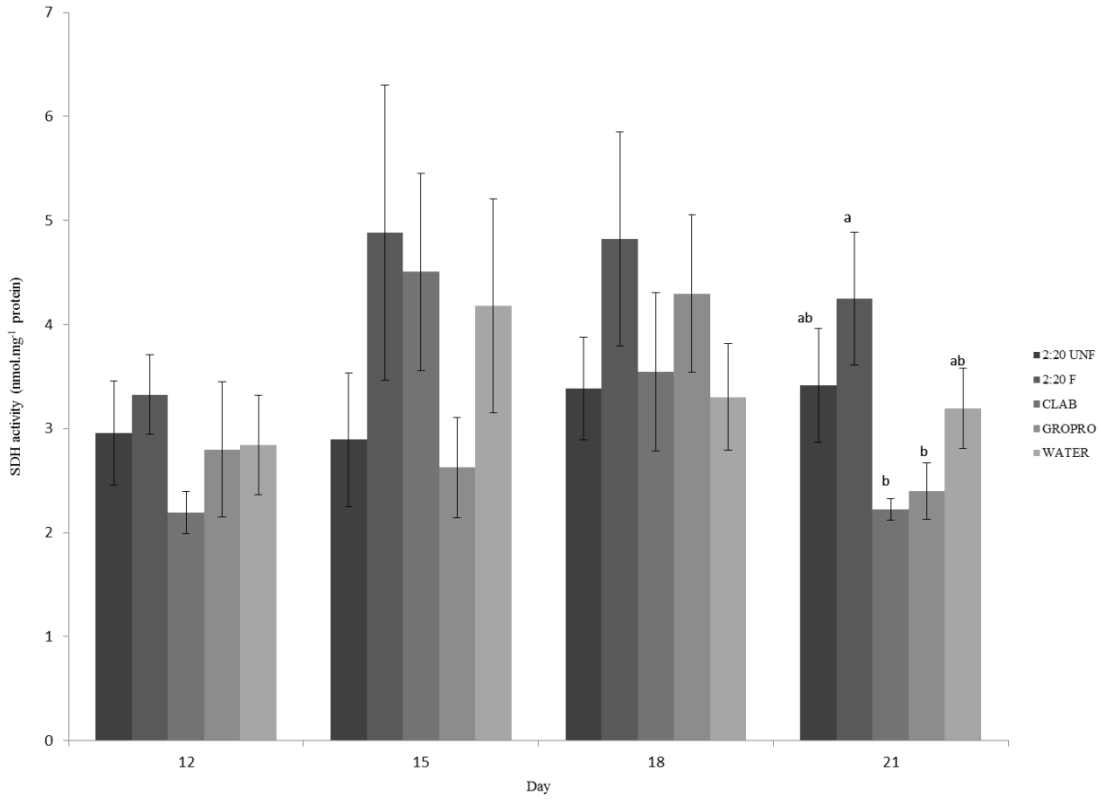

Figure 62: Effect of priming on Succinate dehydrogenase activity (nmol mg-1 protein) of fava beans leaf tissue (12, 15, 18 and 21 d).

6.2.4.3 Proline Dehydrogenase (PDH) Activity and Proline Content in Fava Bean Leaves.

PDH can mediate proline oxidation as a potential alternate energy source via oxidative phosphorylation in the mitochondria. With this rationale the activity of PDH and proline content were investigated (Fig 63). Initial baseline PDH activity on day 12 was similar for all treatments (12.0-14.0 units/min/mg protein) following which on day 15, the activity increased in the fermented elicitor (27%), CLAB (23%) and water (14.5%) treatments whereas it remained constant or slightly decreased for GroPro (9.4%) and 2:20 UNF (0.07%) treatments. On day 18, the activity increased for all treatments except water however; a similar trend was maintained where 2:20 F treatment (18.1 units/min/mg protein) had higher PDH activity than all other treatments (12.6-15.3 units/min/mg protein). Although on day 21, enzyme activity decreased for all treatments except water (increased by 15.8%), and the fermented elicitor treatments had higher PDH activity (16.0 units/min/mg protein as compared to the other treatments (10.2-14.9 units/min/mg protein). Highest PDH activity was found in 2:20 F (18.1 units/min/mg protein) treatment on day 18 whereas lowest activity was found in GroPro (10.2 units/min/mg protein) treatment on day 21.

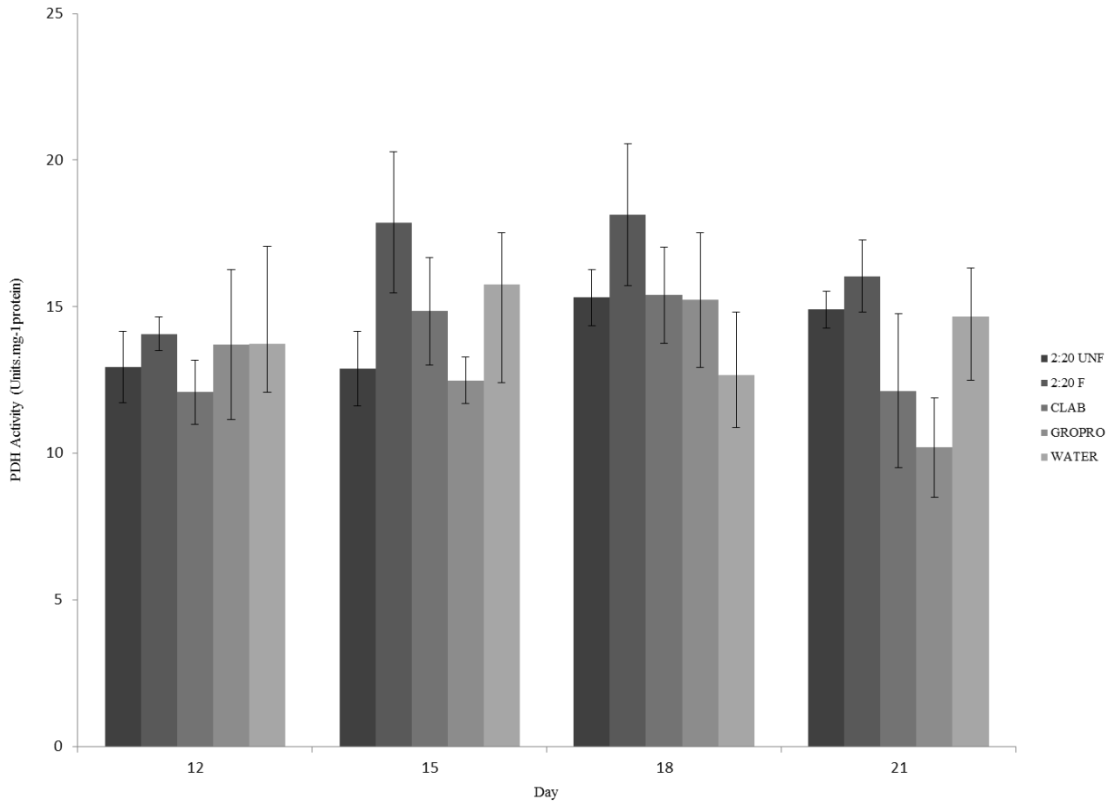

Figure 63: Proline dehydrogenase activity (Units mg-1 protein) of fava beans leaf tissue (12, 15, 18 and 21 d).

On day 12, Proline content was high in GroPro (3.6 mg/g) and water (3.9 mg/g) (Fig 64) treatments. On day 15, it increased for CLAB (3.74 mg/g) treatments whereas it decreased in GroPro (3.1 mg/g) and water (3.3 mg/g) treatments. On day 18, it decreased in all but this decrease was highest in GroPro (49%) treatment. On day 21, it slightly increased for 2:20 UNF (9.1%), 2:20 F (14.2%) and GroPro (6.2%) treatments whereas it decreased for CLAB (16%) and water (31%) treatments. Highest proline

accumulation was found in water on day 12 (3.9 mg/g) whereas lowest was found in GroPro on day 18 (1.6 mg/g) treatments.

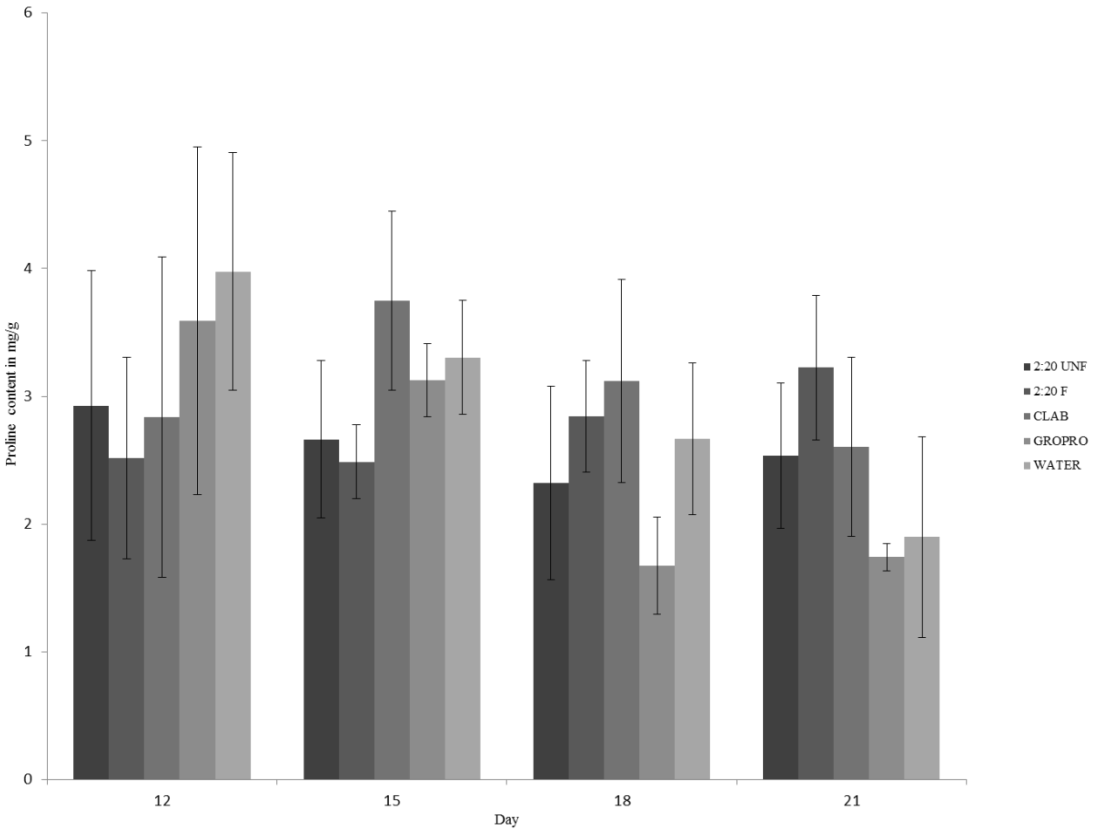

Figure 64: Total proline content (mg g-1 FW) of fava beans leaf tissue (12, 15, 18 and 21 d).

6.2.4.4 Changes in Superoxide Dismutase (SOD), Catalase (CAT), and Guaicol peroxidase (GPX) Activity of Fava Bean Leaves

To investigate the effects of elicitor treatments on inducing antioxidant enzymes, the activity of three key antioxidant enzymes; SOD, CAT and GPX were assayed. SOD activity were similar in all treatments (0.73-0.85 units/mg protein) on day

12, following which on day 15, the activity increased sharply in 2:20 F (33%), CLAB (35%) and water (32%) treatments (Fig 65). On day 18, SOD activity was significantly higher ($P<0.05$) in elicitor treatment as compared to control indicating induction of antioxidant enzyme by elicitors. On day 21, 2:20 F treatment had significantly higher SOD activity ($P<0.05$; 1.09 units/min/mg protein) than all the other treatments (0.77-0.91 units/mg protein). Highest SOD activity was found in 2:20 F treatment on day 21 (1.09 units/mg protein) whereas lowest was found in water treatment on day 12 (0.73 units/mg protein).

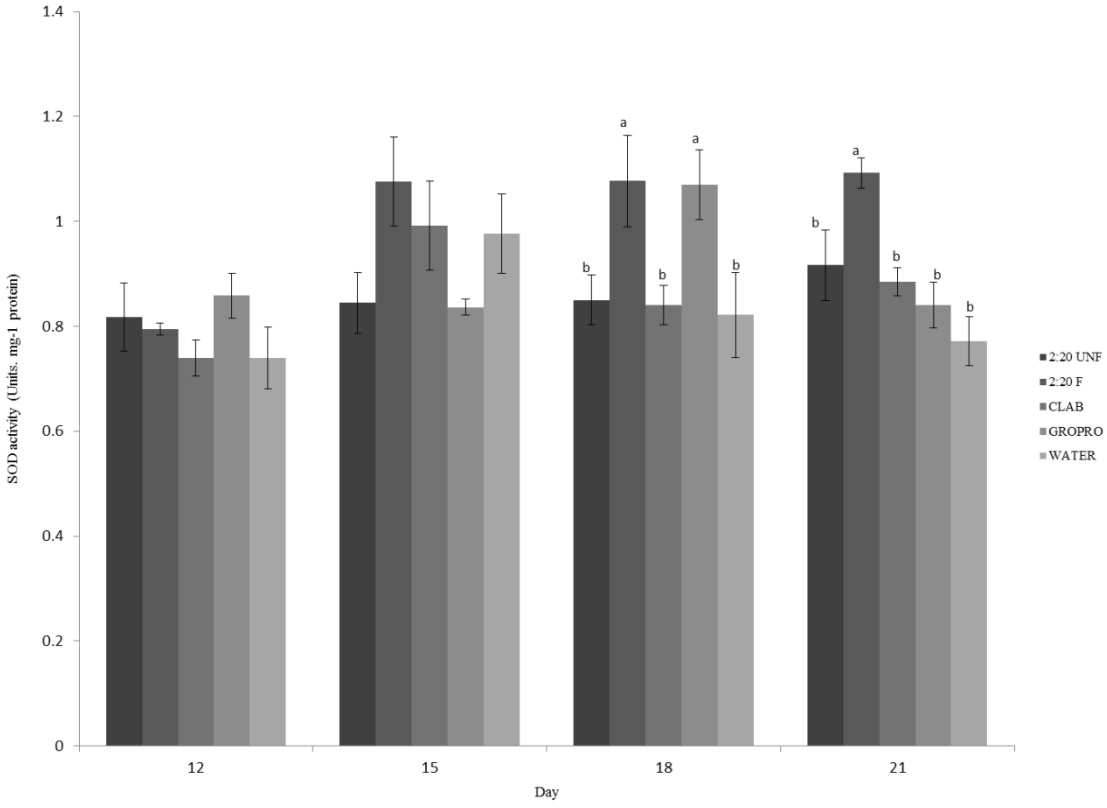

Figure 65: Superoxide dismutase activity (Units mg-1 protein) of fava beans leaf tissue (12, 15, 18 and 21 d).

Initial baseline CAT activity on day 12 was similar among treatments (37-42 units/min/mg protein) (Fig 66). On day 15, CAT activity increased for all treatments (43-48 units/min/mg protein) but was highest for water (54.3 units/min/mg protein) treatment. On day 18, CAT activity decreased slightly (4.4-14.8%) for all treatments except GroPro (61.7 units/min/mg protein) treatment in which case it increased (41%) and had significantly higher ($P<0.05$) activity than all other treatments (38-46 units/min/mg protein). On day 21, CAT activity remained constant for all treatments except GroPro and water treatments where it decreased. Highest CAT activity was found in GroPro (61.7 units/min/mg protein) treatment on day 18, whereas lowest was found in 2:20 UNF (37.7 units/min/mg protein) treatment on day 12.

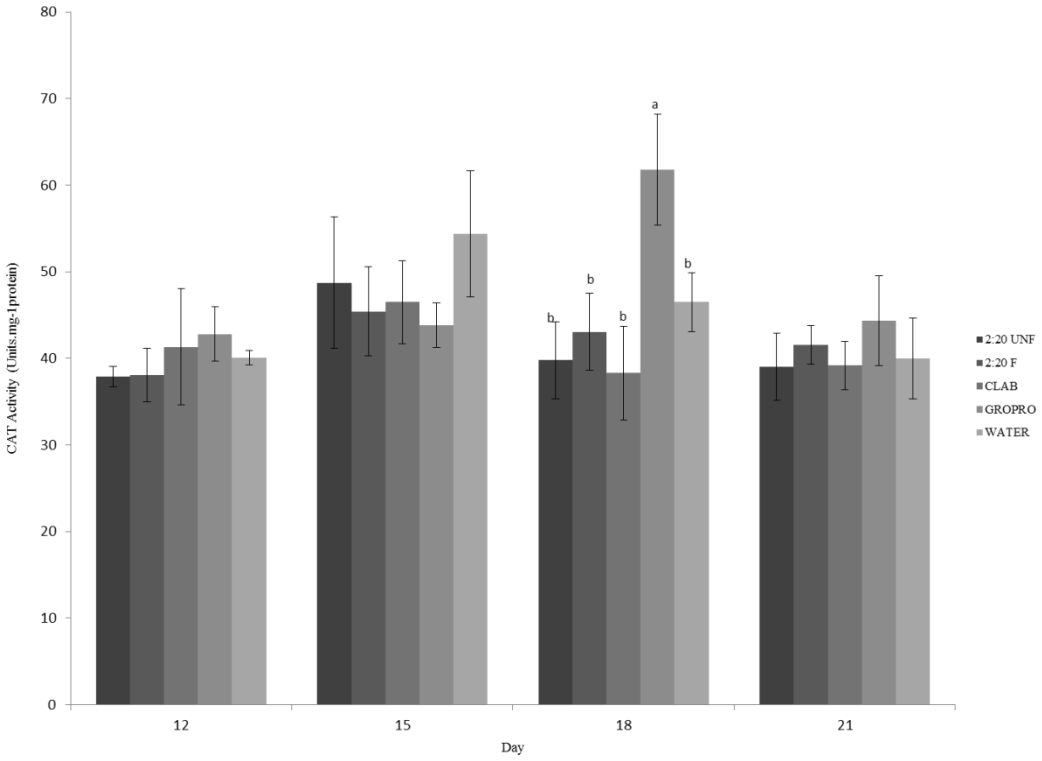

Figure 66: Catalase activity (Units mg-1 protein) of fava beans leaf tissue (12, 15, 18 and 21 d).

GPX converts phenolics to lignin which is required for the structural development of the plant during its growth phase. There was no significant difference ($P>0.05$) in GPX activity between treatments (54-73 nmol/min/mg protein) on day 12 (Fig 67). On day 15 GPX activity decreased for water (30%) and GroPro (49%) treatments whereas for other treatments it remained the same. On day 15, 2:20 UNF and 2:20 F treatments had significantly higher ($P<0.05$) GPX activity compared to GroPro treatment. On day 18, elicitor treatments had higher GPX activity as compared to the controls. On day 21, the activity increased for 2:20 UNF (37.7%) treatment, whereas it decreased for all other treatments (4.4-33.7%). Highest GPX activity was found in 2:20 F (90.4 nmol/min/mg protein) treatments on day 18, whereas lowest was found in GroPro treatments on day 15, (37.3 nmol/min/mg protein).

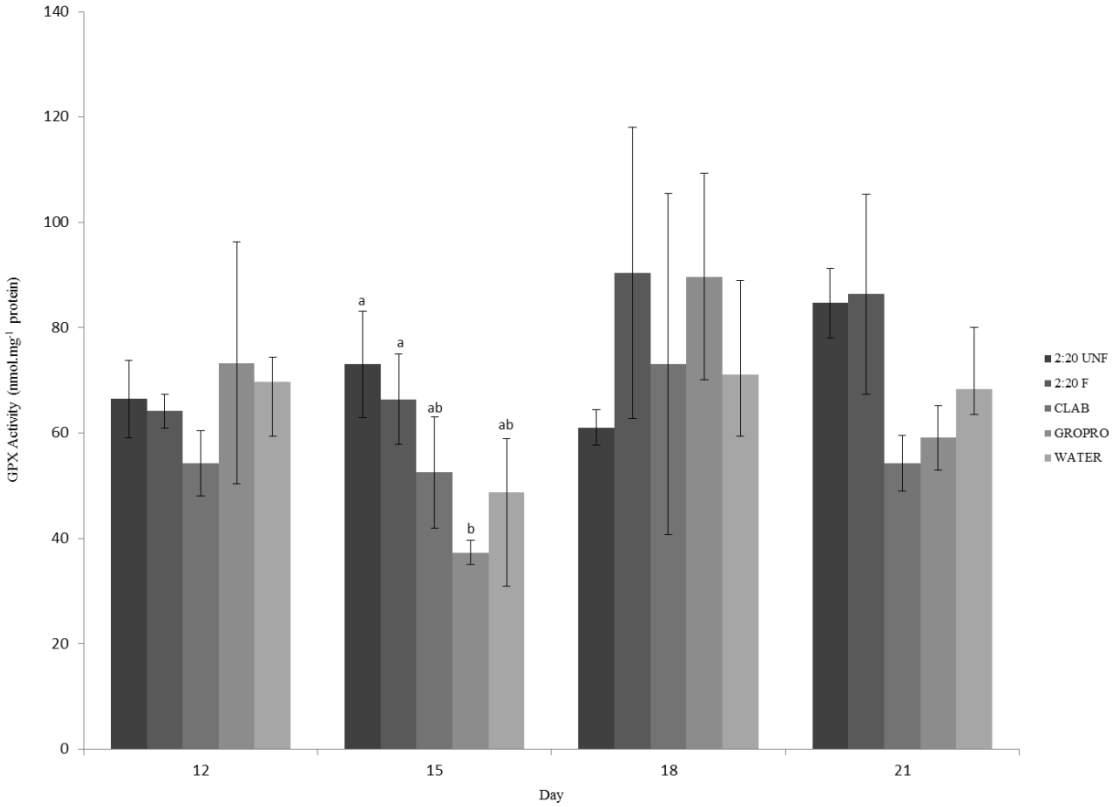

Figure 67: Guaiacol peroxidase activity (µmol mg-1 protein) of fava beans leaf tissue (12, 15, 18 and 21 d).

6.2.5 Discussion

Phenolics are plant secondary metabolites involved in a number of important aspects of plant physiology such as UV protection, constituents of flower pigments and cell walls, modulating interactions between the plant and its environment (Morandi, 1996). Phenolic compounds and antioxidant enzymes may regulate plant defense response by countering ROS species produced during biotic and abiotic stresses (Jiang and Zhang, 2002; Korkina, 2007). Stimulating effect of elicitor signal transduction leading to secondary metabolite synthesis has attracted considerable attention (Zhao et al., 2005). Using this rationale, effects of the phytoelicitors on stimulation of phenolic biosynthesis and antioxidant enzyme response were investigated (Fig. 68).

Elicitor treatments (2:20 F; GroPro) had significantly higher ($P<0.05$) phenolic accumulation and antioxidant enzyme response stimulation as compared to controls (2:20 UNF, CLAB and water), suggesting an increase in stress resistance by elicitor treatments. It has been reported that the elicitors may stimulate phenolic biosynthesis and antioxidant enzyme response by inducing a signaling cascade or by stress like perception due to involvement of ROS, changes in ion fluxes and reversible phosphorylation/dephosphorylation (Randhir et al., 2009; Dixon and Paiva, 1995; Shetty and Wahlqvist, 2004). It is likely that glutamate or proline present in GroPro or small proline mimicking phenolics present in 2:20 F, may accumulate in the seeds during priming and stimulate pathways involved in phenolic synthesis (Randhir and

Shetty, 2003). Overall phenolic content decreased slightly over the growth phase (day 12, 15, 18 and 21). This slight decrease may represent phenolics being portioned towards lignin synthesis, required for plant growth and development (Randhir and Shetty, 2005; Rogers and Campbell, 2004). This is further confirmed by a slight increase in GPX activity on day 18 and day 21. Higher phenolic content on day 12 may represent oxidative stress during early germination due to a high demand for oxygen and therefore an increased need for phenolic antioxidants to scavenge and protect the cells from ROS (Randhir and Shetty, 2003). Our data is in conformation with the previous reports regarding up regulation of phenolic synthesis and biosynthetic pathways involved, in response to various biotic and abiotic elicitors (Conceicao et al., 2006; Daayf et al., 2003; Vattem et al., 2005c; Shetty et al., 2003; Lozoya et al., 1991; Zahringer et al., 1978).

Antioxidant content determined using ABTS assay was high in the fava bean leaves through the growth phase (day 12, 15, 18 and 21) and did not change significantly between treatments. This indicates fava bean is a powerful antioxidant source and elicitor treatments did not have a significant effect in increasing its antioxidant potential. High antioxidant capacity of fava bean has been previously reported (Amarowicz et al., 1996; Berghofer et al., 1998). L-DOPA content was higher in elicitor treatment during the growth phase (day 12, 15, 18, 21). Total phenolic synthesis was stimulated in the elicitor treatment as compared to the control. This indicates an up regulation of the shikimate pathway driving anabolic precursors from glycolysis and pentose phosphate pathway towards synthesis of ring structures of phenylalanine and tyrosine. Tyrosine is hydroxylated to form L-DOPA (Elsworth and

Roth, 1997). Up regulation of shikimate pathway should stimulate L-DOPA synthesis. L-DOPA stimulation in fava bean in response to various peptide and phytochemical elicitors have been reported (Randhir and Shetty, 2003; Randhir et al., 2002; Shetty et al., 2003).

G6PDH catalyzes the rate limiting step of the pentose phosphate pathway and it converts glucose into carbon skeletons required in synthesis of nucleotides, aromatic amino acids and phenolics (Kruger and von Schaewen, 2003). Overall, G6PDH remained constant or slightly decreased over the growth phase which indicates mobilization of carbohydrate reserves to the growing sprouts (Randhir and Shetty, 2005). By contrast, an increase in G6PDH activity during sprouting and initial growth phase has been reported (Panneerselvam et al., 2007; Gosling and Ross, 1980). This might be because the elicitors and/or the conditions used for sprouting in their study were different. In response to various peptide and phytochemical elicitors an initial increase, followed by a decrease in the G6PDH activity during germination has been reported (Randhir and Shetty, 2003; Shetty et al., 2003; Randhir et al., 2002; Randhir et al., 2004; Andarwulan and Shetty, 1999; Randhir and Shetty, 2005). Change in activity of G6PDH prior, during, and after dormancy breaking has been reviewed in a range of seeds (Botha et al., 1992).

Respiration drives carbon flux towards two essential needs of oxidation of substrates to provide energy and provide intermediates required for biosynthesis (Botha et al., 1992). SDH, a key enzyme of the TCA cycle, generates NADH for oxidative phosphorylation and was assayed to investigate the energy demands of the germinating seed. Fermented elicitor (2:20 F) had higher SDH activity throughout the growth phase

as compared to other treatments and this difference was significant on day 21 between CLAB, GroPro and 2:20 F. This suggests that in fava bean seedsprouts treated with 2:20 F there is a higher need for cellular energy and this is achieved by stimulating the TCA cycle. In germinating seeds, an increase in SDH activity in peanut was observed whereas in cucumber activity remained constant (Botha et al., 1992). Phenolic biosynthesis is based on the regulated coordination of complex network of routes from at least five different pathways. Anabolic pathways involved are the glycolytic pathway that provides phosphenol pyruvate and pentose phosphate pathway that provides erythrose-4-phosphate (Randhir et al., 2009). These anabolic precursors then enter the shikimate pathway where phenylalanine is produced which is the starting point for all phenolic compounds. Phenylalanine is then modified through the phenylpropanoid and the flavonoid pathway to produce a spectrum of phenolic compounds commonly consumed in the diet touted for their diverse health beneficial effects (Crozier et al., 2009). In this study, G6PDH activity was not significantly different among treatments however, the 2:20 F treatment had significant phenolic accumulation as compared to other controls. This may be because 2:20 F treatment had higher SDH activity indicating a higher carbon flux through glycolytic pathways providing the required anabolic precursor in the form of phosphenolpyruvate for phenolic synthesis. The combination of higher G6PDH and SDH activity may provide a balance between the anabolic and catabolic needs in elicitor treatments and subsequently help to drive phenolic biosynthesis and antioxidant enzyme response in fava bean leaves. Higher carbon flux through the glycolytic pathway suggests higher proline synthesis through glutamate production (Shetty and Wahlqvist, 2004). Accumulated proline can provide

reducing equivalents to support oxidative phosphorylation in the mitochondria providing an alternate energy source for ATP synthesis (Hare and Cress, 1997). This was further confirmed by higher PDH activity in 2:20 F as compared to all the other treatments throughout the growth phase.

Superoxide radicals ($O_2^{\cdot -}$) are acted upon by SOD and converted to H_2O_2 which are further acted upon by CAT and converted to water and molecular oxygen (Jaleel et al., 2009). Fermented elicitor gave a significantly higher SOD response as compared to treatments on day 18 and day 21. GroPro treatment had significantly higher activity as compared to controls on day 18. 2:20 F treatment increased the catabolic need of the germinating fava bean and stimulated the glycolytic pathway. It has been reported that mitochondrial metabolism can produce ROS (Andreyev et al., 2005) and it is possible that this high amount of ROS may have triggered a higher SOD response. This indicates a better resistance of the fava bean leaves in dealing with oxidative pressure with the 2:20 F and GroPro treatments. Catalase activity was higher in control treatment with water on day 15 and it was significantly higher in GroPro treatment on day 18. Higher CAT activity in water indicates an inherent ability of fava bean to induce CAT response. Additionally elicitor treatment GroPro treatment may induce a higher CAT response in order to stimulate the defense response against peroxides induced oxidative stress produced by the conversion of superoxide radicals to hydrogen peroxides by SOD. On day 15, GroPro treatment had low antioxidant enzyme response as compared to other treatments. This might have led to a build up of ROS and stimulated antioxidant enzyme response which is evident from day 18 results. An increase in SOD and CAT activity during germination in sunflower seeds has been reported by an osmotreatment

with polyethylene glycol (Bailly et al., 2000). Similarly in sunflower seeds an increase in activity of all antioxidant enzymes except SOD has been reported during germination (Bailly et al., 2002) Expression of antioxidant enzymes in relation to ROS produced during seed germination has been reviewed (Bailly, 2004). SOD activity was reported to increase in dark germinated *Mucuna pruriens* sprouts by peptide and phytochemical elicitors (Randhir et al., 2009).

GPX plays an important role in redox reactions in plasma membranes, cell wall modifications and in defense mechanism **(**Sarkar et al., 2009a**)**. GPX activity increased slightly which coincided with the slight decrease in phenolics which indicates phenolics being portioned for lignification. GPX is an isoenzyme and can act as an antioxidant protecting the cells from oxidative pressure. On day 15, 2:20 UNF and 2:20 treatments had significantly higher GPX activity as compared to GroPro treatment. GPX activity has been reported to increase in light and dark germinated fava beans, mung beans, corn and pea by various peptide and phytochemical elicitors (Randhir and Shetty, 2003; Shetty et al., 2003; Randhir et al., 2002; Andarwulan and Shetty, 1999; Randhir and Shetty, 2005).

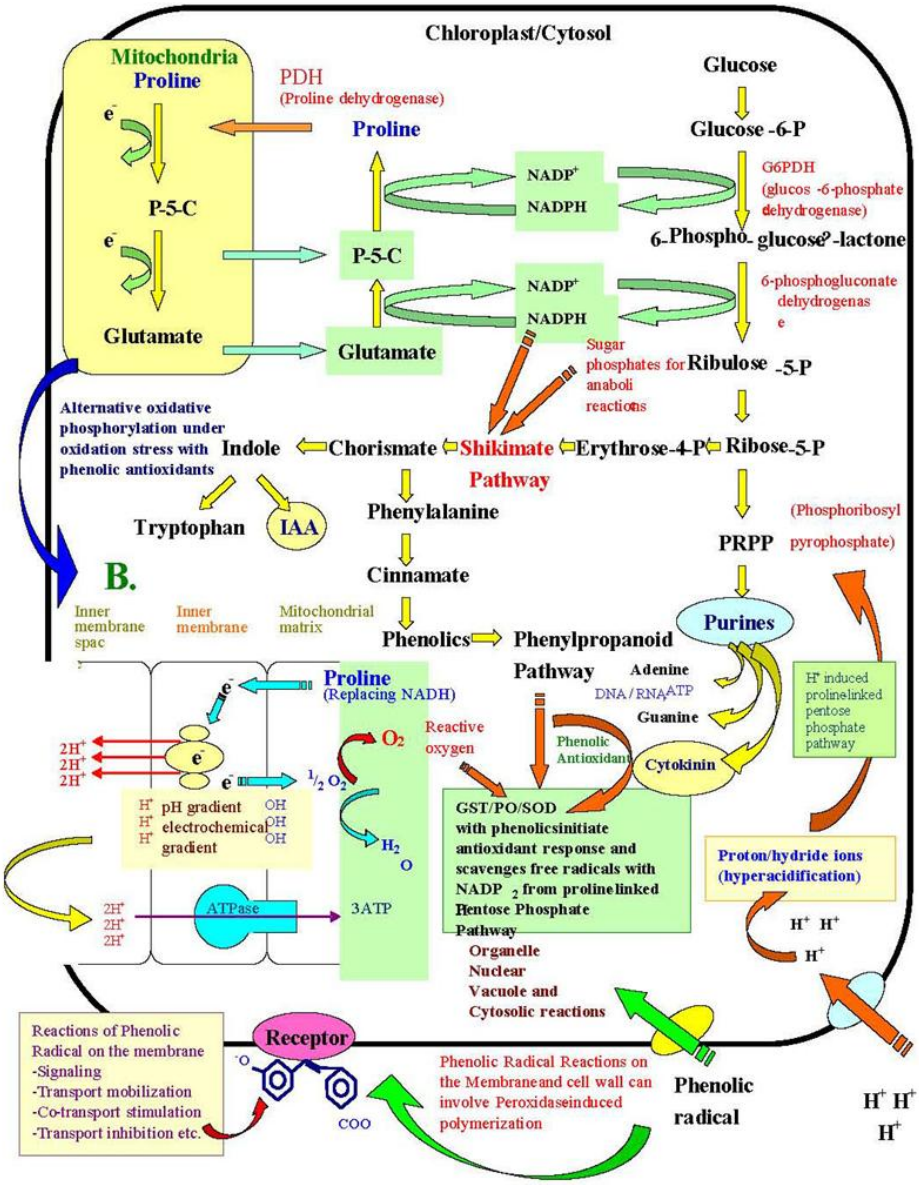

Figure 68: Proline linked Pentose Phosphate Pathway

6.2.6 Conclusion

This study provides insights and strategy into metabolic regulation by energy partitioning using natural elicitors to improve defense responses of the germinating seed during initial growth phase. This study demonstrates the application of a novel fermented elicitor for stimulating phenolic biosynthesis and antioxidant enzyme response in fava bean. Phenolic biosynthesis in the elicitor treatment was likely stimulated by using proline as an alternate energy source and by a stimulated carbon flux through the TCA cycle. This novel approach and innovative strategy provides basis for practical applications of using such elicitors for improving nutritional and health relevant functional value of legumes. This strategy can be the basis of better design of functional foods and ingredients for better health.

6.3 Fermented Apple Extract Mediated Antioxidant Enzyme Response in Oxidatively Stressed *Saccharomyces cerevisiae* and likely Mode of Action Through Proline-Associated Pentose Phosphate Pathway.

6.3.1 Abstract

Understanding the role of phenolic compounds in stimulating antioxidant response is essential in interpreting the recent epidemiological reports of linking lower incidences of oxidation-linked diseases from consumption of a diet rich in fruits and vegetables. In this study the effect of fermented apple extracts with high phenolic antioxidants in stimulating antioxidant response in different yeast treatment models were evaluated. In pretreatment model (A) fermented apple extract was added to the yeast growth media, in concurrent model (B) fermented apple extract was added just before exposure to H_2O_2 and in control no fermented apple extract was added. Concurrent treatment model (B) had the best yeast survival rate after H_2O_2 exposure, followed by control and then pretreatment model (A). Yeast metabolism after H_2O_2 exposure was mainly supported by pentose phosphate pathway which was indicated by no change in activity of G6PDH, key enzyme of PPP, and a decrease in activity of SDH, key enzyme of the TCA cycle. No significant change was found in the activity of antioxidant enzyme SOD and CAT assayed in this study between the different treatment models. However MDA content, a reflection of oxidative damage was lower in pretreatment model reflecting protection from fermented apple juice. Although yeast, after H_2O_2 exposure, shifts to an alternate metabolism involving proline oxidation linked to stimulation of PPP to support its energy needs, the role of fermented apple extracts in simulating the assayed antioxidant enzyme response is not strong enough to

make a case for the role of proline in this study. This may be due to the high concentration of oxidant used in this study may potentially inhibit the alternate energy metabolism model involving proline oxidation, thereby reducing its role in supporting the energy needs and hence survival of yeast.

6.3.2 Introduction

Damage to DNA, lipids and proteins has been proposed to be a causative response in a growing number of diseases including CVD, certain types of cancer, neurodegenerative diseases and age related macular degeneration (Winkler et al., 1999; Halliwell, 2001). Essential as regulatory mediators in the signaling process at moderate concentrations, these free radicals and free radical-derived, nonradical species have the potential to damage all cellular constituents at high concentrations (Droge, 2002). Reactive oxygen species (ROS) may be produced as a result of normal metabolic processes such as respiration or may be induced by exogenous sources such as UV, toxins, and inflammation (Shao et al., 2008). Aerobic organisms have developed multiple defense strategies including antioxidant enzymes and cellular antioxidants in order to minimize and repair the damage caused by oxidative stress (Juhnke et al., 1996). Antioxidant enzymes such as Superoxide dismutase (SOD) acts on Superoxide radicals ($O_2^{\cdot -}$) and converts them to H_2O_2 which are further acted upon by Catalases (CAT) which convert them to water and molecular oxygen (Jaleel et al., 2009). Cellular antioxidants such as Ascorbic acid, Vitamin E and Glutathione play an important role in the defense against oxidative stress as well (Juhnke et al., 1996).

Recent epidemiological studies highlight the lower incidences of oxidation linked diseases such as cancer, diabetes and cardiovascular diseases (CVD) associated with a diet rich in fruits and vegetables (Serdula et al., 1996; Hung et al., 2004). This has been partly attributed to the presence of phenolic phytochemicals (Crozier et al., 2009). Phenolic phytochemicals can act as antioxidants due to the presence of a phenolic ring and the side chain hydroxyl substituents. Therefore, dietary phenolic phytochemicals can scavenge harmful free radicals and chelate pro-oxidant metal ions thereby alleviating increased oxidative damage, causative in the development of many age related diseases (Barja, 2004; Petti and Scully, 2009). Phenolic secondary metabolites are commonly synthesized and accumulated by plants in response to environmental and biological stress such as high energy radiation exposure, bacterial infection or fungal attacks (Dixon and Paiva, 1995; Briskin, 2000). Flavonoids, the most common source of phenolic compounds in the diet, may modulate stress response by several different mechanisms including acting as an antioxidant, inducing antioxidant enzyme response, screening harmful radiation, binding phytotoxins and regulating auxin transport (Winkel-Shirley, 2002; Shetty and Wahlqvist, 2004).

Cellular antioxidant homeostasis is maintained by antioxidant enzymes such as superoxide dismutase (SOD), Catalase (CAT), Glutathione peroxidase (GPX), Glutathione S-transferase (GST) and molecular antioxidants such as tocopherols, ascorbate, glutathione by a series of redox reactions (Foyer and Noctor, 2005; Mates et al., 2008). Regeneration of these antioxidant defense systems, an energy intensive process, is essential to maintain efficiency and requires cellular reducing equivalents in the form of NADPH and $FADH_2$ (Mates and Sanchez-Jimenez, 1999; Mates et al.,

1999; Nordberg and Arner, 2001). NADH and $FADH_2$ provide reducing equivalents for reduction of molecular oxygen to water which results in ATP synthesis via oxidative phosphorylation in the mitochondria. These reducing equivalents for mitochondrial oxidative phosphorylation can also be provided by proline replacing NADH (Shetty and Wahlqvist, 2004). Imposition of biotic and abiotic stresses lead to accumulation of free proline by reduction of pyroline-5-carboxylate (P5C) using reducing equivalents in the form of NADPH which leads to formation of $NADP^+$ (Verbruggen and Hermans, 2008). $NADP^+$ is a co-factor of the Glucose-6-Phosphate Dehydrogenase (G6PDH), an enzyme which drives the rate limiting step of the pentose phosphate pathway. Using this rationale, an alternate model for proline metabolism was proposed where proline biosynthesis is linked to stimulation of Pentose Phosphate Pathway (PPP) through $NADP^+$/NADPH redox balance (Shetty and Wahlqvist, 2004). This up-regulated PPP provides excess metabolic intermediates for anabolic reactions including secondary metabolite synthesis whereas proline and P5C can act as a redox couple for oxidative phosphorylation providing a non-NADH route for ATP synthesis (Hare and Cress, 1997; Shetty and Wahlqvist, 2004). Further, phenolic antioxidants may mediate the antioxidant response of the cell, not only by acting as direct scavengers of ROS but also by stimulating proline biosynthesis which provides excess NADPH to aide cellular antioxidant defense response (Shetty and Wahlqvist, 2004).

Yeast model is an inexpensive and reliable way to simulate free radical biology in eukaryotic systems with the additional advantage of easy delivery of desired food extracts. The antioxidant defense systems in yeast are well elucidated and it gives us an ideal platform for physiological studies of oxidant effects because of its microbial

habitat (Collinson and Dawes, 1992). Results from this study will help us evaluate the effectiveness of high phenolic antioxidant food extracts in protecting eukaryotic cells against oxidative stress which can have potential applications as a part of dietary strategy to prevent chronic oxidation-linked diseases in humans. Lactic acid bacteria mediated mobilization of phenolics using fermentation based processing has been shown in soymilk (McCue and Shetty, 2005; Wang et al., 2006). Liquid state /submerged fermentation involves reduced or lack of oxygen and hence represents a reductive state for maintaining redox protective bioactive compounds. Here we investigated the effect of mobilized phenolic from fermented apple juice extracts to stimulate cellular antioxidant response in oxidatively stressed yeast cells.

6.3.3 Materials and Methods

6.3.3.1 Total Phenolics Assay

The total phenolics was determined by an assay modified from Shetty et al (1995). Briefly, one mL of fermented apple extract extract was transferred into a test tube and mixed with 1 mL of 95 % ethanol and 5 mL of distilled water. To each sample 0.5 mL of 50 % (v/v) Folin-Ciocalteu reagent was added and mixed. After 5 min, 1 mL of 5 % Na_2CO_3 was added to the reaction mixture and allowed to stand for 60 min. The absorbance was read at 725 nm. The absorbance values were converted to total phenolics and were expressed in micrograms equivalents of gallic acid per milliliter of

the sample. Standard curves were established using various concentrations of gallic acid in 95 % ethanol.

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

To 3 mL of 60 μM DPPH in ethanol, 250 μL of fermented apple extract was added, the decrease in absorbance was monitored at 517 nm until a constant reading was obtained. The readings were compared with the controls, which contained 250 μL of 95 % ethanol instead of the extract. The % inhibition was calculated by:

$$\%\ \text{inhibition} = \left(\left[\frac{A_{517}^{Control} - A_{517}^{Extract}}{\left[A_{517}^{Control} \right]} \right] \right) x100$$

6.3.3.3 Fermentation

One cultivar of Apple: Red delicious; was obtained from Stop and Shop, Hadley, MA. Whole Apples were washed with water and then cut into small pieces. The edible pieces were homogenized using a Waring blender for 5 min. The thick pulp was then centrifuged for 15 min at 15,000g, supernatants were collected and kept at -20^{0}C during the period of the study. Apple juice was then fermented using *Lactobacillus helveticus* (LA) provided by Rosell Institute Inc. Montreal, Canada (Lot#: XA 0145, Seq#:00014160). Five mL of the overnight grown culture of LA was added to 45 mL of apple juice and fermented for 24 h at 37 ^{o}C. Fermented apple juice was then filter

sterilized and stored in sterile tubes at 4 ^{o}C during the period of the study. Unless noted, all chemicals also were purchased from Sigma Chemical Co. (St. Louis, MO)

6.3.3.4 Preparation of Starter Culture of *Saccharomyces cerevisiae*

A 125-mL Erlenmeyer flask containing 50 mL potato dextrose broth (PDB; 4 g of potato starch per liter and 20 g of dextrose per liter of water, Difco Laboratories, Detroit, MI) was used for yeast growth (Baker's yeast; ATCC 32167). A volume of 0.1 mL of stock *S. cerevisiae* was added into 125-mL Erlenmeyer flask containing 50 mL of broth media. A volume of 1 mL yeast culture was added to 49 ml of PDB, mixed and then incubated at 37 ^{o}C for 24 h in non-shaking conditions for the concurrent model (B) and control (C). Ten mL fermented apple juice was added to 39 mL of PDB for the pre-treatment model (A) and incubated at 37 ^{o}C for 24 h in non-shaking conditions after inoculating it with one mL yeast culture. In this study, pre-treatment model is represented by letter A; Concurrent model by letter B and the control by C.

6.3.3.5 Sensitivity of *Saccharomyces cerevisiae* extract to H_2O_2 induced Oxidative Stress

After 24 h cells were harvested by centrifugation, 50 mL culture was distributed into two sterile 25 mL tubes and washed twice with water, resuspended in 9 mL sterile water. Resuspension volumes were different for different treatments**.** One mL of 2.5 M H_2O_2 was added to a final volume of 10 mL for each treatment model. For concurrent

treatment model (B), 2 mL fermented apple extract was added just before adding one mL H_2O_2. Cells were exposed to H_2O_2 for 0 ~ 20 min at 25 °C with intermittent shaking every 2-3 min. For all experiments, untreated and treated cells were immediately diluted and plated on Potato Dextrose agar. Colonies were scored after 48 h of growth at 37 °C. All experiments were independently carried out four times.

6.3.3.6 Enzyme Extraction

The culture broth of *S. cerevisiae* was harvested after H_2O_2 exposure by centrifuging at 12,000 x g for 10 min at 2-5 ºC and resuspended in 2.0 mL of 50 mM potassium phosphate buffer of pH 7.5 (0.15 M NaCl and 1 mM EDTA) and stored on ice. This was then thoroughly vortexed with 2.5 g bead ball (0.4 - 0.6 mm) for 6 min at 2-5 ºC with intermittent mixing every 1 min and then 1 min storage in ice for heat management (Vortexer; Biospec products, OK). The sample was again centrifuged at 12000 x g for 15 min at 2-5 ºC and stored on ice. The supernatant was used for further analysis.

6.3.3.7 Total Protein Assay

Protein content was measured by the method of Bradford assay (Bradford, 1976). The dye reagent concentrate (Bio-Rad protein assay kit II, Bio-Rad Laboratory, Hercules, CA) was diluted 1:4 with distilled water. A volume of 5 mL of diluted dye reagent was added to 50 µL of the yeast extract. After vortexing and incubating for 3

min, the absorbance was measured at 595 nm against a 5 mL reagent blank and 50 µL buffer solutions using a UV-VIS Genesys spectrophotometer (Milton Roy, Inc., Rochester, NY).

6.3.3.8. Glucose-6-Phosphate Dehydrogenase (G6PDH) Assay

A modified version of the assay described by Deutsch, (1983) was followed. The enzyme reaction mixture containing 5.88 µmol ß-NADP, 88.5 µmol $MgCl_2$, 53.7 µmol glucose-6-phosphate, and 0.77 mmol maleimide was prepared. This mixture was used to obtain baseline (zero) of the spectrophotometer reading at 340 nm wavelength. To 1 mL of this mixture, 100 µL of the enzyme sample was added. The rate of change in absorbance per minute was used to quantify the enzyme in the mixture using the extinction co-efficient of NADPH (6.22 $mM^{-1}cm^{-1}$).

6.3.3.9. Succinate Dehydrogenase (SDH) Assay

Modified method described by Bregman, (1987) was used to assay the activity of succinate dehydrogenase. The assay mixture consisted of the following: 1.01 mL of 0.4 M potassium phosphate buffer (pH 7.2); 40 µL of 0.15 M sodium succinate (pH 7.0); 40 µL of 0.2 M sodium azide; and 10 µL of 6.0 mg/mL DCPIP. This mixture was used to obtain baseline (zero) of the spectrophotometer reading at 600 nm wavelength. To 1.0 mL of this mixture, 200 µL of the enzyme sample was added. The rate of change in absorbance per minute was used to quantify the enzyme in the mixture using the extinction co-efficient of DCPIP (19.1 $mM^{-1}cm^{-1}$).

6.3.3.10. Superoxide Dismutase (SOD) Assay

A competitive inhibition assay was performed that used xanthine-xanthine oxidase-generated superoxide to reduce nitroblue tetrazolium (NBT) to blue formazan. Spectrophotometric assay of SOD activity was carried out by monitoring the reduction of NBT at 560 nm (Oberley and Spitz, 1985). The reaction mixture contained 13.8 mL of 50 mM potassium phosphate buffer (pH 7.8) containing 1.33 mM DETAPAC; 0.5 mL of 2.45 mM NBT; 1.7 mL of 1.8 mM xanthine and 40 IU/mL catalase. To 0.8 mL of reagent mixture 100 μL of phosphate buffer and 100 μL of xanthine oxidase was added. The change in absorbance at 560 nm was measured every 20 sec for 2 min and the concentration of Xanthine oxidase was adjusted to obtain a linear curve with a slope of 0.025 absorbance per min. The phosphate buffer was then replaced by the enzyme sample and the change in absorbance was monitored every 20 sec for 2 min. One unit of SOD was defined as the amount of protein that inhibits NBT reduction to 50% of the maximum.

6.3.3.11. Catalase (CAT) Assay

A method originally described by Beers and Sizer, (1952), was used to assay the activity of catalase. To 1.9 mL of distilled water 1 mL of 0.059 M hydrogen peroxide (Merck's Superoxol or equivalent grade) in 0.05 M potassium phosphate, pH 7.0 was added. This mixture was incubated in a spectrophotometer for 4-5 min to achieve temperature equilibration and to establish blank rate. To this mixture 0.1 mL of diluted enzyme sample was added and the disappearance of peroxide was followed

spectrophotometrically by recording the decrease in absorbance at 240 nm for 2-3 min. The change in absorbance ΔA_{240}/min from the initial (45 sec) linear portion of the curve was calculated. One unit of catalase activity was defined as amount that decomposes one micromole of H_2O_2

$$\text{Units /mg} = \frac{\Delta A_{240}/\text{min} \times 1000}{43.6 \times mg\ enzyme/mL\ of\ reaction\ mixture}$$

6.3.3.12 Malondialdehyde (MDA) Assay

MDA content was measured by following all the steps untill yeast extraction except that it was resuspended in 2.5 mL 70% ethanol instead of 50 mM potassium phosphate buffer (Tamagnone et al., 1998). During the homogenization step with glass beads 25 μL of butylated hydroxyl toluene was added. The extract was then centrifuged at 12,000 x g for 5 min. A volume of 200 μL of the *S. cerevisiae* extract was mixed with 800 μL of water, 500 μL of 20 % (w/v) trichloroacetic acid and 1 mL of 10 mM thiobarbutyric acid. The test mixture were incubated for 30 min at 100 ^{o}C and then centrifuged at 12,000 x g for 10 min. The absorbance of the supernatant was measured at 532 nm, 600 nm and 44 nm and the concentration of MDA was calculated from its molar extinction coefficient (ε) 156 $\mu mol^{-1}cm^{-1}$.

6.3.3.13. HPLC Analysis of Proline

High performance liquid chromatography (HPLC) analysis was performed using an Agilent 1100 liquid chromatograph equipped with a diode array detector (DAD 1100). The analytical column was reverse phase Nucleosil C18, 250 nm x 4.6 mm with a packing material of 5 µm particle size. The extract samples were eluted out in an isocratic manner with a mobile phase consisting of 20 mM potassium phosphate (pH 2.5 by phosphoric acid) at a flow rate of 1 mL min^{-1} and detected at 210 nm. L-Proline (Sigma chemicals, St. Louis, MO) dissolved in the 20 mm potassium phosphate solution was used to calibrate the standard curve. The amount of proline in the sample was calculated as mg of proline per milliliter.

6.3.3.14. Statistical Analysis

All experiments were performed with a minimum of four replications for each treatment. The effect of each treatment was determined by the analysis of variance (ANOVA) of SAS (version 8.2; SAS Institute, Cary, NC). Differences among different treatments were determined by the Fishers least significant difference (LSD) test at the 0.05 probability level. Standard error was calculated using Microsoft Excel 2010.

6.3.4 Results

6.3.4.1 Total Phenolics and Antioxidant activity by DPPH

The total phenolic content was analyzed by the Folin-Ciocalteu method. Total phenolic content was found to be 0.2 mg/mL fermented extracts. The free radical linked antioxidant activity of the extracts was monitored using the DPPH radical inhibition (DRI) assay. Fermented apple extracts had 60% inhibition indicating fermented apple juice has good free radical linked antioxidant activity.

6.3.4.2 Changes in CFU during H_2O_2 Induced Oxidative Stress.

Total colony forming units (CFU) of yeast pre-treatment model (A), concurrent treatment model (B) and control (C) were evaluated to determine the potential protective effects of fermented apple juice extract against H_2O_2 induced oxidative stress (Fig. 69). At 0 min the growth range was around 10^7 CFU/mL for all three models. After 10 min the CFU/mL value was significantly lower ($P<0.05$) for pretreatment model (A) than the concurrent model (B) and the control (C). After 20 min, CFU/mL for concurrent model (B) was significantly higher ($P<0.05$) than pretreatment model (A) and control (C). This suggests that in the pretreatment model fermented apple does not counter the death of yeast cells induced by H_2O_2 whereas in the concurrent model fermented apple extracts may be better in protecting the yeast cells from H_2O_2 induced oxidative stress as compared to the control.

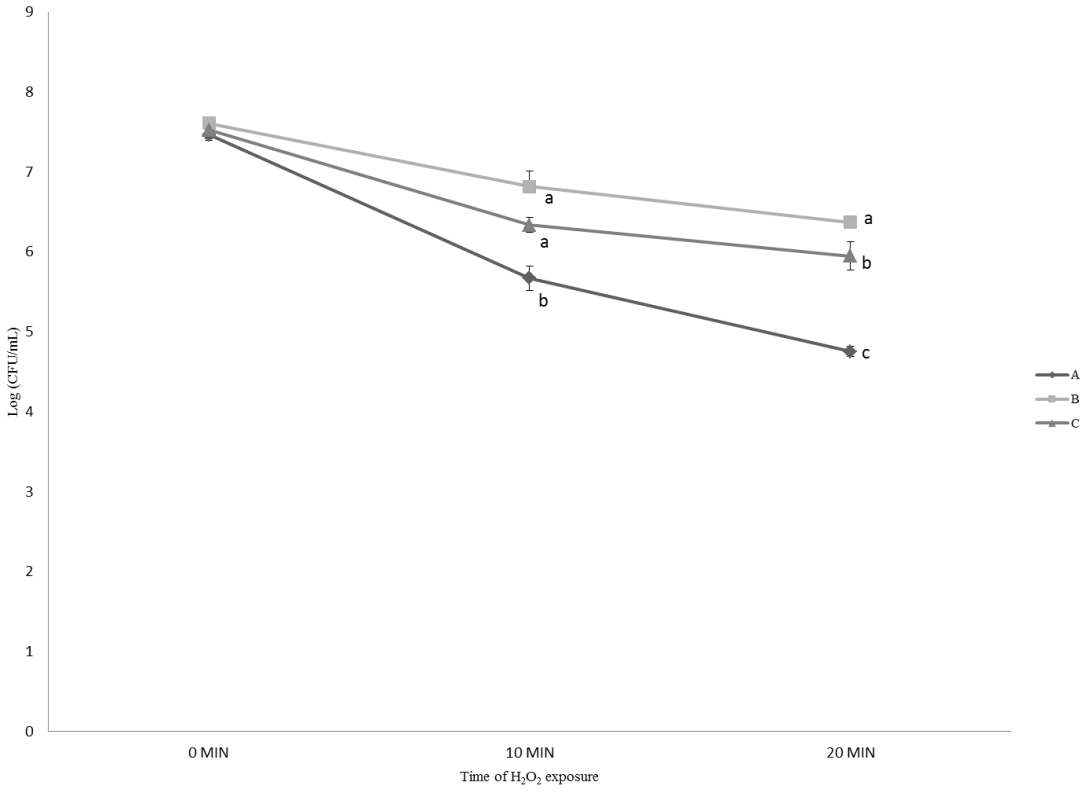

Figure 69: Changes in CFU/mL of *Saccharomyces cerevisiae* during exposure to H_2O_2 with different treatment models of fermented apple extract.

6.3.4.3 Glucose-6-Phosphate Dehydrogenase (G6PDH) and Succinate Dehydrogenase (SDH) Activity in Oxidatively Stressed Yeast Cells

The G6PDH activity of *S. cerevisiae* was assayed in order to measure the stimulation of pentose phosphate pathway in response to H_2O_2 induced oxidative stress in the presence and absence of fermented apple extracts (Fig 70). Although the concurrent model (B) and control (C) had higher activity than pretreatment model (A),

the differences were not significant (P>0.05). G6PDH activity was higher for yeast cells treated with peroxide for the pretreatment model than without peroxide. This resulted in a corresponding higher accumulation of proline in peroxide treated yeast cells in the pretreatment model indicating a coupled PPP response.

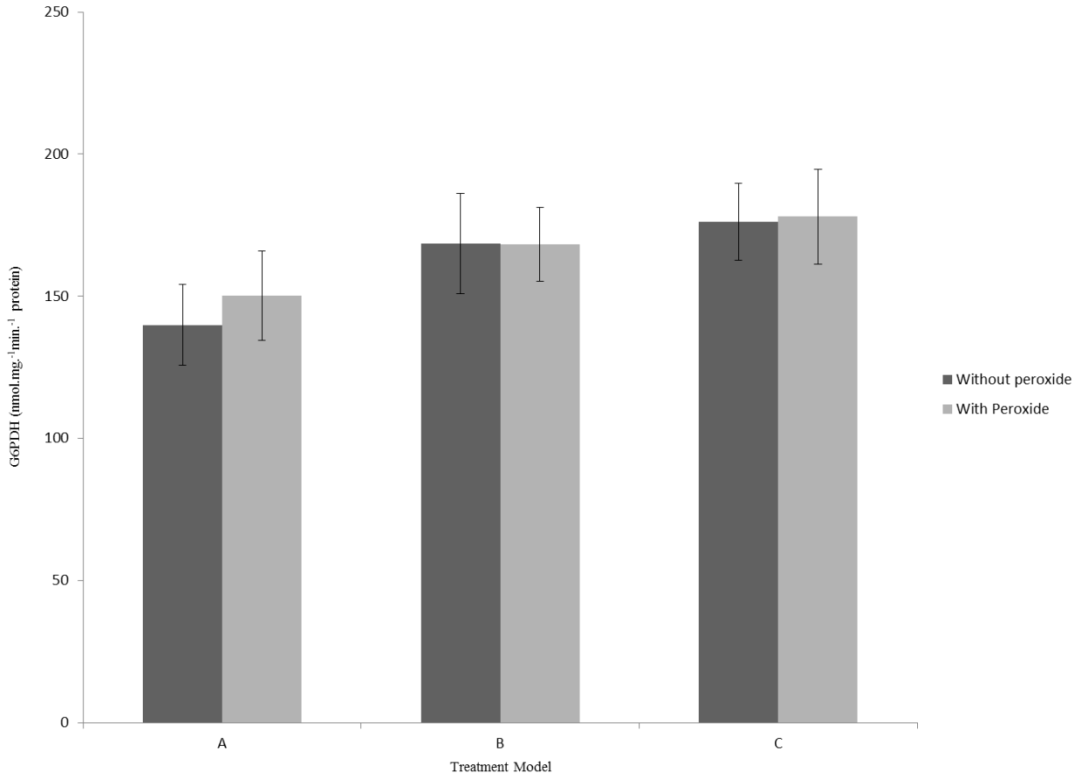

Figure 70: Changes in Glucose-6-phosphate dehydrogenase activity (nmol mg-1 protein) of *Saccharomyces cerevisiae* before and after exposure to H_2O_2 with different treatment models of fermented apple extract.

To confirm if H_2O_2 induced oxidative stress was able to modulate tricarboxylic acid (TCA) cycle activity, the key enzyme SDH was evaluated (Fig 71). SDH activity

decreased significantly ($P<0.05$) for all treatment models following 20 min of H_2O_2 exposure. SDH activity after 20 min of H_2O_2 exposure was maintained at a significantly higher level ($P<0.05$) in control as compared to the pretreatment model (A) and the concurrent model (B). However, the extent of decrease for all treatments was the same. This may indicate a moderate metabolic shift towards a non NADH route to ATP synthesis in the presence of fermented apple extracts as compared to the control with or without the presence of oxidative stress.

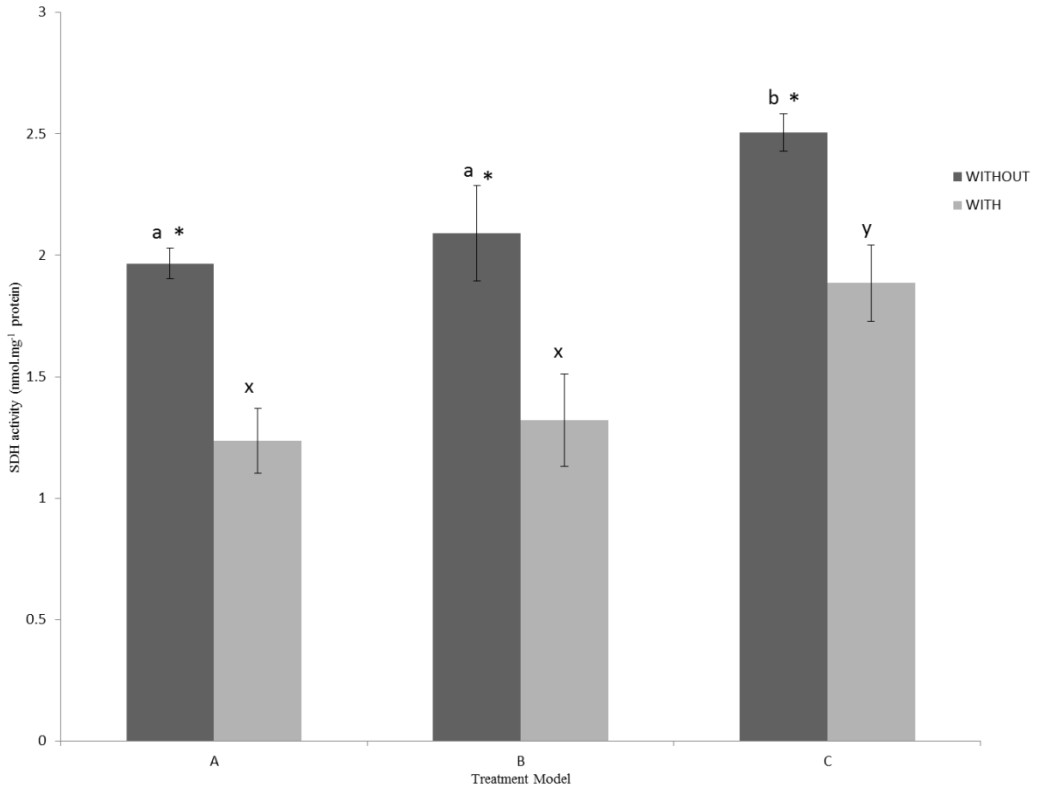

Figure 71: Succinate dehydrogenase activity (nmol mg-1 protein) of *Saccharomyces cerevisiae* before and after exposure to H_2O_2 with different treatment models of fermented apple extract.

6.3.4.4 Proline Content and Proline Dehydrogenase (PDH) activity in Oxidatively Stressed Yeast Cells

To confirm if peroxide treatments were able to modulate cellular proline oxidation for alternative energy production, activity of PDH was investigated (Fig 72). PDH activity decreased significantly ($P<0.05$) for all models following 20 min of H_2O_2 exposure. PDH activity was higher with fermented apple extracts as compared to control before treatments.

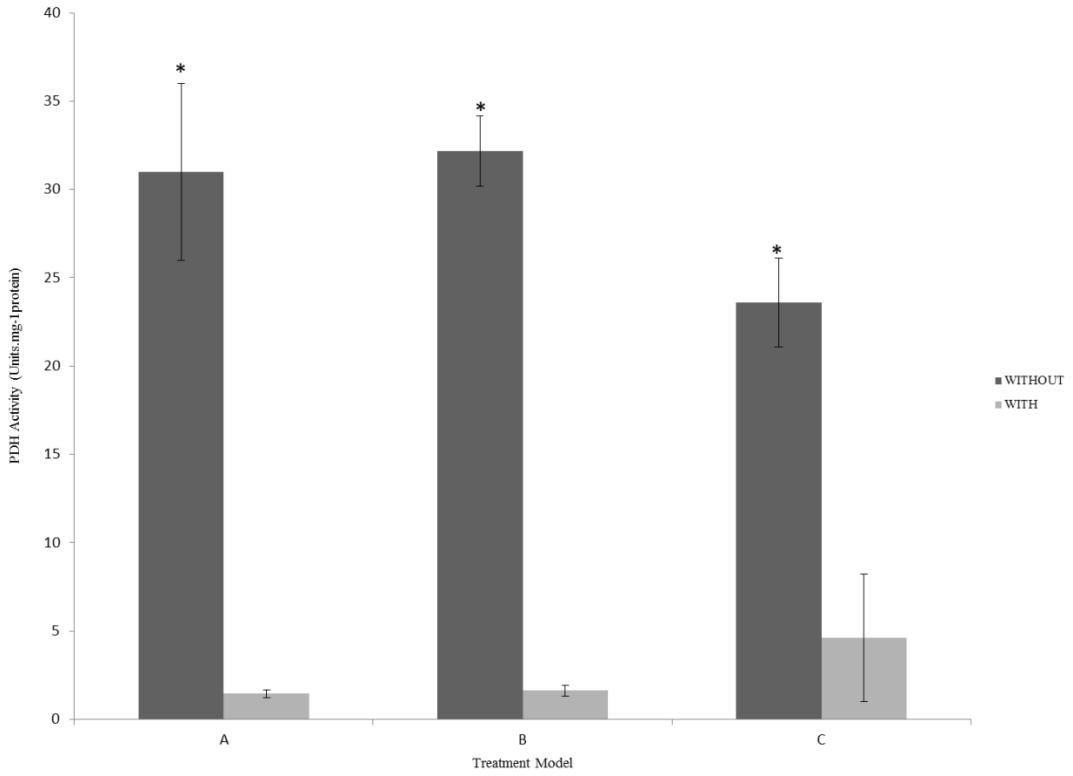

Figure 72: Proline dehydrogenase activity (Units mg-1 protein) of *Saccharomyces cerevisiae* before and after exposure to H_2O_2 with different treatment models of fermented apple extract.

By contrast, PDH activity was higher in control following H_2O_2 treatment as compared to the pretreatment model (A) and the concurrent model (B) however, none of these differences were significant ($P>0.05$).

Higher proline accumulation in pretreatment model (A) may be correlated to lower PDH activity following 20 min of H_2O_2 treatment (Fig 73). There was no significant difference in proline accumulation amongst different models following 20 min of H_2O_2 exposure.

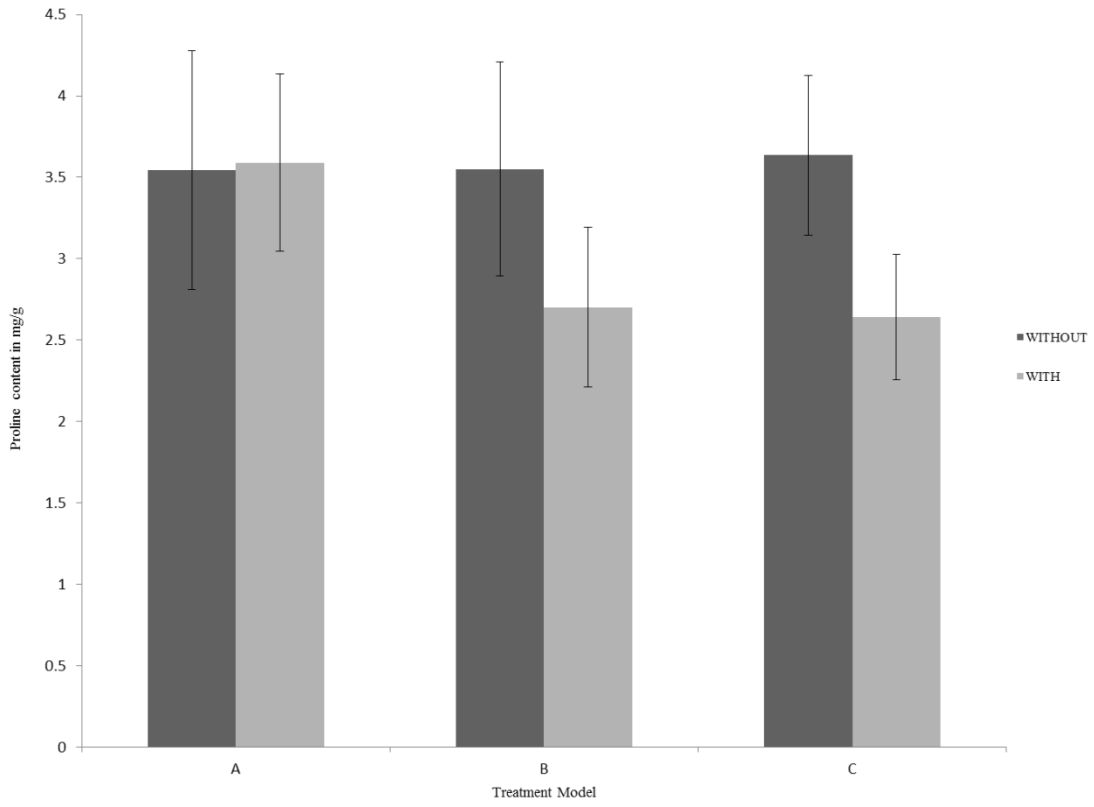

Figure 73: Total proline content (mg/mL FW) of *Saccharomyces cerevisiae* before and after exposure to H_2O_2 with different treatment models of fermented apple extract.

6.3.4.5 Changes in Superoxide Dismutase (SOD), Catalase (CAT) Activity Oxidatively Stressed Yeast Cells

To confirm if peroxide treatments were able to modulate antioxidant enzymes and therefore influence cellular redox environment, the activities of antioxidant enzymes SOD and CAT was investigated. SOD activity was slightly higher in concurrent model (B) as compared to pretreatment model (A) and the control (C) (Fig 74).

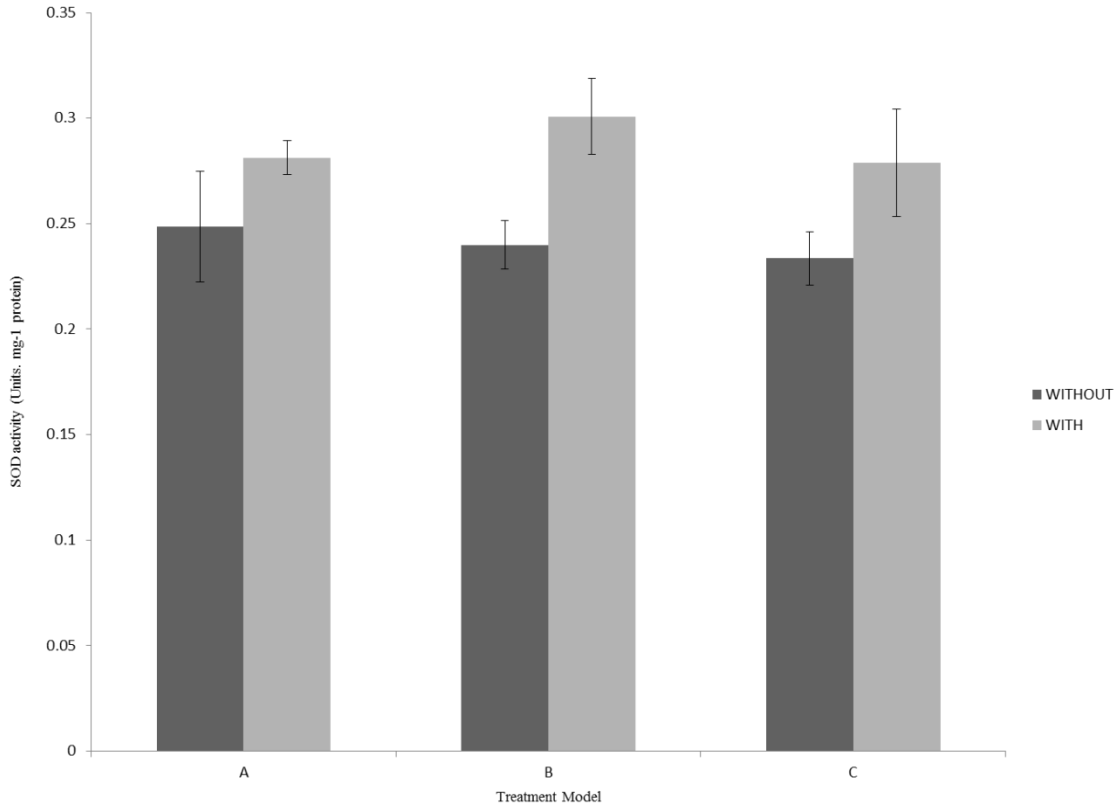

Figure 74: Superoxide dismutase activity (Units mg-1 protein) of *Saccharomyces cerevisiae* before and after exposure to H_2O_2 with different treatment models of fermented apple extract.

As expected SOD activity was higher in all three models following 20 min of H_2O_2 exposure indicating an increase in oxidative pressure. These differences however were not significant ($P<0.05$).

CAT activity was not found following 20 H_2O_2 exposure (Fig 75). This may be due to a very high load of H_2O_2 concentration used in this study.

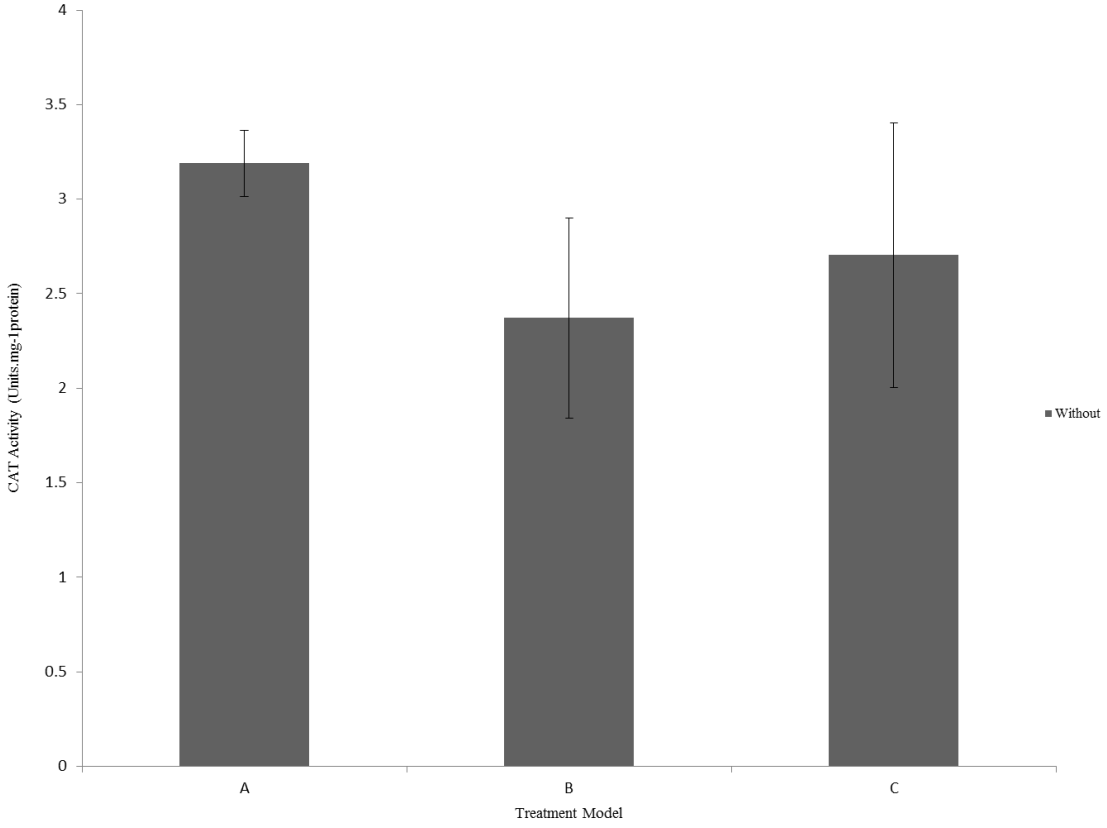

Figure 75: Catalase activity (Units mg-1 protein) of *Saccharomyces cerevisiae* before exposure to H_2O_2 with different treatment models of fermented apple extract.

At 0 min, pretreatment model (A) had the highest CAT activity indicating oxidative pressure can be better managed using fermented apple extracts in the

pretreatment model. This is further confirmed by lower MDA contents in pretreatment model (A) as compared to concurrent model (B) and the control (C).

6.3.4.6 Malondialdehyde (MDA) Content in Oxidatively Stressed Yeast Cells

The malondialdehyde (MDA) content reflects breakdown of membrane due to likely effects of ROS and was therefore evaluated to indicate the protective effect of fermented apple extracts on *S. cerevisiae* over the course of the H_2O_2 treatment (Fig 76).

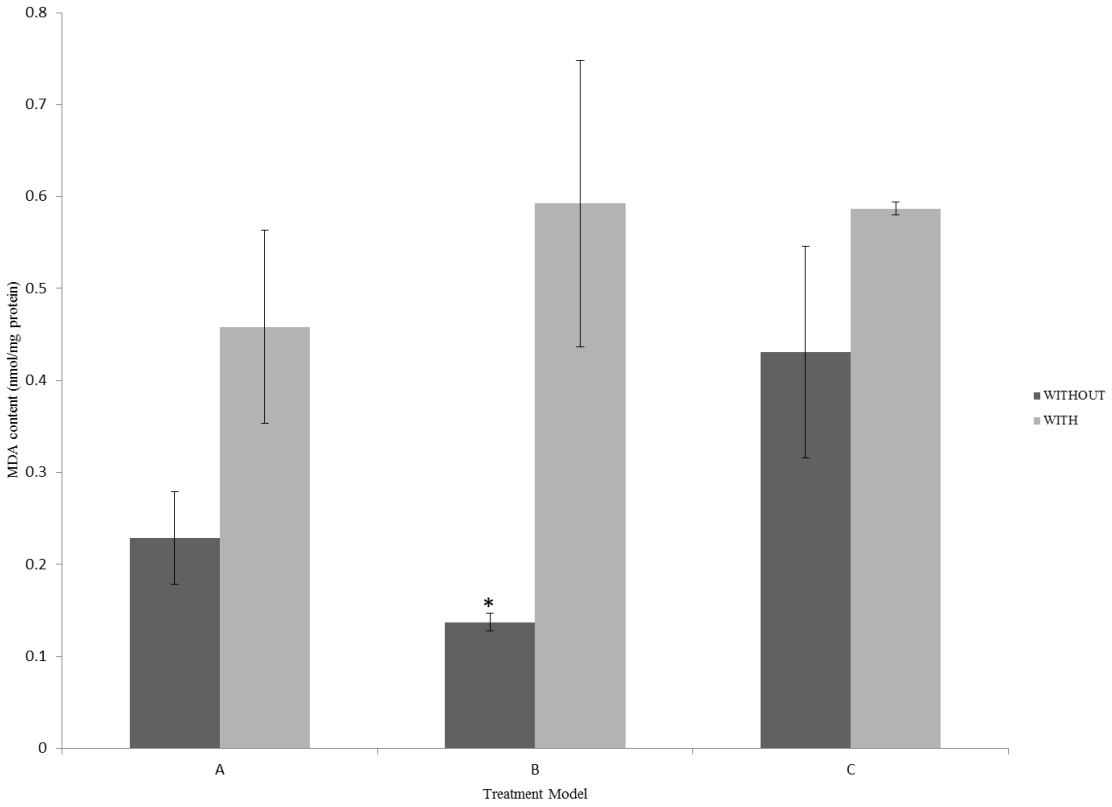

Figure 76: Changes in formation of malondialdehyde (MDA) in *Saccharomyces cerevisiae* before and after exposure to H_2O_2 with different treatment models of fermented apple extract.

Significant increase ($P<0.05$) was found in concurrent model following H_2O_2 treatment. Pretreatment model (A) had lower MDA content as compared to concurrent model and control indicating better protection with fermented apple extracts when it is incorporated as a part of the growth media for yeast. However, the survival rate of these yeast in response to H_2O_2 is lower than concurrent model (B).

6.3.5 Discussion

Hydrogen peroxide is one of the reactive oxygen species implicated in cell damage associated with initiation and progression of many oxidative linked diseases. Although essential as a regulator of eukaryotic signal transduction, excess of these ROS can have deleterious effects (Valko et al., 2007; Veal, 2007). Antioxidant enzymes such as superoxide dismutase, Catalase and Peroxidases play an important role in maintaining ROS balance and protect the cells from damage (Mokni et al., 2007). Dietary phenolics have been shown to not only act as molecular antioxidants but also modulate up-regulation of antioxidant enzymes such as SOD, CAT, GSHPX which is required to regulate ROS homeostasis (COSkun et al., 2005; Williams et al., 2004; Martin et al., 2010).

In this study, H_2O_2 induced oxidative stress mediated inhibition of yeast growth and enhanced cell death. This was reflected in the significant decrease in the total colony count in all the treatment models. A common characteristic of fruit juices is high free radical-linked antioxidant activity which may be related to the presence of specific phenolic compounds. In the concurrent model (B) fermented apple juice with high antioxidant activity may have immediately scavenged free radicals reducing the potency

of H_2O_2 in inducing cell death. Lower cell numbers in the pretreatment model (A) is not completely understood at this point. It is possible that in the pretreatment model (A) apple juice regulates metabolism in yeast during growth by coupling proline biosynthesis with the stimulation of PPP. This is evident from higher or increased activities of PDH and G6PDH activity after H_2O_2 exposure. As the cells shift towards an alternate metabolism involving proline oxidation, combination of H_2O_2 exposure and simple mono phenolics present in the fermented apple juice may inhibit PDH to a greater extent causing cell death by starvation. This is further evident from the higher proline accumulation in the pretreatment model after H_2O_2 exposure. PDH inhibition by mono phenolics has been reported in *Helicobacter pylori* and *Listeria monocytogenes* (Lin et al., 2005; Apostolidis et al., 2008; Ankolekar et al., 2011). However among the cells that survive the extent of membrane damage is much reduced indicating a better overall antioxidant response among survivors.

Proline associated PPP is a proposed alternative energy metabolism model where proline synthesized under stress stimulates the PPP, which provides metabolic precursors for various anabolic needs under stress, whereas the proline synthesized can provide the reducing equivalents for a fast, non-NADH route to ATP synthesis (Fig. 77). According to the results from this study, H_2O_2 exposure of yeast induced some key changes in the enzymes of the PLPPP and antioxidant enzyme response. Activity of G6PDH, the enzyme catalyzing the rate limiting step of the PPP was not affected by H_2O_2 exposure whereas activity of SDH, key enzyme of the TCA cycle decreased significantly ($P<0.05$) for all treatment models. This suggests yeast metabolism following H_2O_2 exposure is supported by PPP. Further, the decrease in activity of SDH

and PDH may not provide enough reducing equivalents for oxidative phosphorylation essentially starving the cells of energy. This could to some extent be overcome by fermented apple juice added at the same time as H_2O_2 in model B.

Major Antioxidant enzymes involved in detoxification of ROS are Superoxide dismutase (SOD), Catalase (CAT), Glutathione peroxidase (GPOX) and Glutathione reductase (GR). SOD converts superoxide radicals to H_2O_2 and CAT converts H_2O_2 to H_2O and O_2, GPOX converts lipid hyroperoxide (LOOH) to lipid hydroxide (LOH) oxidizing Glutathione (GSH) to Glutathione disulfide (GSSG) which is reduced back to Glutathione by GSR (Ahmad, 1992).

Following H_2O_2 exposure SOD activity slightly increased whereas no CAT activity was found following exposure. Absence of CAT activity may be due to inactivation of CAT due to excess H_2O_2 (Kirkman and Gaetani, 1984, Ying et al., 2008). Antioxidant enzyme activity assayed in this study was not significantly affected in response to fermented apple extracts. It is possible that other antioxidant enzymes expressed in response to oxidative stress were potentially involved (Ahmad, 1992). Better yeast protection in terms of CFU/mL in the concurrent model (B) may be due to direct scavenging of ROS by antioxidant compounds in the fermented apple extracts, resulting in better maintenance of redox environment during H_2O_2 exposure.

The malondialdehyde (MDA) content reflects breakdown of membrane due to likely effects of ROS and was therefore evaluated to indicate the protective effect of fermented apple juice extracts on *S. cerevisiae* over the course of the H_2O_2 treatment (Nielsen et al., 1997; Moore et al., 1998). Overall there was no significant difference in the MDA contents between the different treatment models after H_2O_2 exposure although

the pretreated cells (model A) had reduced MDA reflecting better protection of membranes in surviving cells due to to pretreatment with fermented apple extracts. This correlates to a lack of significant difference in the stimulation of antioxidant enzyme found in different treatments. It is also possible that the lower amount of MDA content found in the pretreatment model is due to the stimulation of other antioxidant enzymes such as NADPH oxidase or glutathione peroxidases that were not assayed in this study.

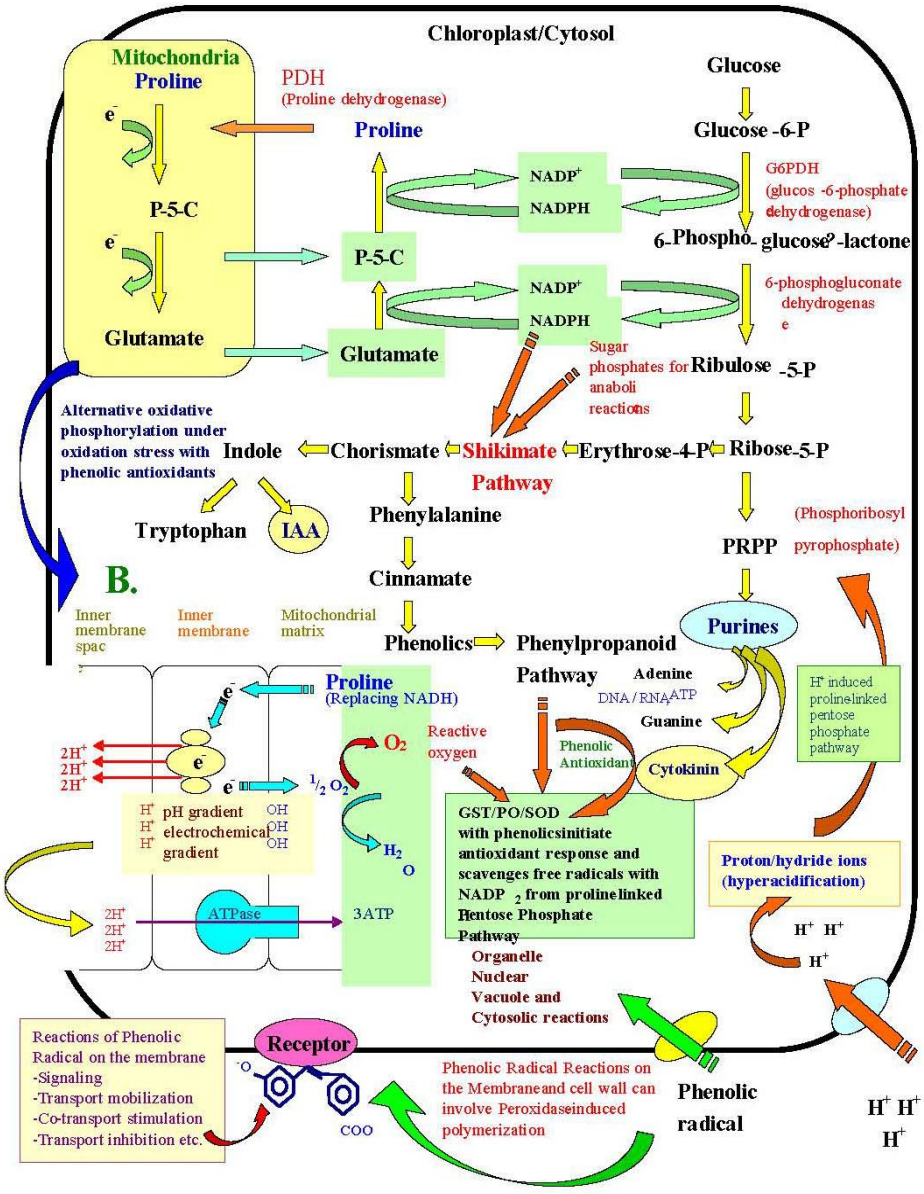

Figure 77: Proline Linked Pentose Phosphate Pathway

CHAPTER 7

SCALD PREVENTION IN APPLES USING CHITOSAN TREATMENTS

7.1 Evaluation of Superficial Scald Reduction using DPA and Chitosan Oligosaccharide Treatments in Relation to Phenolic Linked Metabolic Regulation and its Effect on *in vitro* Anti-Hyperglycemia Models

7.1.1 Abstract

Chitosan oligosaccharide dipping was evaluated as an alternative treatment for inhibiting superficial scald as compared to diphenylamine (DPA) in 'Cortland' apple stored at 4 ^{0}C for 14 weeks. Stimulation of pentose phosphate pathway and associated antioxidant enzyme response as well as phenolic biosynthesis was investigated. Further the effect of storage on *in vitro* inhibition of α-amylase and α-glucosidase by stored apple extracts were evaluated to determine the effect of treatment and storage on these carbohydrate metabolizing enzymes associated with hyperglycemia. Activity of glucose-6-phosphate dehydrogenase and succinate dehydrogenase could not be related to superficial scald development. However, this related to higher phenolic content in chitosan treatments as compared to DPA and control. Overall chitosan oligosaccharide treatments had higher superoxide dismutase activity and guaiacol peroxidase activity but no difference was detected in catalase activity. A direct correlation between activity of antioxidant enzyme activity and superficial scald development could not be concluded. A higher proline dehydrogenase activity was detected in chitosan oligosaccharide treatments, which may provide a non-NADH route to ATP synthesis in response to these treatments by rapid catabolism of proline. In ethanolic and water

extracts of peel, chlorogenic acid, p-coumaric acid and quercetin derivatives were the major phenolic compounds detected whereas in ethanolic and water extracts of pulp, gallic acid and chlorogenic acid were the major phenolic compounds detected. A treatment related change in inhibition of carbohydrate metabolizing enzymes during storage was not detected. No significant difference in the content of polyphenol oxidase or malondialdehyde was detected whereas DPA treatments had significantly lower conjugated triene content at 14 weeks of storage. DPA treatments had significantly lower 'mild, 'moderate' and 'severe' superficial scald as compared to control and chitosan oligosaccharide treatments. Although an overall stimulation of pentose phosphate pathway, TCA cycle, antioxidant enzyme response and phenolic biosynthesis was observed in chitosan oligosaccharide treatment, superficial scald prevention was similar to control.

7.1.2 Introduction

Darkening and browning of skin after cold storage also referred to as superficial scald is an important physiological disorder in apples. Currently diphenylamine or 1-MCP is used to prevent scald development in apples after cold storage (Ju and Bramlage, 2000). Diphenylamine (DPA) used in postharvest treatments is one of the chemicals detected in highest concentration in apple peel and was reported to be detected in 20% of the samples tested (Iñigo-Nuñez et al., 2010). Drzyzga (2003) presents an excellent review of chemistry, application, toxicity, metabolism and microbial degradation of DPA and its derivatives. Recently, concerns over the residues of DPA in fruits has led to many countries banning usage of DPA, making it necessary

to look for more natural alternative treatments (Bauchot et al., 1995). In animals, DPA has been shown to cause increase in organ weights as well as damage to liver, spleen and kidney (Drzyzga, 2003). Scald development in apples involves oxidation of α-farnesene to conjugated triene oxidation products (Rowan et al., 2001). Since scald development involves oxidation, antioxidants present in the fruit cuticle may play a crucial role in preventing scald development. It has been reported that α-farnesene concentration is minimum in the pre-climacteric stage after which starts increasing during cold storage, peaking at first 8-12 weeks of storage (Lurie and Watkins, 2012). α-Farnesene is present in the lipid phase of cellular environment and so the focus has been on lipid soluble molecular antioxidants. An increase in molecular antioxidants such as α-tocopherol, carotenoids and ascorbic acid did not correlate to scald development (Barden and Bramlage, 1994). Phenolics have been reported to have a dual role in scald development (Treutter, 2001). They may contribute to browning due to polymerization by polyphenol oxidase (PPO); whereas they may prevent scald development by acting as antioxidants and preventing oxidation of α-farnesene (Lurie and Watkins, 2012). Recently, reports suggest that scald development may be due to oxidative stress and oxidation of α-farnesene may be a consequence of unchecked free radical reactions (Rao et al., 1998; Rupasinghe et al., 2000; Whitaker, 2004).

Apple consumption has been inversely linked to many chronic diseases including certain types of cancer, cardiovascular diseases, asthma and pulmonary function, diabetes, and inhibition of lipid oxidation (Boyer and Liu, 2004) and this is mainly attributed to the phenolic phytochemicals such as flavonoids, and phenolic acids present in them (Ross and Kasum, 2002). In the USA it is estimated that apple alone

contributes to about 22% of total phenolics consumed from fruits making it the largest source of these health beneficial phenolic phytochemicals (Vinson et al., 2001).

Chitosan, the deacylated form of chitin has a broad range of applications in pre-harvest and post-harvest applications including edible coating for fruits for preservation and as antimicrobials to prevent fungal infection (Shahidi et al., 1999). Chitosan coating delayed changes in contents of anthocyanins, flavonoids and total phenolics as well as delayed the increase in PPO and peroxidase activity (Zhang and Quantick, 1997). Further it was reported that chitosan post-harvest coatings as well as a pre-harvest chitosan spray in fruits reduced ethylene production, delayed ripening, allowed retention of firmness and controlled decay (Reddy et al., 1999; No et al., 2007; Meng et al., 2008). Chitosan is a well-established elicitor of phenolics phytochemicals and antioxidant enzyme response and controlled release of antioxidants may have relevance in preventing scald development (Fajardo et al., 1995; Shahidi et al., 1999; Kim and Rajapakse, 2005; Sarkar et al., 2009a). Scald development is linked to total phenolic content, antioxidant enzymes, ethylene production as well as ripening and chitosan coatings have been reported to affect each of these intrinsic parameters which warrant investigation of chitosan oligosaccharide as a replacement of DPA.

Reactive oxygen species (ROS) such as superoxide radical, hydrogen peroxide and hydroxyl radicals are constantly produced as a result of aerobic respiration and may also be induced by exogenous sources such as UV, toxins, and inflammation (Shigenaga et al., 1994). Reducing equivalents in the form of NADH and $FADH_2$ is needed to regenerate antioxidant defense systems such as tocopherols, ascorbate, glutathione and antioxidant enzymes such as superoxide dismutase, catalase and glutathione peroxidase

in order to maintain cellular antioxidant homeostasis (Mates and Sanchez-Jimenez, 1999; Mates, et al, 1999; Nordberg and Arner, 2001; Foyer and Noctor, 2005). Proline synthesis under stress takes place by the reduction of pyroline-5-carboxylate (P5C) leads to the accumulation of $NADP^+$ (Verbruggen and Hermans, 2008) which is a co-factor for the enzyme glucose-6-phosphate dehydrogenase that catalyzes the rate limiting step of the pentose phosphate pathway (PPP) (Kruger and von Schaewen, 2003). Using this rationale, an alternate model for proline metabolism was proposed where proline biosynthesis is linked to stimulation of Pentose Phosphate Pathway (PPP) through $NADP^+$/NADPH redox balance (Shetty and Wahlqvist, 2004).

The aim of the present study was to examine the effect of chitosan oligosaccharide as a potential inhibitor of superficial scald in apple as compared to the more conventional DPA treatments. Increased involvement of pentose phosphate pathway and its link to proline synthesis was evaluated in trying to understand and explore the biochemical rationale involved in phenolic biosynthesis, antioxidant enzyme response system and scald development. While studying the biochemistry, total antioxidant activity including antioxidant enzymes as well as phenolic content, phenolic profile was determined to investigate oxidative stress management and protection delivered by antioxidants in relation to superficial scald development. Further the changes in inhibition of carbohydrate metabolizing enzymes, α-glucosidase and α-amylase, were evaluated during storage to determine the effect of post-harvest treatment on physiological disorders and its consequences in phenolics for potential dietary management of hyperglycemia.

7.1.3 Materials and Methods

7.1.3.1 Harvest

Apple cultivar ‘Cortland’ was selected to investigate the effect of natural treatments as a potential inhibitor of superficial scald in this study. Harvesting was done at University of Massachusetts Cold Spring Orchard, Belchertown, MA on September 19, 2011. Early harvests increase the pre-disposition of apples to superficial scald (Zanella, 2003). Eighty to hundred fruits from one tree were collected in a bushel. Each bushel was considered a replicate. For five treatments a total of 30 bushel was harvested.

7.1.3.2 Treatment

A total of five treatments were selected for evaluating scald prevention. The treatments are described as follows a) T1- Control—Dipped for 1 min in water b) T2-DPA Dipped for 1 min in DPA 1000ppm c) T3-DPA – Dipped for 1 min in DPA 2000ppm d) T4-COS - Dipped for 1 min in COS-C 2000ppm e) T5-COS – Dipped for 1 min in COS-C 4000ppm. After dipping, the bushels were allowed to dry before being stacked on a pellet and put in cold storage. Samples (about 15 fruits) were taken out at 0, 1, 2, 3 (Sept., Oct., Nov., Dec., Jan.) months for analysis. COS referes to chitosan oligosaccharide and DPA to diphenylamine.

7.1.3.3 Extraction

Extractions for the pentose phosphate pathway assays and the health benefits linked functionality assays were carried out in two different ways and are described as follows

For Pentose Phosphate Pathway Assays: A weight of 2.5 g of apple peel was cut into small pieces and added to 5 mL of cold enzyme extraction buffer (0.5 % polyvinylpyrrolidone (PVP), 3 mM EDTA, 0.1 M potassium phosphate buffer of pH 7.5). Tissue tearor (Biospec Products, Bartleville, OK) was used to homogenize and blend the peel with the buffer. The sample was centrifuged at 12000 x g for 30 min at 2-5 ºC and stored on ice. The supernatant was used for further analysis.

For total phenolic and ABTS assay, 100 mg of peel tissue was immersed in 2.5 mL of 95% ethanol and kept in the freezer for 48-72 h. Samples were homogenized using a tissue tearor (Biospec Products, Bartleville, OK) and centrifuged at 12000 x g for 20 min. Supernatant was used for further analysis.

Health Benefits linked Functionality Assays (total phenolics, DPPH, α-glucosidase and α-amylase): Apples were first peeled then cut and weighed. Peel and Pulp were weighed separately then extracted using distilled water and 12% ethanol. Extractions were carried out by homogenizing using a Waring blender for 3 min. The samples were centrifuged at 15,000 *g* for 15 min. Supernatants were collected and stored at -20 ^{0}C during the period of study. pH of the extracts was adjusted to 5.8-6.2 and analysis was carried out within a week of extraction.

Peel **e**xtractions were performed by using 20 g of peel in 50 mL of water and 5 g of peel in 15 mL of 12% ethanol. The peel refers to outer skin only.

Pulp extractions were performed by using 100 g of pulp in 50 mL of water and 10 g of pulp in 20 mL of 12% ethanol. This included no outer skin.

7.1.3.4 Total Protein Assay

Protein content was measured by the method of Bradford assay (Bradford, 1976). The dye reagent concentrate (Bio-Rad protein assay kit II, Bio-Rad Laboratory, Hercules, CA) was diluted 1:4 with distilled water. A volume of 5 mL of diluted dye reagent was added to 50 µL of the extract. After vortexing and incubating for 3 min, the absorbance was measured at 595 nm against a 5 mL reagent blank and 50 µL buffer solutions using a UV-VIS Genesys spectrophotometer (Milton Roy, Inc., Rochester, NY).

7.1.3.5 Glucose-6-Phosphate Dehydrogenase (G6PDH) Assay

A modified version of the assay described by Deutsch (1983) was followed. The enzyme reaction mixture containing 5.88 µmol ß-NADP, 88.5 µmol $MgCl_2$, 53.7 µmol glucose-6-phosphate, and 0.77 mmol maleimide was prepared. This mixture was used to obtain baseline (zero) of the spectrophotometer reading at 340 nm wavelength. To 1 mL of this mixture, 100 µL of the enzyme sample was added. The rate of change in absorbance per minute was used to quantify the enzyme in the mixture using the extinction co-efficient of NADPH (6.22 $mM^{-1}cm^{-1}$).

7.1.3.6 Succinate Dehydrogenase (SDH) Assay

Modified method described by Bregman (1987) was used to assay the activity of succinate dehydrogenase. The assay mixture consisted of the following: 1.01 mL of 0.4 M potassium phosphate buffer (pH 7.2); 40 µL of 0.15 M sodium succinate (pH 7.0); 40 µL of 0.2 M sodium azide; and 10 µL of 6.0 mg/mL DCPIP. This mixture was used to obtain baseline (zero) of the spectrophotometer reading at 600 nm wavelength. To 1.0 mL of this mixture, 200 µL of the enzyme sample was added. The rate of change in absorbance per minute was used to quantify the enzyme in the mixture using the extinction co-efficient of DCPIP (19.1 $mM^{-1}cm^{-1}$).

7.1.3.7 Superoxide Dismutase (SOD) Assay

A competitive inhibition assay was performed that used xanthine-xanthine oxidase-generated superoxide to reduce nitroblue tetrazolium (NBT) to blue formazan. Spectrophotometric assay of SOD activity was carried out by monitoring the reduction of NBT at 560 nm (Oberley and Spitz, 1985). The reaction mixture contained 13.8 mL of 50 mM potassium phosphate buffer (pH 7.8) containing 1.33 mM DETEPAC; 0.5 mL of 2.45 mM NBT; 1.7 mL of 1.8 mM xanthine and 40 IU/mL catalase. To 0.8 mL of reagent mixture 100 µL of phosphate buffer and 100 µL of xanthine oxidase was added. The change in absorbance at 560 nm was measured every 20 sec for 2 min and the concentration of Xanthine oxidase was adjusted to obtain a linear curve with a slope of 0.025 absorbance per min. The phosphate buffer was then replaced by the enzyme sample and the change in absorbance was monitored every 20 sec for 2 min. One unit of

SOD was defined as the amount of protein that inhibits NBT reduction to 50% of the maximum.

7.1.3.8 Catalase (CAT) Assay

A method originally described by Beers and Sizer, (1952), was used to assay the activity of catalase. To 1.9 mL of distilled water 1 mL of 0.059 M hydrogen peroxide (Merck's Superoxol or equivalent grade) in 0.05 M potassium phosphate, pH 7.0 was added. This mixture was incubated in a spectrophotometer for 4-5 min to achieve temperature equilibration and to establish blank rate. To this mixture 0.1 mL of diluted enzyme sample was added and the disappearance of peroxide was followed using a spectrophotometer by recording the decrease in absorbance at 240 nm for 2-3 min. The change in absorbance ΔA_{240}/min from the initial (45 sec) linear portion of the curve was calculated. One unit of catalase activity was defined as amount that decomposes one micromole of H_2O_2

$$\text{Units /mg} = \frac{\Delta A_{240}/\text{min} \times 1000}{43.6 \times mg\ enzyme/mL\ of\ reaction\ mixture}$$

7.1.3.9 Guaiacol Peroxidase (GPX) Assay

Modified version of assay developed by Laloue et al., (1997), was used. Briefly, the enzyme reaction mixture contained 0.1 M potassium phosphate buffer (pH 6.8), 56

mM guaiacol solution and 0.2 mM hydrogen peroxide. To 1 mL of this reaction mixture, 50 µL of enzyme sample was added. The absorbance was noted at zero time and then after 5 min. The rate of change in absorbance per minute was used to quantify the enzyme in the mixture using the extinction co-efficient of the oxidized product tetraguaiacol (26.6 $mM^{-1}cm^{-1}$).

7.1.3.10 HPLC Analysis of Proline

High performance liquid chromatography (HPLC) analysis was performed using an Agilent 1100 liquid chromatograph equipped with a diode array detector (DAD 1100). The analytical column was reverse phase Nucleosil C18, 250 nm x 4.6 mm with a packing material of 5 µm particle size. The extract samples were eluted out in an isocratic manner with a mobile phase consisting of 20 mM potassium phosphate (pH 2.5 by phosphoric acid) at a flow rate of 1 mL min^{-1} and detected at 210 nm. L-Proline (Sigma chemicals, St. Louis, MO) dissolved in the 20 mM potassium phosphate solution was used to calibrate the standard curve. The amount of proline in the sample was calculated as mg of proline per milliliter and converted and reported as mg g^{-1} FW.

7.1.3.11 Proline Dehydrogenase (PDH) Assay

A modified method described by Costilow and Cooper (1978) was carried out to assay the activity of proline dehydrogenase. The enzyme reaction mixture containing 100 mM sodium carbonate buffer (pH 10.3), 20 mM L-proline solution and 10 mM

NAD was used. To 1 mL of this reaction mixture, 200 µL of extracted enzyme sample was added. The increase in absorbance was measured at 340 nm for 3 min, at 32 ^{0}C. The absorbance was recorded at zero time and then after 3 min. In this spectrophotometric assay, one unit of enzyme activity is equal to the amount causing an increase in absorbance of 0.01 per min at 340 nm (1.0 cm light path).

7.1.3.12 Total Soluble Phenolics Assay

The total phenolic content in apple peel and pulp leaves was analyzed by the Folin-Ciocalteu method (Shetty et al., 1995). One milliliter of supernatant was transferred into a test tube and mixed with 1 mL of 95 % ethanol and 5 mL of distilled water. To each sample 0.5 mL of 50 % (v/v) Folin-Ciocalteu reagent was added and mixed. After 5 min, 1 mL of 5 % Na_2CO_3 was added to the reaction mixture and allowed to stand for 60 min. The absorbance was read at 725 nm. The absorbance values were converted to total phenolics and were expressed in milligrams equivalents of gallic acid per grams Fresh weight (FW) of the sample. Standard curves were established using various concentrations of gallic acid in 95 % ethanol.

7.1.3.13 Antioxidant Activity by DPPH Radical Inhibition Assay

The antioxidant activity was determined by the DPPH radical scavenging method modified from Kwon et al. (2006b). A 250-µL aliquot of the sample extract was mixed with 1,250 µL of DPPH (60 µM in ethanol). Absorbance was measured at 517

nm using the Genesys UV/Visible spectrophotometer. The readings were compared with the controls, containing 95% ethanol instead of sample extract. The percentage inhibition was calculated by:

$$\% \text{ inhibition} = \frac{(Absorbance_{\text{control}} - Absorbance_{\text{extract}})}{Absorbance_{\text{control}}} \times 100$$

7.1.3.14 α-Glucosidase Inhibition Assay

The α-glucosidase inhibitory activity was determined by an assay modified from McCue et al. (2005a). α-Glucosidase was assayed by using 50 μL of sample extracts and 100 μL of 0.1 M phosphate buffer (pH 6.9) containing α-glucosidase solution (1 U/mL) and was incubated in 96-well plates at 25°C for 10 min. After preincubation, 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 incubation, absorbance readings were recorded at 405 nm by a microplate reader (Thermomax, Molecular Devices Co., Sunnyvale, CA) and compared to a control that had 50 μL of buffer solution in place of the extract. The α-glucosidase inhibitory activity was expressed as percentage inhibition and was calculated with the same equation as for percentage inhibition in the DPPH radical inhibition assay. Dose dependency was tested using 25 μL and 10 μL of the sample, the volume made up to 50 μL using 0.1 M phosphate buffer (pH 6.9) and same protocol was followed.

7.1.3.15 α-Amylase Inhibition Assay

The α-amylase inhibitory activity was determined by an assay modified from McCue and Shetty (2004). A total of 500 µL of 1:10 dilution (due to the high sugar content) of sample extract and 500 µL of 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M NaCl) containing α-amylase solution (0.5 mg/mL) were incubated at 25°C for 10 min. After preincubation, 500 µL of a 1% starch solution in 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M NaCl) was added to each tube at timed intervals. The reaction was stopped with 1.0 mL of dinitrosalicylic acid color reagent. The test tubes were incubated in a boiling water bath for 5 min and cooled to room temperature. The reaction mixture was then diluted after adding 15 mL of distilled water, and the absorbance was measured at 540 nm using the Genesys UV/Visible spectrophotometer. The readings were compared with the controls, containing buffer instead of sample extract. The percentage α-amylase inhibitory activity was calculated with the same equation as for percentage inhibition in the DPPH radical inhibition assay.

7.1.3.16 High Performance Liquid Chromatography (HPLC) Analysis of Phenolic Profiles

The extracts (2 mL) were filtered through a 0.2 µm filter. A volume of 5 µL of extract was injected using an Agilent ALS 1100 auto sampler into an Agilent 1100 series HPLC (Agilent Technologies, Palo Alto, CA) equipped with a 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, 25 min). The analytical column used was Agilent Zorbax SB-C_{18}, 250 x 4.6 mm i.d., with packing material of 5 μm particle size at a flow rate of 1 mL/min at room temperature. During each run the absorbance was recorded at 226 and 306 nm and the chromatogram was integrated using Agilent Chemstation enhanced integrator. Pure standards of phenolic compounds in 100% methanol were used to calibrate the standard curves and retention times.

7.1.3.17 Malondialdehyde (MDA) Assay

Method for measurement of MDA was adapted from Tamagnone et al., (1998). A volume of 200 μl of the peel extract was mixed with 800 μL of water, 500 μL of 20 % (w/v) trichloroacetic acid and 1 mL of 10 mM thiobarbutyric acid. The test tubes were incubated for 30 min at 100 ^{0}C and then centrifuged at 12,000 x g for 10 min. The absorbance of the supernatant was measured at 532 nm, 600 nm and 440 nm and the concentration of MDA was calculated from its molar extinction coefficient (ε) 156 $\mu mol^{-1}cm^{-1}$.

7.1.3.18 Measurement of Conjugated Trienes (CT) in Apple Peel

Method for measurement of conjugated trienes (CT) was adapted from Rowan et al., (2001). Briefly, heptane 20 mL was added to glass evaporating dish (90 mm i.d. x 50 mm deep) and apples were placed in the dish such that a part of the apple was

immersed in heptane for 20 min. Apples were removed and the area immersed in heptane was calculated by measuring the diameter of the area exposed to heptane. The volume of the heptane remaining in the dish was measured. CT content was determined using the UV spectrum of the heptane recovered at A_{281}-A_{292} with $€_{281}$-$€_{292}$ = 25000. The results were expressed as nanomoles per square centimeters.

7.1.3.19 Polyphenol Oxidase

Method for estimation of PPO activity was adapted from Dawson and Magee, (1955). Briefly, 2.6 mL of 50 mM potassium phosphate buffer, pH 6.5, was taken in a cuvette. To that 100 µL of L-DOPA (5mM), 100 µL ascorbic acid (2.1mM), and 100 µL of EDTA (0.065 mM) was added and mixed. To this 100 µL of the peel extract was added and the change in absorbance was monitored at A_{265}nm until constant. δ A_{265}nm/ minute was calculated using the maximum linear rate for both the test and the blank. Enzyme activity was calculated by using the definition of one unit of PPO which is the change in A_{265}nm of 0.001 per minute at pH 6.5 in a 3 mL reaction mixture containing L-DOPA and L-ascorbic acid.

7.1.3.20 Scald Evaluations

Scald evaluations was carried out with a leading expert in this field. After taking the samples for analysis at three months of storage the remaining apples were stored in the cold room for another three weeks. Bushels were taken out and stored at room temperature for allowing scald to develop. After about a week of storage at room

temperature when it determined that about 50% of the control group had scald development, a comprehensive scald evaluation was undertaken. Every apple was evaluated for the developed scald and it was further classified as ‘mild’, ‘moderate’ and ‘severe’ depending on the degree and/or the area of the scald on the apple surface.

7.1.3.21 Statistical Analysis

All experiments were performed with six replications. The effect of each treatment was determined by the analysis of variance (ANOVA) of SAS (version 8.2; SAS Institute, Cary, NC). Differences among different treatments were determined by the Fishers least significant difference (LSD) test at the 0.05 probability level. Different letters on bar in the graph indicate statistical significant difference. Standard error was calculated using Microsoft Excel 2010.

7.1.4 Results

7.1.4.1 Glucose-6-Phosphate Dehydrogenase (G6PDH) and Succinate Dehydrogenase (SDH) activity in Apple Peel

The oxidative phase of the pentose phosphate pathway generates NADPH by converting glucose-6-phosphate to ribose-5-phosphate with G6PDH catalyzing the rate limiting step (Puskas et al., 2000; Velasco et al., 1995). At 0 month, G6PDH activity ranged from 627-864 nmol/min/mg protein and it was slightly higher in control treatment. However, this difference was not significant (P>0.05) (Fig 78).

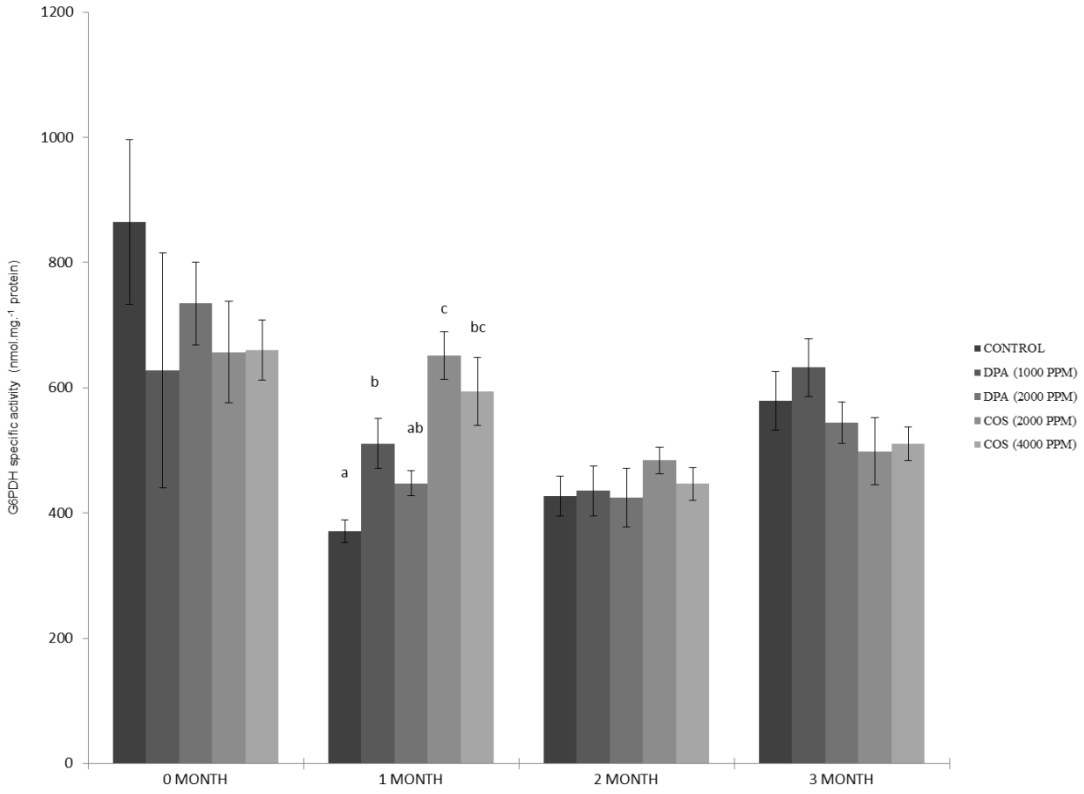

Figure 78: Effect of postharvest treatments on changes in Glucose-6-phosphate Dehydrogenase activity (nmol mg-1 protein) in apple peel during 3 three month storage.

At one month time point chitosan oligosaccharide 2000 ppm treatment had significantly higher ($P<0.05$) activity than DPA and control treatments. At two month and three month time point's G6PDH activity for all treatments were not significantly different.

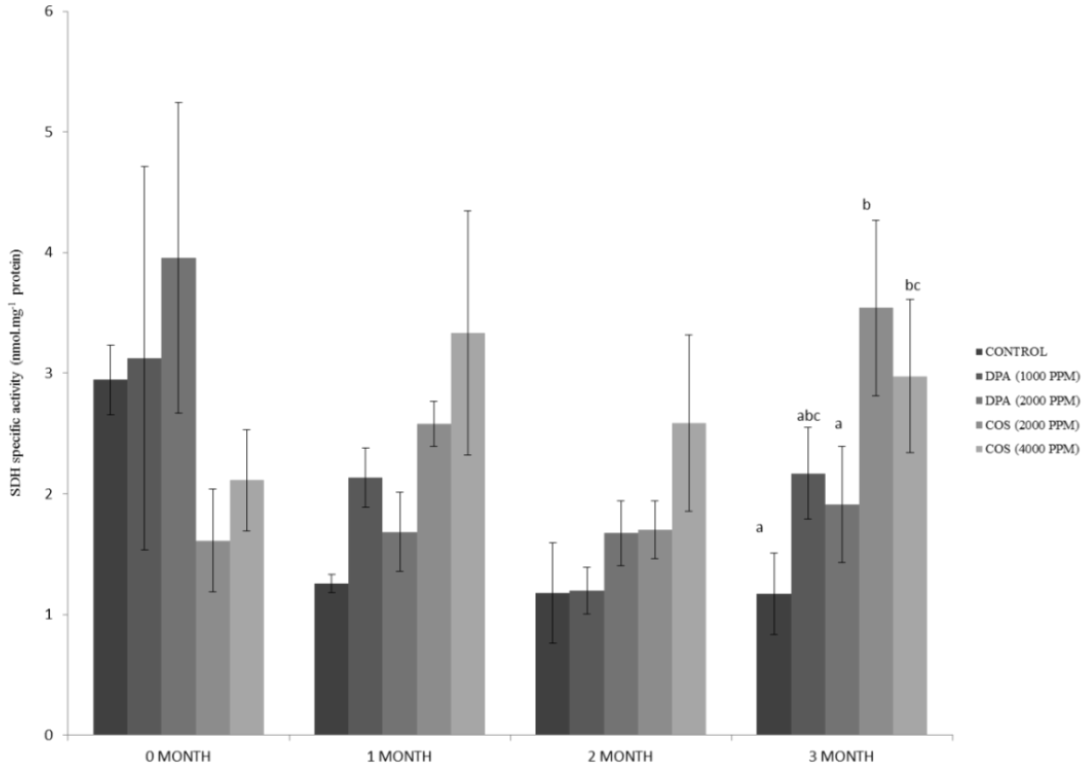

Figure 79: Effect of postharvest treatments on changes in Succinate dehydrogenase activity (nmol mg-1 protein) in apple peel during 3 three month storage.

In order to investigate the effect of the treatment in modulating energy metabolism using the Tricarboxylic Acid Cycle (TCA), the activity of the key mitochondrial TCA cycle enzyme SDH was assayed (Selak et al., 2005). SDH activity was not significantly different ($P>0.05$) at 0 month with a range of 1.6-7.4 nmol/min/mg

protein (Fig. 79). Although the difference was not significant, DPA and control treatments had higher activity than COS. The difference at 0 months cannot be explained at this time.

At one month time point, DPA treatments reduced the activity of TCA cycle enzyme whereas it slightly increased for chitosan oligosaccharide treatments. At two month time point a similar trend was observed with chitosan oligosaccharide 4000 ppm having the highest activity. Significant difference was not observed ($P>0.05$) between treatments at one month and two months time of storage. At three months of storage chitosan oligosaccharide (2000 and 4000 ppm) had significantly higher ($P<0.05$) activity than DPA 4000 ppm and control.

7.1.4.2 Proline Dehydrogenase (PDH) activity and Proline content in Apple Peel

PDH can mediate proline oxidation as a potential alternate energy source via oxidative phosphorylation in the mitochondria. With this rationale the activity of PDH and proline content were investigated (Fig 80). PDH activity ranged from 116-141 units/mg protein at 0 month. At one month of storage time PDH activity for DPA and control treatments decreased whereas it remained the same for chitosan oligosaccharide treatment. PDH activity in chitosan treatment was significantly higher ($P>0.05$) than DPA and control treatments. At two months of storage PDH activity decreased for all treatments and it remained the same for 3 months of storage.

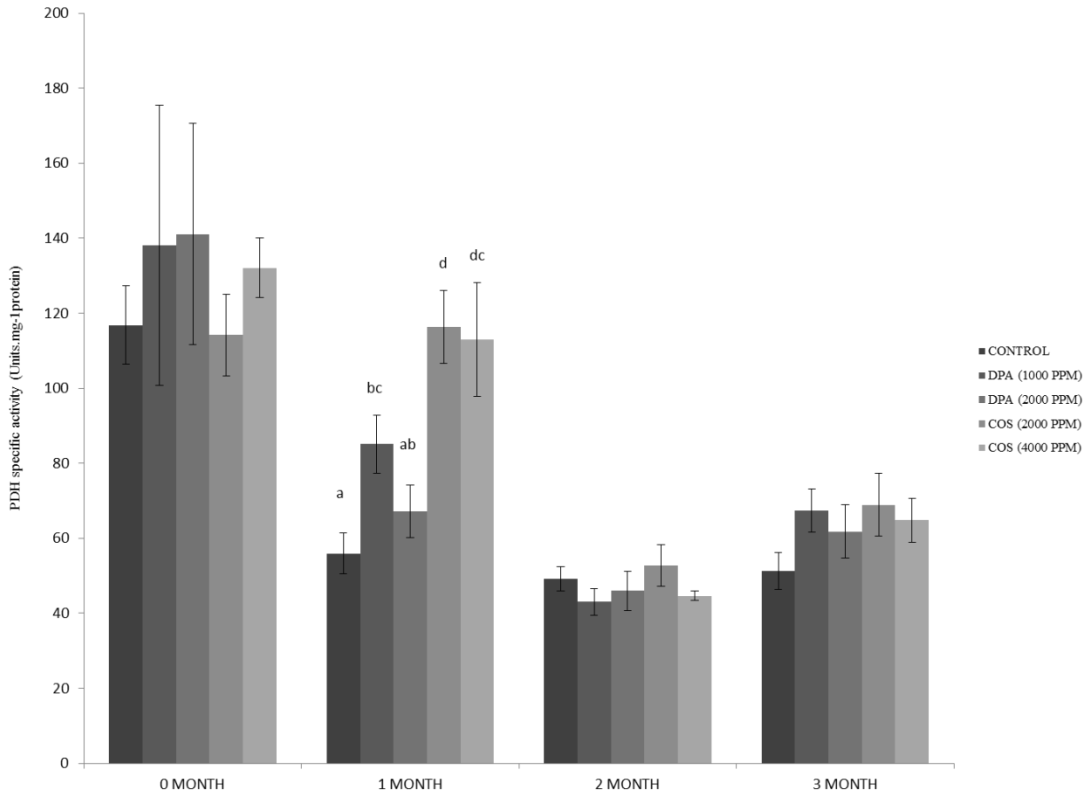

Figure 80: Effect of postharvest treatments on changes in Proline dehydrogenase activity (nmol mg-1 protein) in apple peel during 3 three month storage.

Initial baseline proline content at 0 month was not significantly different between treatments. Proline content was in the range of 7.5-8.7 mg/g of apple peel (Fig. 81). Proline content decreased after one month of storage and no significant changes were observed between treatments during remaining period of the study. Adyanthaya et al., (2007) reported a decrease in proline and PDH content for two and three month time period after an initial increase at one month. They further suggested a switch to TCA cycle from the the proline metabolism pathway which leads to likely generation of free radicals leading to an accelerated deterioration and loss of preservation.

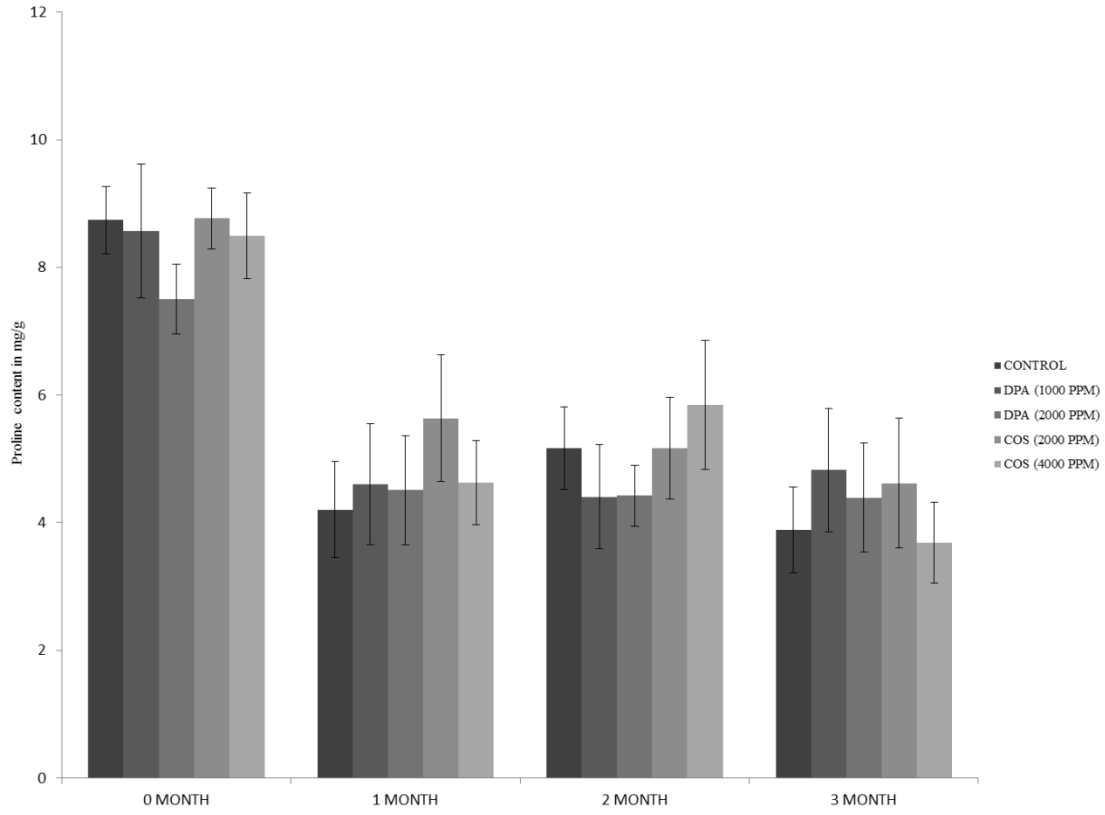

Figure 81: Effect of postharvest treatments on Total proline content (mg g-1 FW) in apple peel during 3 three month storage.

7.1.4.3 Changes in Superoxide Dismutase (SOD), Catalase (CAT), and Guaiacol peroxidase (GPX) Activity in Apple Peel

To investigate the effects of treatments on inducing antioxidant enzymes, the activity of three key antioxidant enzymes; SOD, CAT and GPX were assayed. SOD activity was higher in DPA treatments at 0 months as compared to control and COS treatments. However, this difference was not significant ($P>0.05$) at 0 months. SOD activity ranged from 8.9-13.4 units/mg protein (Fig 82). After one month of storage,

significant difference (P<0.05) in SOD activity was observed between treatments. Control had the lowest activity whereas COS treatment had the highest activity.

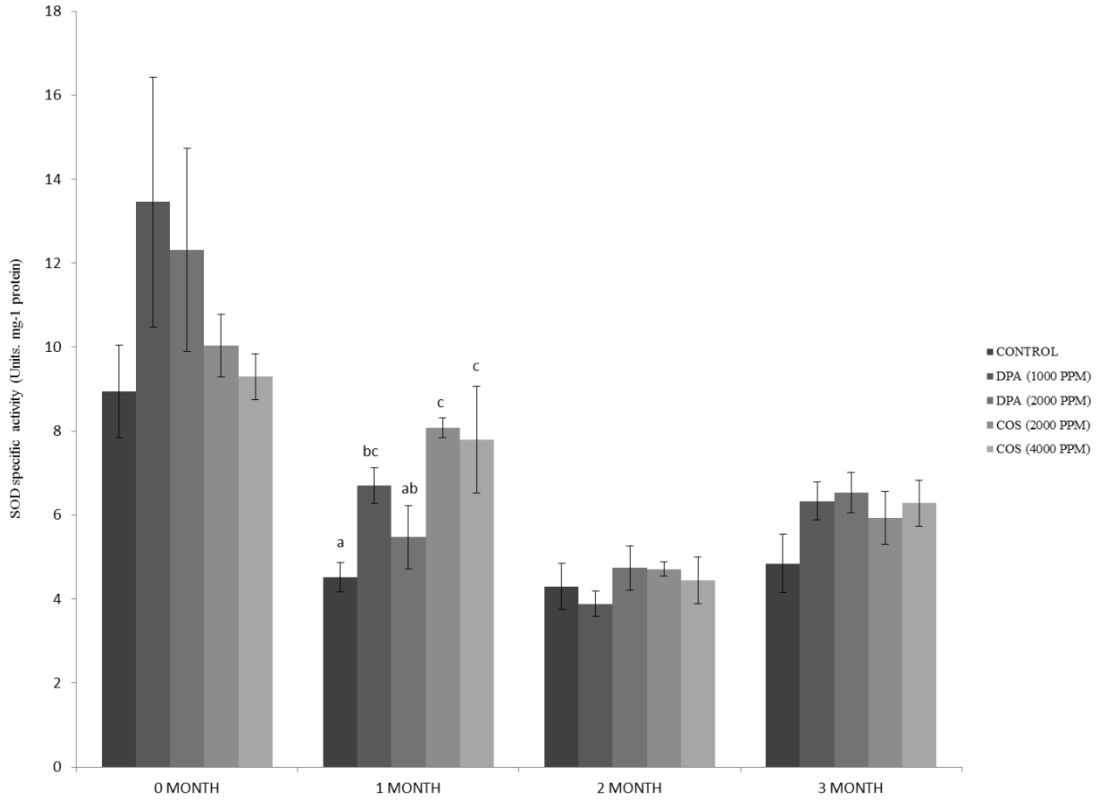

Figure 82: Effect of postharvest treatments on changes in Superoxide dismutase activity (Units mg-1 protein) in apple peel during 3 three month storage.

At two months of storage SOD activity decreased for all treatments activity and no significant difference (P>0.05) was observed between treatments either at two or three months of storage. This is in contrast to results reported by Adyanthaya et al. (2007) where they reported an initial increase in activity in apple varieities at one month followed by a decrease at two and three month storage time. Further they reported that

higher SOD content related to better presererva tion in apples. This is in contrast to our results where we did not find a correlation between antioxidant function and scalding which has also been reported to be linked to oxidative reactions. Other factors such as type of antioxidants; lipophilic or hydrophilic, the stage at which they are overexpressed and the time of storage may also be important.

At 0 months of storage, CAT activity ranged from 15.9-33.5 units/mg protein (Fig 83). No significant difference was found (P>0.05) between treatments for CAT activity throughout the period of the study.

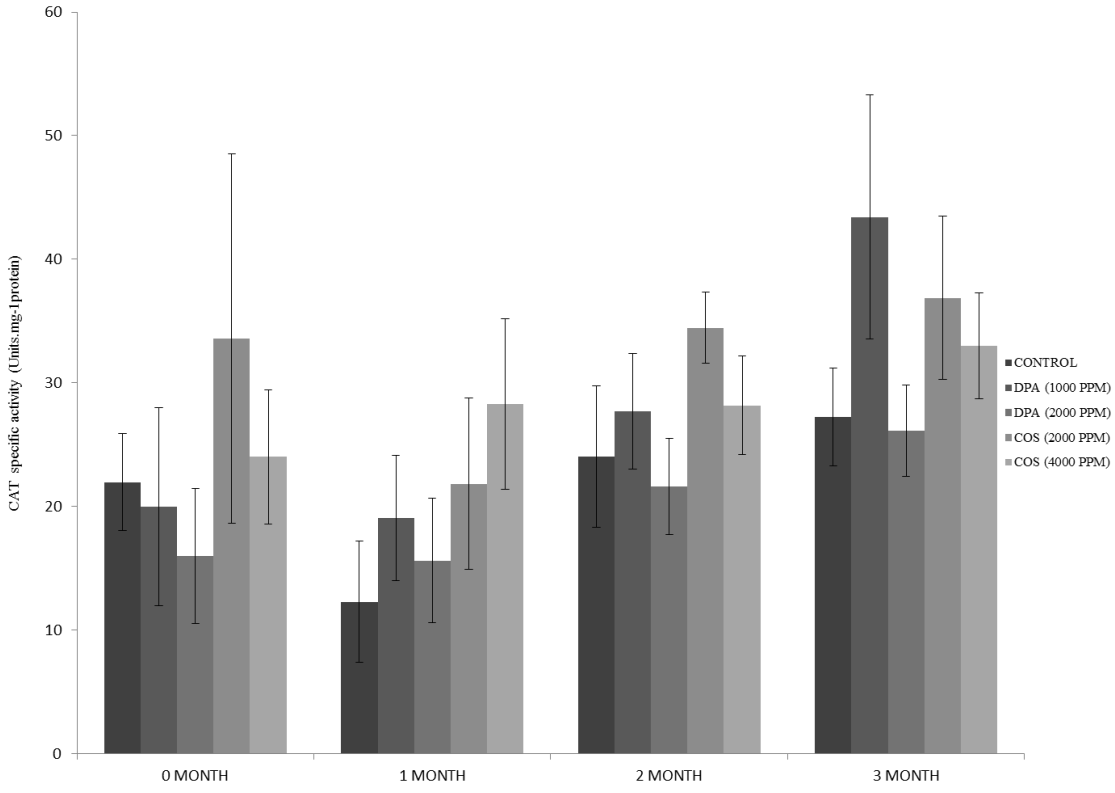

Figure 83: Effect of postharvest treatments on changes in Catalase activity (Units mg-1 protein) in apple peel during 3 three month storage.

This indicates that although scald development may be related to oxidative stress, hydrogen peroxide may not be involved in the pathogenesis of this physiological disorder.

DPA treatments had significantly lower ($P<0.05$) GPX activity than chitosan oligosaccharide at 0 month of storage (Fig 84). The significant difference at 0 months between treatments cannot be explained at this time. A similar trend was observed at one month of storage except that control also had significantly lower activity ($P<0.05$) as compared to chitosan oligosaccharide treatment. At two months of storage there was no difference between DPA and chitosan oligosaccharide treatments. However, a significant difference was observed between chitosan oligosaccharide and control treatments ($P<0.05$). No significant difference between treatments was observed at the final time point of analysis. Adyanthaya et al., (2007), reported an initial increase at one month in GPX activity and a decrease in activity in all vairieties after that. The difference in results here may be due to the different treatments used.

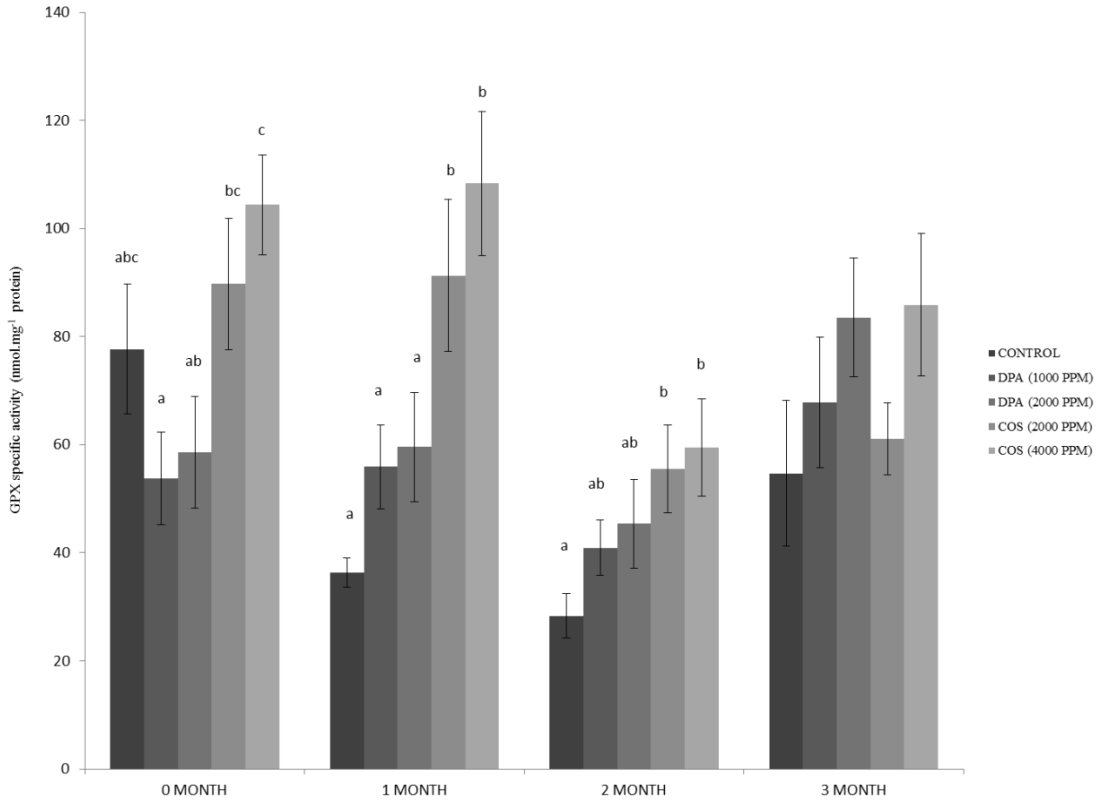

Figure 84: Effect of postharvest treatments on changes in Guaiacol peroxidase activity (µmol mg-1 protein) in apple peel during 3 three month storage.

7.1.4.4 Changes in Total Soluble Phenolics and Free Radical-linked Antioxidant Activity of Apple Peel

Total soluble phenolic content in apple peel was assayed using the Folin-Ciocalteu method. Total phenolic content ranged from 2.4-4.8 mg/g at 0 month of storage (Fig. 85). No significant difference was observed at 0 and one month of storage although DPA 1000 ppm and COS 2000 ppm treatments had higher phenolic content

than other treatments.

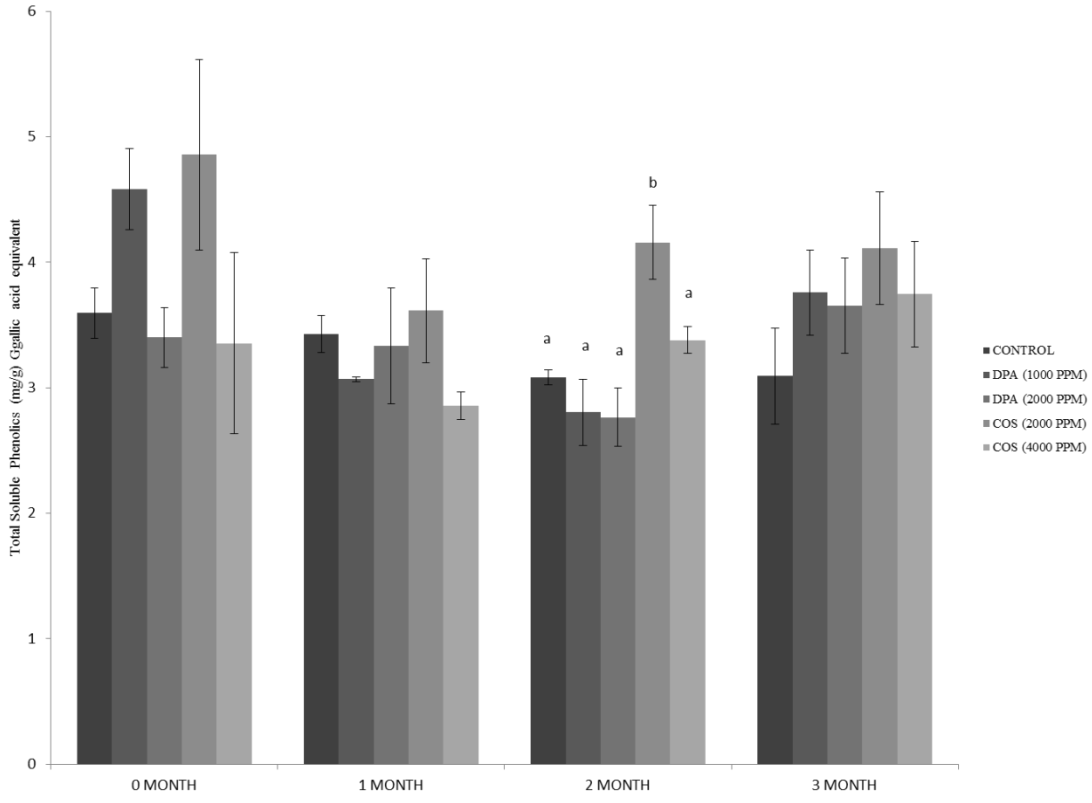

Figure 85: Effect of postharvest treatments on changes in Total phenolic content (mg/g of sample DW) in ethanolic extracts of apple peel during 3 three month storage.

At two months of storage, chitosan oligosaccharide 2000 ppm had significantly higher content ($P<0.05$). At three months of storage control had the lowest phenolics whereas chitosan oligosaccharide 2000 ppm treatment had the highest phenolics. However, this difference was not significant ($P>0.05$). Overall total phenolics did not change significantly over a three month storage period for the same treatment.

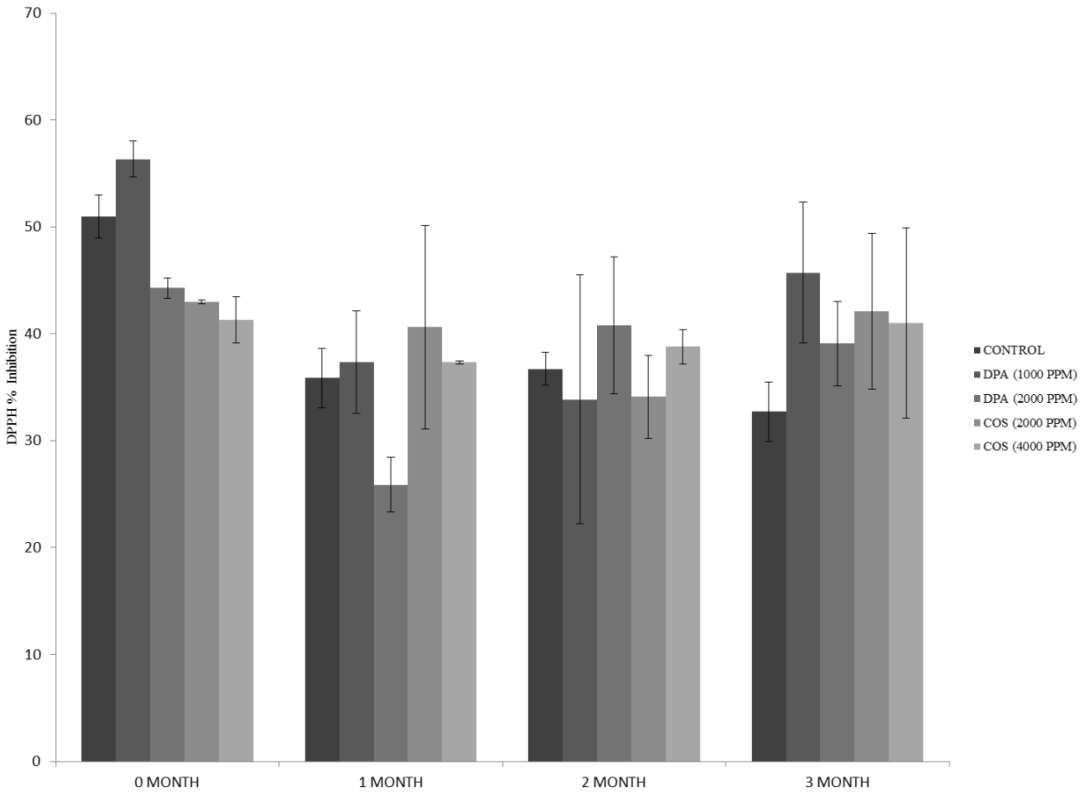

Figure 86: Effect of postharvest treatments on changes in free radical linked DPPH scavenging activity (percentage inhibition) in ethanolic extracts of apple peel during 3 three month storage.

Free Radical-linked antioxidant activity in peel was not significantly different ($P>0.05$) (Fig. 86). At 0 month DPA 1000 ppm treatment had the highest free radical linked antioxidant activity however this difference was not significant ($P>0.05$). No significant difference was found between treatments throughout the period of this study.

7.1.4.5. Changes in Total Soluble Phenolics and Free Radical-linked Antioxidant Activity of Apple Peel and Pulp for Health Benefits linked Functionality Assays

Aqueous and ethanolic extracts of apple peel and pulp were evaluated to investigate the changes in apple phenolics and free radical linked antioxidant activity during storage which has relevance in management of early stage type 2 diabetes. Ethanolic extracts of peel had slightly higher phenolics as compared to water extracts (Fig. 87).

Phenolic content peaked for all treatments at two months of storage. Similar results have been reported in Macintosh varieties over a three month storage time (Adyanthaya et al., 2007). Further the changes in phenolic content during storage were culivar dependent; Macintosh, Empire and Mutsu varieties increased over a three month storage time whereas Cortland remained the same in water extractions of peel. In ethanolic extractions phenolic content increased for Empire, Cortland and Macintosh whereas it remained constant for Mutsu (Adyanthaya et al., 2007). The results for phenolic content here are slightly different as compared to the phenolic content observed in apple peel data in post harvest stage. This could be because the process and concentration of apple peel used here to determine the phenolic content in the functionality stage and post harvest stage are different. More severe extraction procedures are used in the functionality to determine the phenolic content. Overall phenolic content for water extracts remained the same after three months of storage. For ethanolic extracts phenolic content increased untill two months of storage before decreasing at the three months storage period. Lowest phenolic content was observed for DPA 1000 ppm at one month of storage (538 µg GAE/g FW) whereas highest value

(1444 µg GAE/g FW) was observed for chitosan oligosachharide treatment 4000 ppm at two months of storage.

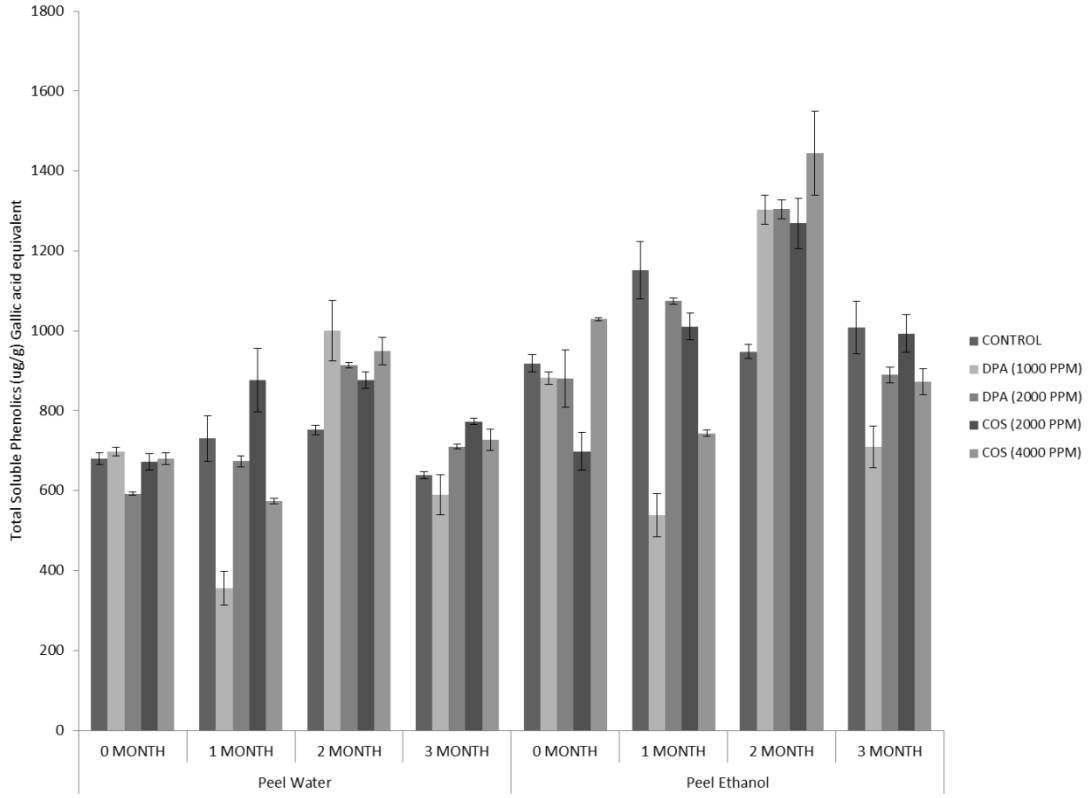

Figure 87: Effect of postharvest treatments on changes in Total phenolic content (mg/g of sample DW) in ethanolic and aqueous extracts of apple peel during 3 three month storage.

For water extracts, pulp phenolics slightly increased over three month storage period (Fig. 88). Change in phenolics content of ethanolic extracts was more treatment dependent.

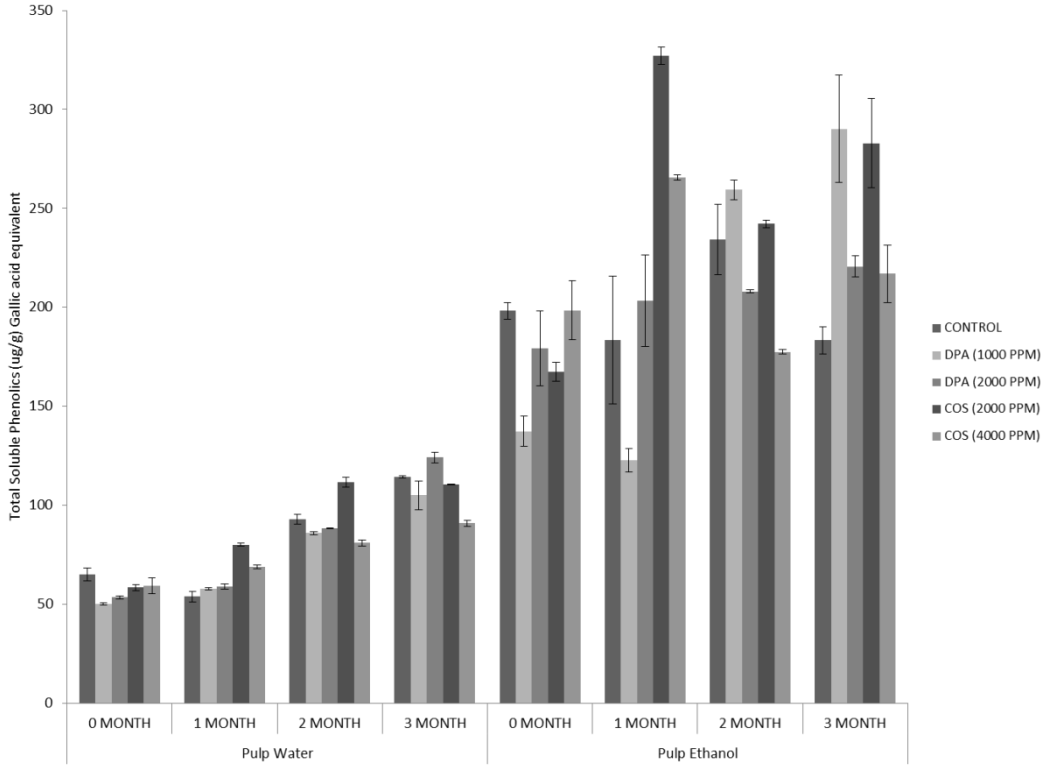

Figure 88: Effect of postharvest treatments on changes in Total phenolic content (mg/g of sample DW) in ethanolic and aqueous extracts of apple pulp during 3 three month storage.

Highest phenolics was observed in chitosan oligosachharide 2000 ppm treatment at one month of storage (327 µg GAE/g FW) whereas lowest was found in DPA 1000 ppm (122 µg GAE/g FW).

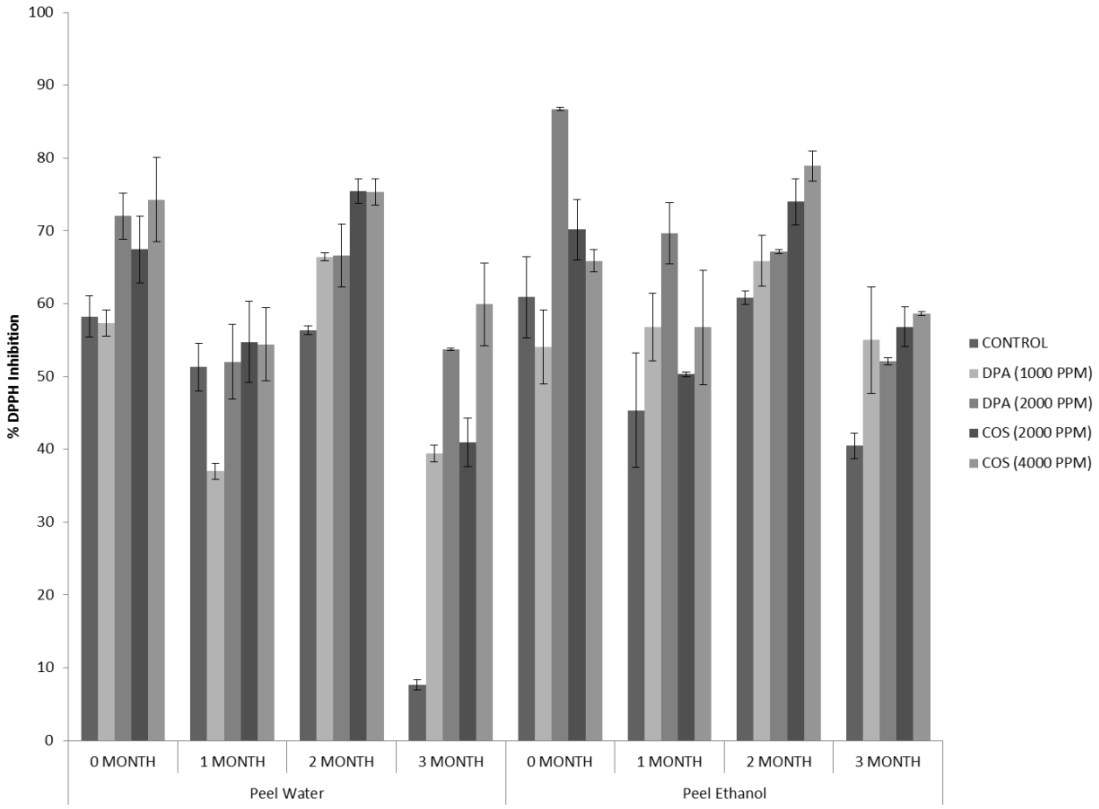

Figure 89: Effect of postharvest treatments on changes in free radical linked DPPH scavenging activity (percentage inhibition) in ethanolic and aqueous extracts of apple peel during 3 three month storage.

Overall there was not a significant difference in the antioxidant activity of water and ethanolic extracts (Fig. 89). Antioxidant activity for both water and ethanolic

extracts slightly decreased over a three month storage period. A wave like trend was observed for treatments, over a period of 3 months. Treatments that had lower free radical antioxidant activity at one month had lower activity at 2 months and it again increased at 3 months. This cyclical rise and fall was observed for all treatments over the course of this study. Water extracts of control at 3 months of storage had the lowest free radical linked antioxidant activity (7%). Adyanthaya et al., (2007) reported a wave like trend in ethanolic extracts whereas in water extracts they reported a decrease in antioxidant activity at one month followed by a graduall increase in phenolic content. It has been reported that higher phenolic content may be linked to better post harvest preservation and this link may be explained through its antioxidant function, amongst many other potential mechanisms. Higher antioxidant activity will help tissues overcome oxidative stress linked post harvest deterioration which will lead to better preservation during storage.

For water and ethanolic extracts of pulp, free radical linked antioxidant activity slightly decreased over a three months storage period (Fig. 90). Overall peel extracts had high free radical linked antioxidant activity as compared to pulp extracts.

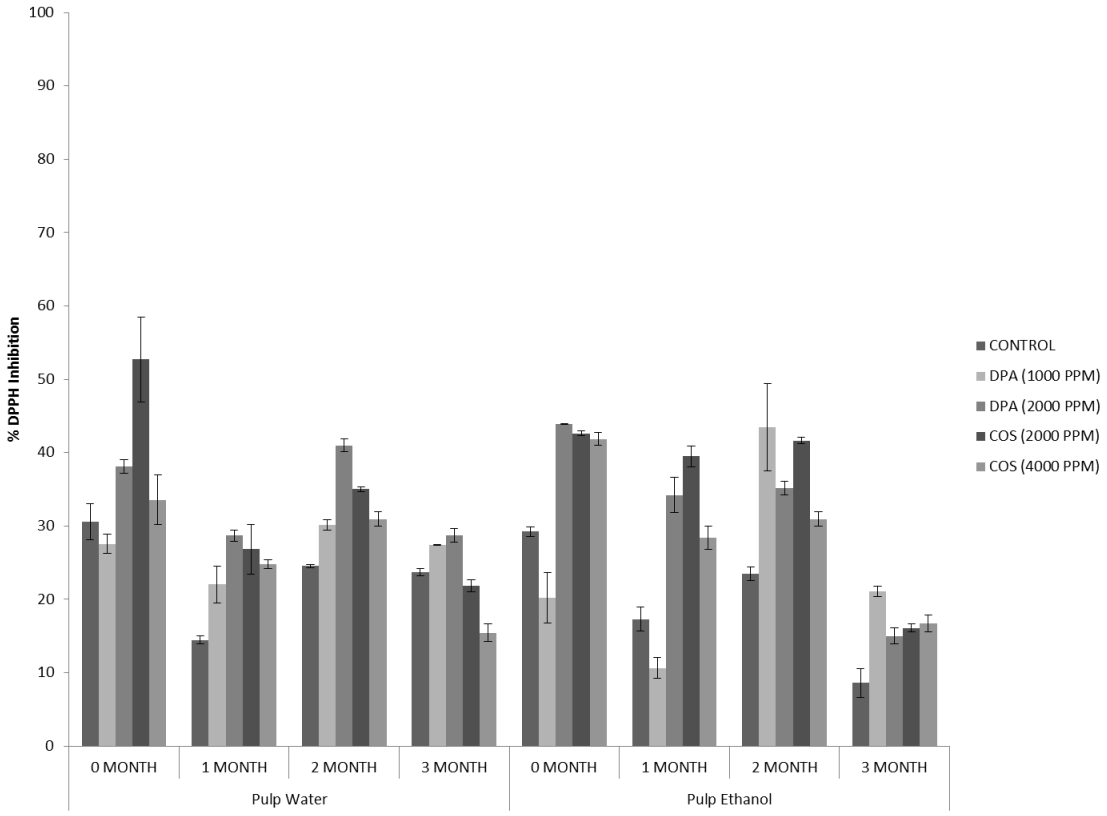

Figure 90: Effect of postharvest treatments on changes in free radical linked DPPH scavenging activity (percentage inhibition) in ethanolic and aqueous extracts of apple pulp during 3 three month storage.

7.1.4.6 α-Amylase Inhibition Assay and α-Glucosidase Inhibition Assay of Apple Peel and Pulp for Health Benefits linked Functionality

α-Amylase and α-Glucosidase are the major carbohydrate metabolizing enzymes in human digestive process and the changes in activity of these enzymes were evaluated to determine the effect of treatments and storage on management of early stages of type 2 diabetes by potential apple phenolic bioactives.

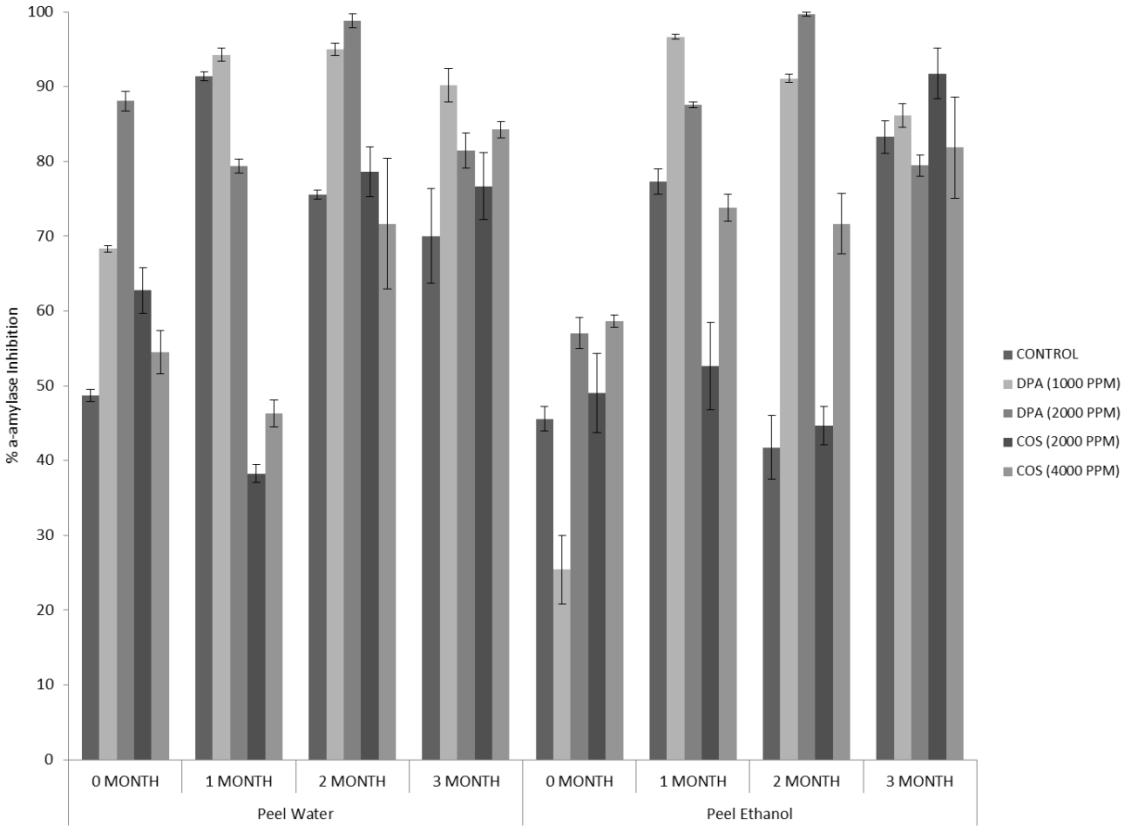

Figure 91: Effect of postharvest treatments on changes in percentage α-amylase inhibitory activity in ethanolic and aqueous extracts of apple peel during 3 three month storage.

Overall there was not a significant difference in the α-amylase inhibitory (Fig. 91) activity of water and ethanolic extracts of peel. Adyanthaya et al., (2007) did not detect any α-amylase inhibitory activity in any samples which is in contrast to the results obtained in this study. Barbosa et al., (2011) reported higher α-amylase inhibitory activity in pulp than peel in both water and ethanolic extractions for all

varieties in long term stored apples. Further a high correlation was reported between phenolic content and α-amylase inhibitory in long term stored apple.

COS and control treatments had lower activity than DPA treatments at 0 month. At one month COS treatmnets remained constant whereas the activity of control and COS treatments increased at 2 month period. This could be because a higher GPX activity was observed in COS treatmnets which may have resulted in polymerization of phenolics overtime resulting in an increase in α-amylase inhibitory activity of COS treatmnets as compared to other treatments. in peel ethanol COS had a wave like cyclical increase and decrease in activity. Activity slightly increased over a 3 month period of storage. Lowest activity amongst treatments was found in DPA 1000 ppm (25%) at 0 months whereas highest activity was observed in DPA 2000 ppm (99%) at two months of storage.

It has been previously reported that apple pulp has significantly higher activity α-amylase than peel so the pulp was appropriately diluted (1:2) to get the inhibition values in an acceptable range. Water extracts of pulp had high inhibition (70-100%) (Fig. 92). Storage period of three months or the effect of treatments had no difference on α-amylase inhibitory effect.

Ethanolic extracts of pulp had lower α-amylase inhibitory activity as compared to water extracts. Overall the activity remained constant over a three month storage period except for a sharp decrease for DPA 2000 ppm treatment at 2 months. α-Amylase inhibitory activity for COS treatments decreased at 2 month period after an initial increase at one month time period

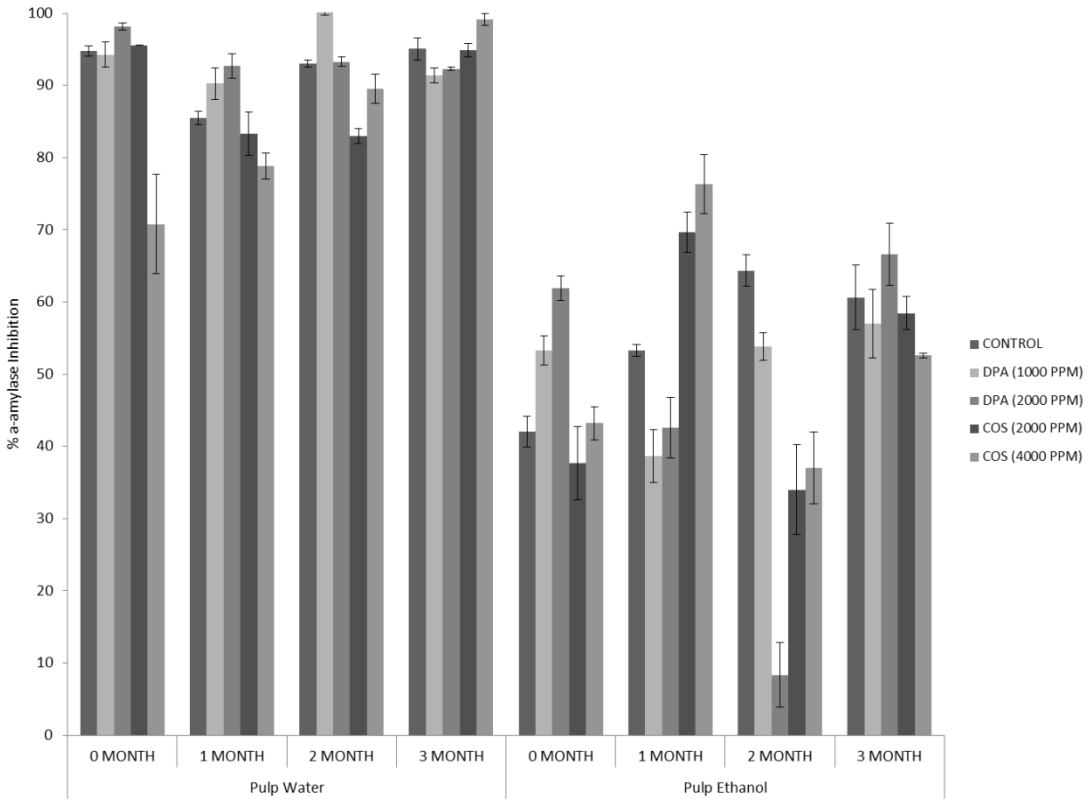

Figure 92: Effect of postharvest treatments on changes in percentage α-amylase inhibitory activity in 1:2 diluted ethanolic and aqueous extracts of apple pulp during 3 three month storage.

α-Glucosidase inhibitory activity was high in water and ethanolic extracts of peel (47-100%) (Fig. 93). Except for lower inhibitory activity of DPA treatment (1000 ppm) at one and three month, α-glucosidase inhibitory activity remained constant over a three month period of storage. In peel water DPA 1000 ppm had lower activity at 1 and 3 months whereas DPA 2000 ppm treatment had lower activity at 0 month. COS 2000

ppm treatment had lower activity at 0 month as compared to other treatments. There was not a significant difference between (P>0.05) water and ethanolic extracts of peel.

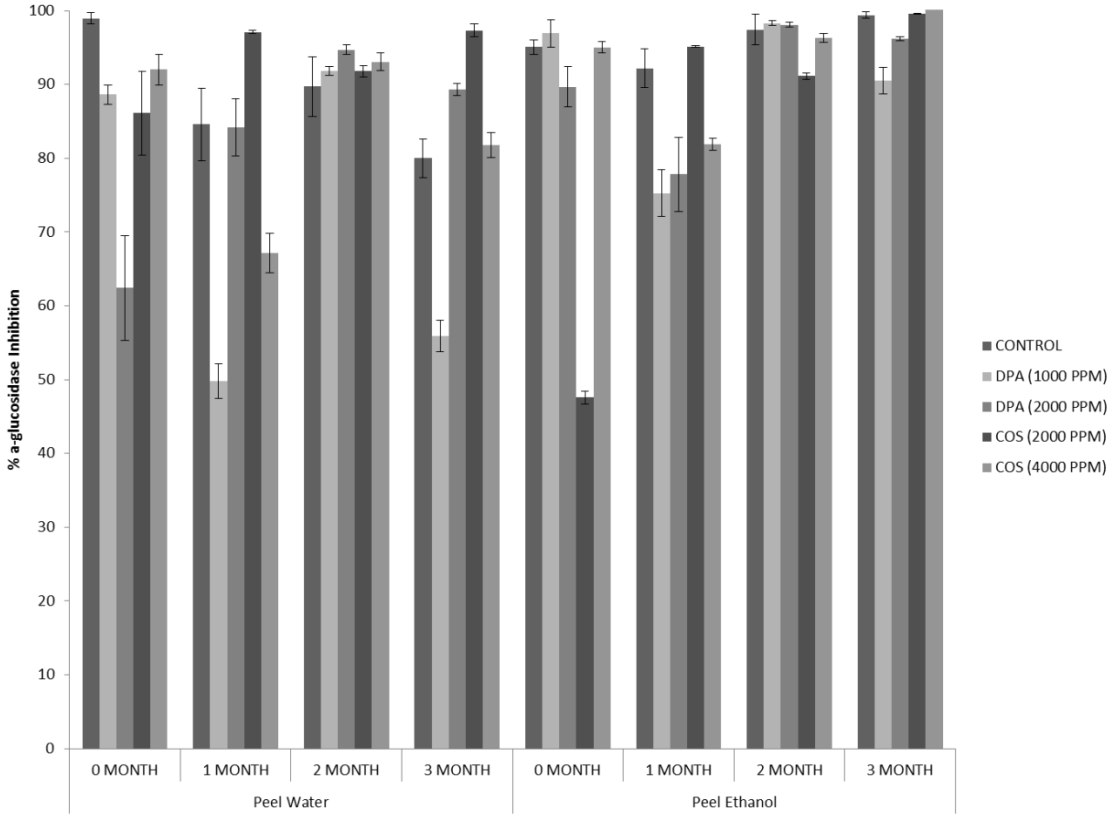

Figure 93: Effect of postharvest treatments on changes in percentage α-glucosidase inhibitory activity in ethanolic and aqueous extracts of apple peel during 3 three month storage.

Adyanthaya et al., (2007) reported higher α-glucosidase inhibitory activity in peel as compared to pulp. Barbosa et al., (2011) reported higher α-glucosidase inhibitory activity in pulp in water extracts in contrast to higher α-glucosidase

inhibitory activity in peel in ethanolic extracts. Further α-glucosidase inhibitory activity had moderate correlation to phenolic content. Adyanthaya et al., (2007) reported that α-glucosidase inhibitory activity was maintained in Macintosh and Cortland cultivars over a three month storage time whereas it decreased for Empire and Mutsu cultivars. Storage had more effect on α-glucosidase inhibitory activity of pulp especially in ethanolic extracts. They further reported a high correlation between phenolic content and α-glucosidase inhibitory activity. Changes in α-glucosidase inhibitory was treatment dependent (Fig. 94). In pulp water DPA 2000 ppm treatment had lower activity at 0 month and DPA 1000 ppm had lower activity at 2 month as compared to toher treatment. Pulp had slightly lower α-glucosidase inhibitory as compared to peel. In pulp ethanol DPA 1000 ppm and 2000 ppm treatments had a cyclical rise and fall over 3 month whereas COS had a cyclical rise and fall in activity after 1 month. The differences in 0 month cannot be explained at this time. At 3 months of storage in both pulp water and pulp ethanol, DPA 2000 ppm treatment and COS 1000 ppm treatment had higher activities which suggests that these treatments may have a better potential in maintaining sustained α-glucosidase inhibitory activity in post harvest stored apples. Adyanthaya et al., (2007) reported that α-glucosidase inhibitory activity in pulp was low to moderate during storage and it was maintained. Changes in α-glucosidase inhibitory activity during storage and its linked to phenolic content is important to understand since varieities that have higher phenolic content have been reported to be preserved better which also suggests they will have better potential in potentially inhibiting this carbohydrate digesting enzyme and therefore may have potential in management of oxidation linked chronic diseases.

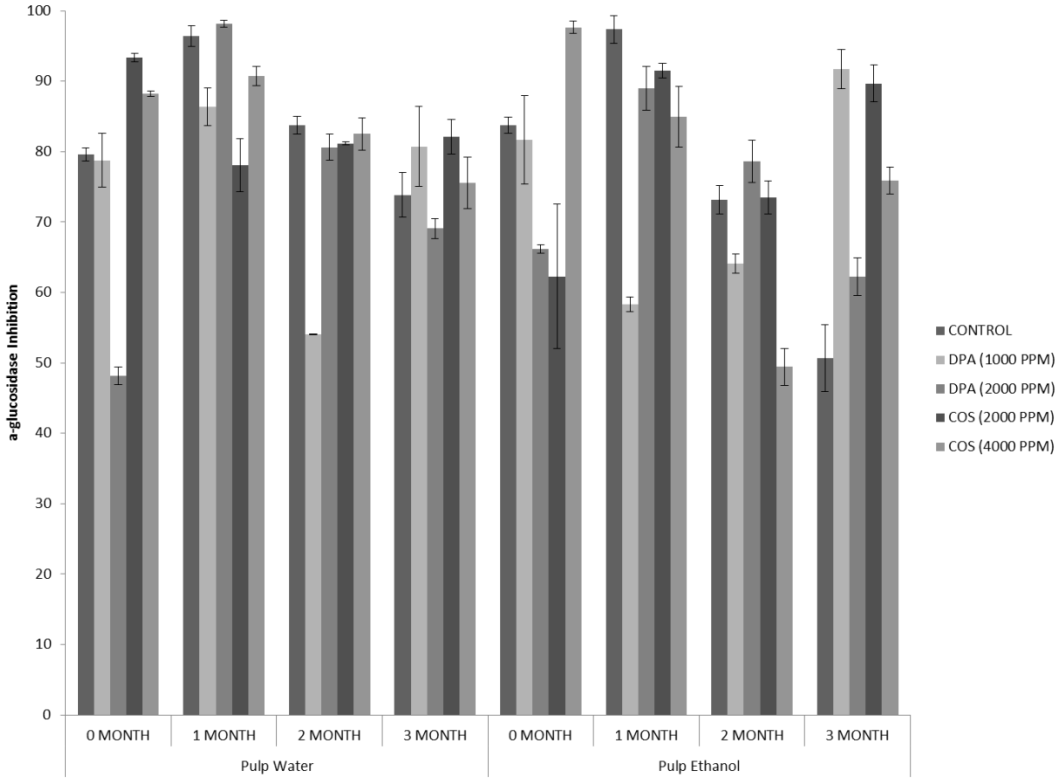

Figure 94: Effect of postharvest treatments on changes in percentage α-glucosidase inhibitory activity in ethanolic and aqueous extracts of apple pulp during 3 three month storage.

7.1.4.7 Changes in Profile of Phenolic Compounds in Apple Peel and Pulp using HPLC

Phenolic profile was determined using HPLC. In ethanolic and water extracts of peel, chlorogenic acid, p-coumaric acid and quercetin derivatives were the major phenolic compounds detected (Fig. 95). In ethanolic and water extracts of pulp, gallic acid and chlorogenic acid were the major phenolic compounds detected.

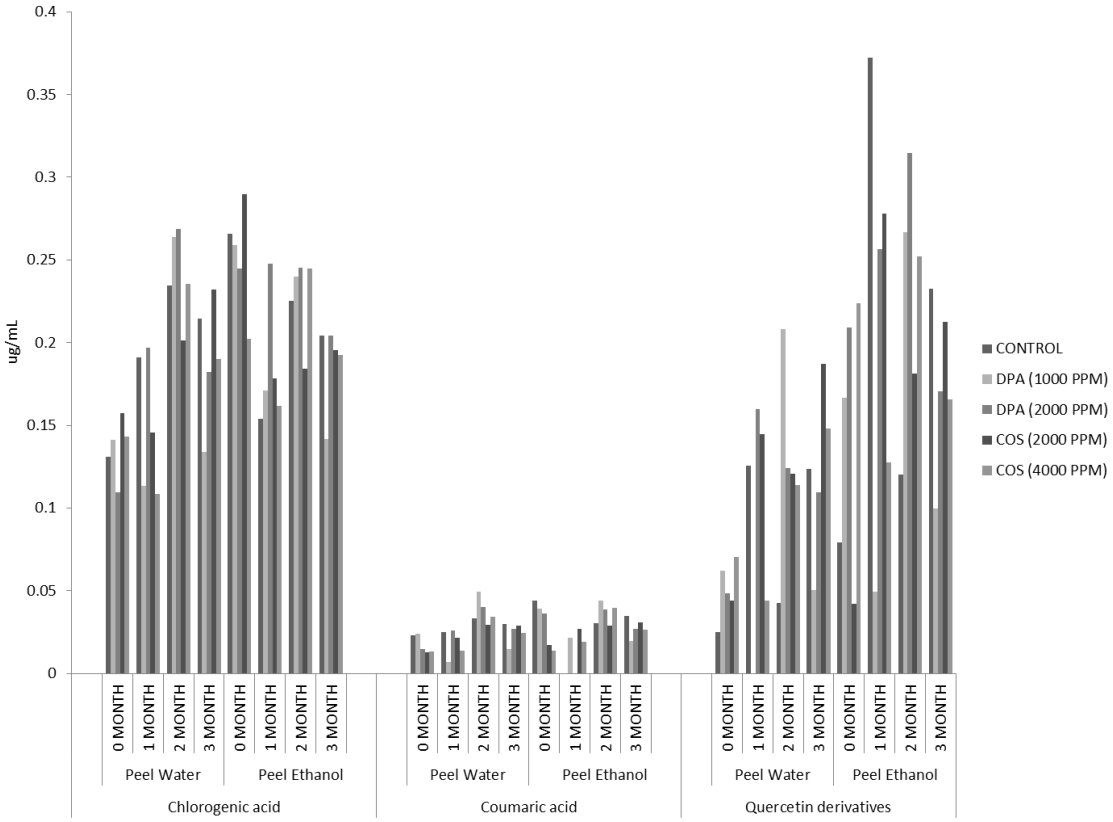

Figure 95: Effect of postharvest treatments on changes in individual phenolic compounds in ethanolic and aqueous extracts of apple peel during 3 three month storage.

The changes in individual phenolics in ethanolic and water extracts of peel and pulp during storage period were treatment dependent and a general trend could not be detected in the changes (Fig. 96).

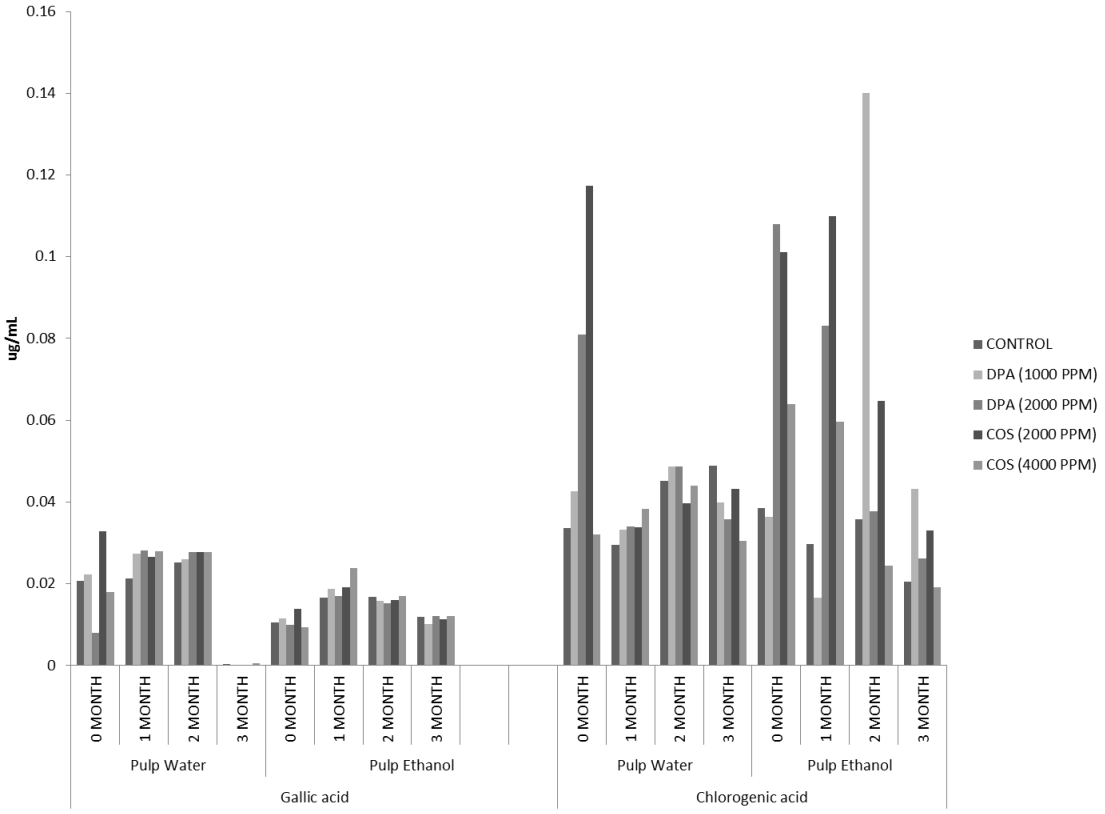

Figure 96: Effect of postharvest treatments on changes in individual phenolic compounds in ethanolic and aqueous extracts of apple pulp during 3 three month storage.

7.1.4.8 Malondialdehyde (MDA), CT Content, and PPO activity

The malondialdehyde (MDA) content reflects breakdown of membrane due to likely effects of ROS and was therefore evaluated to indicate the protective effect of treatments on apple peel cellular breakdown. No significant difference ($P>0.05$) was observed between treatments at 3 months storage time (Fig. 97). This result is slightly

surprising given that scald development has been related to cellular tissue damage which results into polymerization of phenolics by PPO leading to development of brown color.

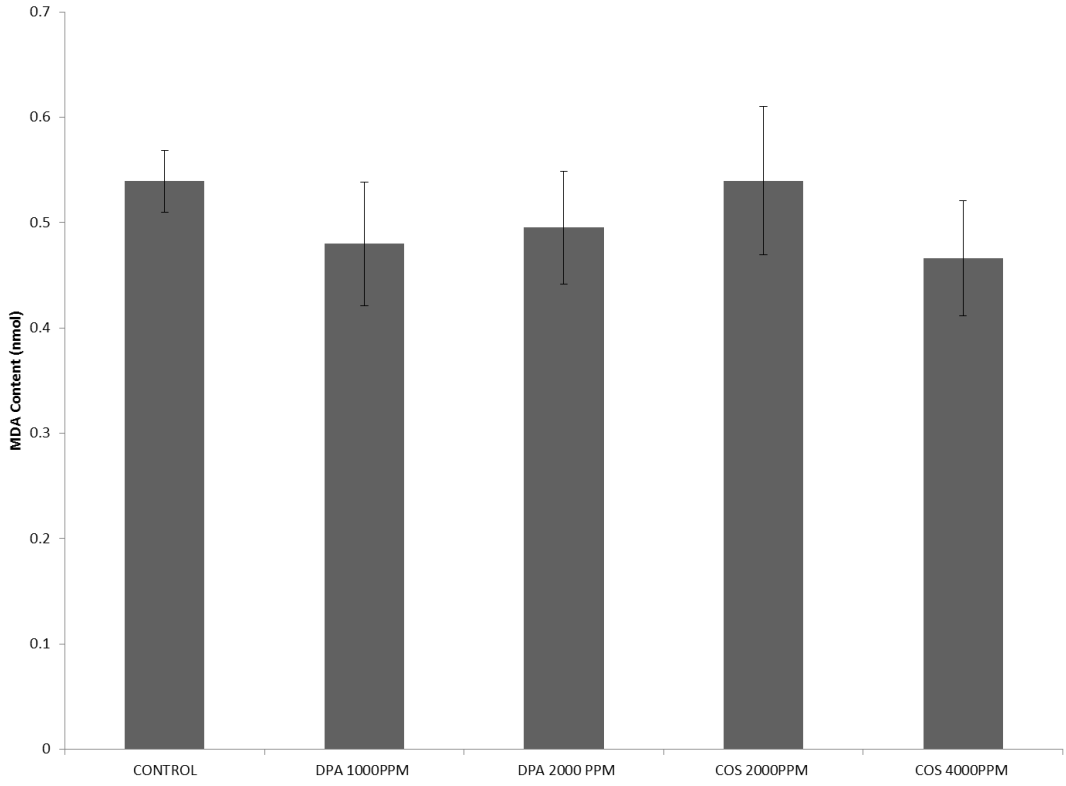

Figure 97: Effect of postharvest treatments on Changes in formation of malondialdehyde (MDA) in apple peel evaluated at three months of storage.

α-Farnesene is oxidized to conjugated triene (CT) oxidation products which has been reported to be the primary cause of scald development. DPA treatments (1000 and 2000 ppm) had significantly lower ($P<0.05$) CT content at three months of storage as compared to control and chitosan oligosaccharide treatments (Fig. 98). Chitosan

oligosaccharide 4000 ppm treatment had more CT content than control which indicates it may potentially accelerate scalding. Interestingly CT content in the two DPA treatments was the same even though the treatment doses were different.

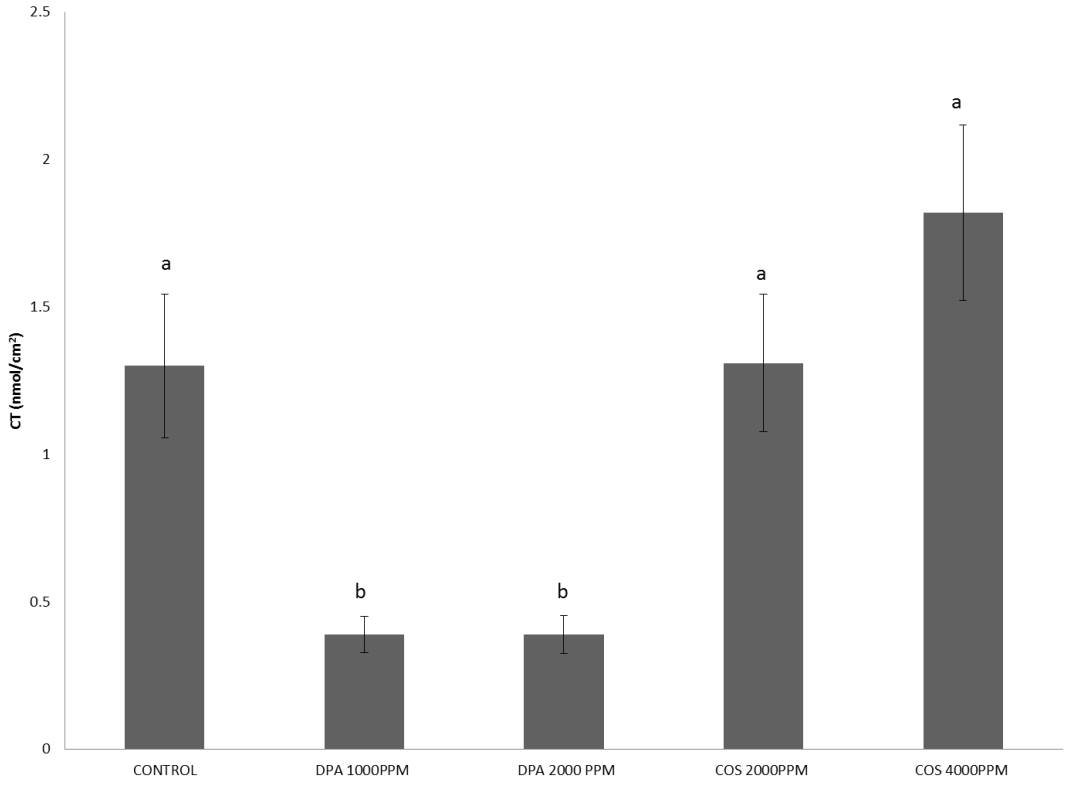

Figure 98: Effect of postharvest treatments on Changes in formation of Conjugated Triene (nmol/cm^2) in apple peel evaluated at three months of storage.

PPO activity was determined which has relevance in linking phenolic content and scald development (Fig. 99). Interestingly, no significant difference was observed in PPO content between treatments at three months of storage. This suggests PPO activity may be more relevant during or just prior to developing scald when apples are stored at room temperature which correlates with MDA content.

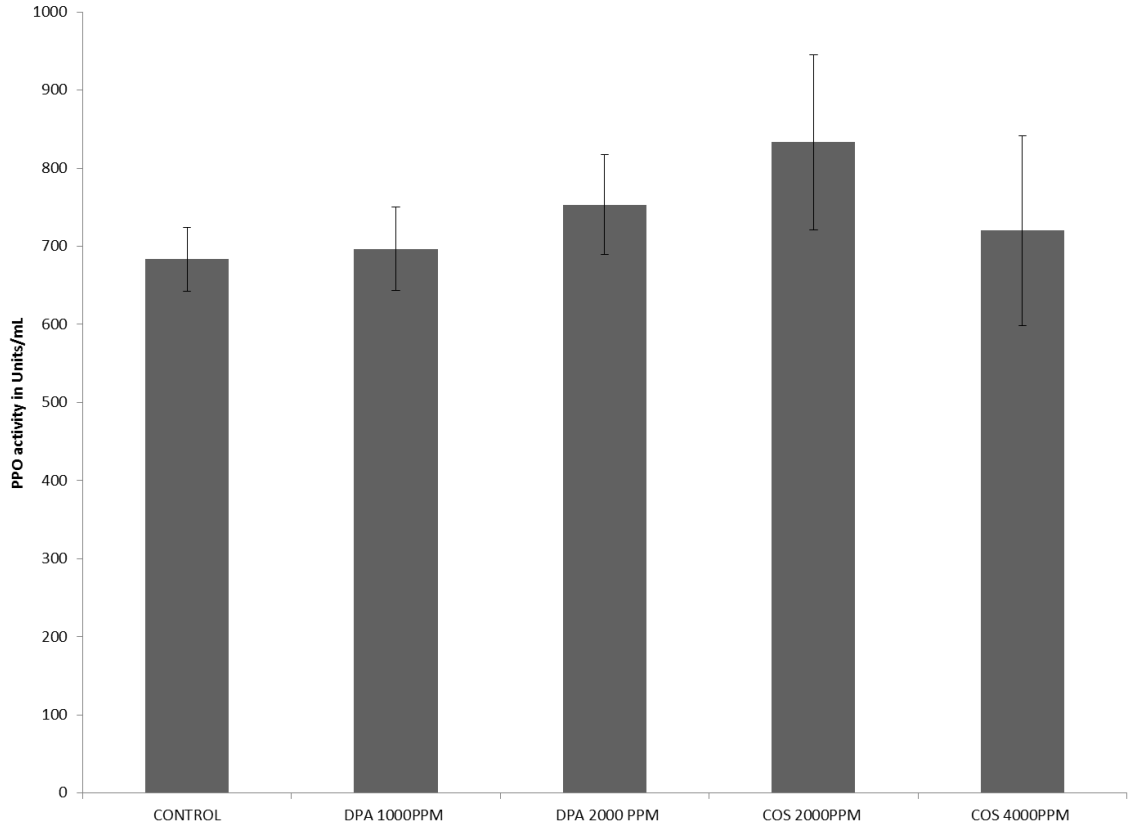

Figure 99: Effect of postharvest treatments on Polyphenol Oxidase Activity (Units/mL) in apple peel evaluated at three months of storage.

7.1.4.9 Superficial Scald Evaluation

Superficial Scald evaluation was carried out after one week of storage at room temperature. Results were expressed as percentage (%) superficial scald (Fig. 100). DPA treatments had significantly lower ($P<0.05$) visual superficial scald as compared to control and chitosan oligosaccharide treatments. Further DPA had lower 'mild', 'moderate' and 'severe' scald as compared to control and chitosan oligosaccharide

treatment. This suggests chitosan oligosaccharide may not be effective at the tested doses in preventing superficial scald.

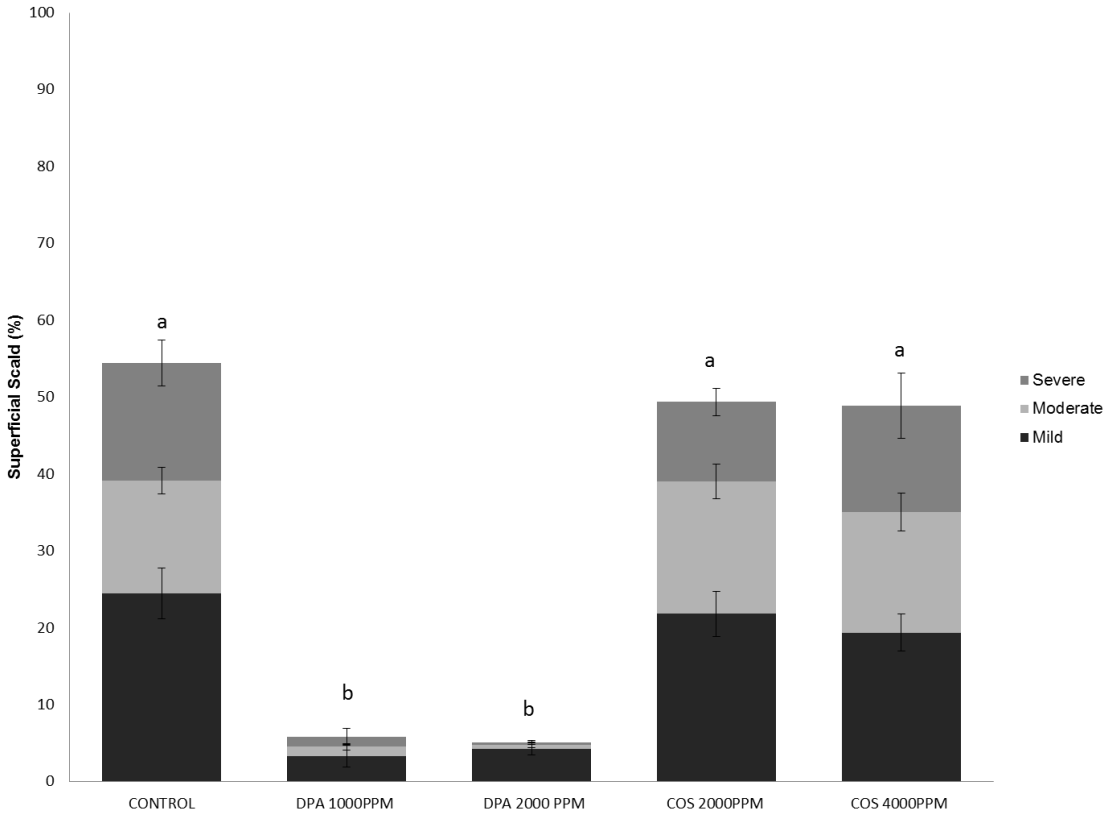

Figure 100: Effect of postharvest treatments on superficial scald development in apples evaluated at three months of storage.

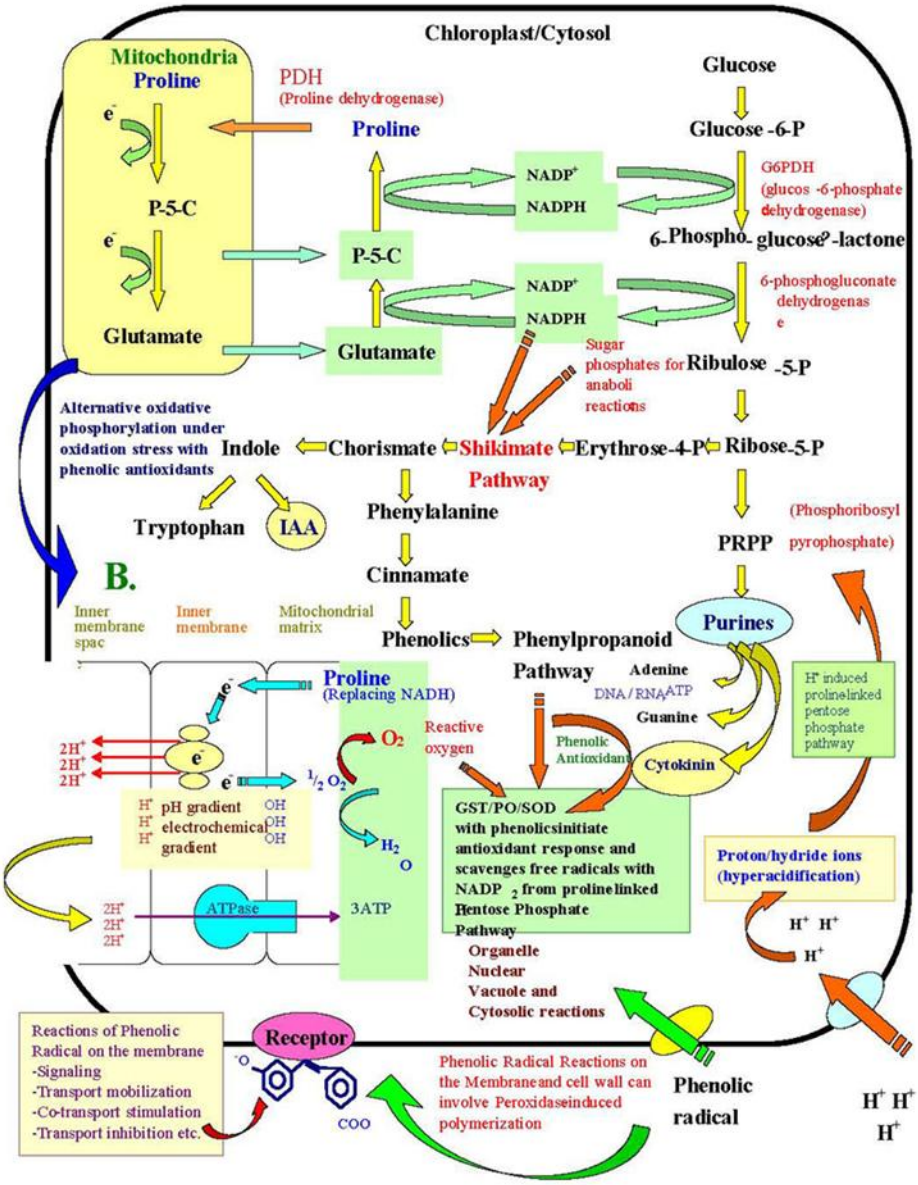

Figure 101: Proline Linked Pentose Phosphate Pathway

7.1.5 Discussion

Stimulation of pentose phosphate pathway involving generation of NADPH and the coupling of this redox cycle to proline synthesis and other anabolic pathways including antioxidant enzyme response pathways and phenolic biosynthesis was investigated considering the role of antioxidants and phenolics in scald development (Shetty, 2004; Treutter, 2001; Lurie and Watkins, 2012) (Fig. 101).

Chitosan oligosaccharide 2000 ppm treatment had significantly higher ($P<0.05$) phenolic accumulation during the two month storage period as compared to other treatments. Overall, control and DPA had lower phenolic accumulation as compared to chitosan oligosaccharide treatments. Decrease in flavonoid synthesis by DPA treatment has been reported previously (Duvenage and Deswardt, 1973). Phenolic content in apple peel during storage has been reported to be cultivar dependent over a three month storage period (Adyanthaya et al., 2007). Chitosan coatings have been shown to increase phenolic compounds in fruits and prevent postharvest decay by activating plant defense response (Zhang et al., 2011). An increase in accumulation of hydroxycinnamic acid derivatives in dates (Hassni et al., 2004) and in wheat an increase in ferulic acid along with lignin synthesis was observed (Reddy et al., 1999). An increase in the key enzyme, phenyl ammonia lyase of the phenolic pathway that converts L-phenylalanine to trans-cinnamate was also observed with chitosan treatments (Romanazzi et al., 2002; Zhang et al., 2011). No significant difference ($P>0.05$) in free radical linked antioxidant activity, as determined by DPPH assay, was detected between treatments. Although many externally applied antioxidants and induction of antioxidant response have been tested none of them have been proven consistent. The effectiveness of externally

applied antioxidants as scald inhibitors not only depended on its solubility characteristics but also on functional group position and characteristics (Rudell et al., 2005). Ascorbic acid was ineffective in both water and lipid soluble form whereas BHT was as effective as DPA only at higher concentrations (Watkins et al., 1988; Chellew and Little, 1995). Lipid soluble α-tocopherol was not only completely ineffective but acted as a pro-oxidant (Ju and Curry, 2000; Lurie and Watkins, 2012). Higher phenolic content has been linked to better post harvest preservation (Adyanthaya et al. 2007). Higher phenolic content can not only serve as a marker for selecting varieities that can preserve better but also deliver better potential in management of early stages of chronic oxidation linked diseases.

GPX is an isoenzyme of peroxidases that cross-links the phenolic moieties from the phenylpropanoid pathway for the biosynthesis of lignins and lignans (Morales and Barcelo, 1997). It is also involved in number of other biological processes including ethylene production, host defense response and aromatic compound degradation (Dijana et al., 1996). Increase in phenolic compounds possibly induced stimulation of GPX production reflecting the need in apple peel for lignification and structural development during storage. DPA treatments have been reported to prevent scald development by a general decrease in metabolic activity (Lurie et al., 1989; Lurie et al., 1990). An increase in GPX should inversely correlate to scald development since it may make individual phenolic moieties unavailable for polymerization by PPO. Both control and chitosan oligosaccharide treatments had no significant difference in scald however there was significant difference in GPX activity. At this point the correlation between GPX activity and scald development is not clear and warrants further investigation.

G6PDH activity in chitosan oligosaccharide and DPA treatments were significantly higher ($P<0.05$) than control at one month of storage period. This increase in G6PDH activity should activate the pentose phosphate pathway to drive the carbon flux towards erthyrose-4-phosphate for biosynthesis of shikimate and phenylpropanoid metabolites (Shetty and Wahlqvist, 2004) and perhaps other intermediates. Increase in G6PDH activity in chitosan oligosaccharide treatments was reflected in higher phenolics in these treatments. Faust et al., (1967) reported that DPA prevented scald development by an increase in activity of pentose phosphate pathway. This is further supported by the fact that recently it has been reported that scald development is related to oxidative stress and pentose phosphate pathway can provide NADPH required for antioxidant enzyme response and other metabolites for scald prevention. In this study, we observed that chitosan oligosaccharide treatment had higher stimulation of the pentose phosphate pathway than DPA treatments however this did not correlate to scald inhibition. At the three month time storage period G6PDH activity for DPA 1000 ppm increased. However, there was not a significant difference between treatments. It is possible that involvement of pentose phosphate pathway may be more relevant when apples are stored at room temperatures after removal from cold storage when scald development is in its preliminary stages or measuring the activity after scald development may present a clearer picture about the involvement of this pathway and the activity of the rate limiting enzyme of this pathway.

Respiration drives carbon flux towards two essential needs; oxidation of substrates to provide energy and provide intermediates required for biosynthesis (Botha et al., 1992). Chitosan oligosaccharide treatments had higher SDH activity throughout

the period of the study and this difference ($P<0.05$) was significant at three month storage time. This suggests chitosan oligosaccharide treated apples had a higher need for cellular energy and this was achieved by stimulating the TCA cycle. High SDH activity indicates a higher carbon flux through glycolytic pathways providing the required phosphenolpyruvate needed for phenolic synthesis through shikimate pathway. The combination of higher G6PDH and SDH activity delivers a balance between the anabolic and catabolic needs in chitosan oligosaccharide treatments and subsequently helps to drive phenolic biosynthesis and antioxidant enzyme response. Higher SDH activity may also relate to higher proline accumulation through glutamate synthesis and stimulation of pentose phosphate pathway through $NADP^+$/NADPH redox cycling. However, proline accumulation was similar between treatments during the storage period and a significant difference ($P<0.05$) was not found. However this could be due to rapid catabolism of proline by PDH providing reducing equivalents in the mitochondria for oxidative phosphorylation.

Superoxide radicals ($O_2^{\cdot -}$) are acted upon by SOD and converted to H_2O_2 which are further acted upon by CAT and converted to water and molecular oxygen. SOD activity slightly decreased over the storage period. Chitosan oligosaccharide and DPA treatments had significantly higher activity than control. This could be due to higher SDH activity in these treatments producing ROS and it is possible that this high amount of ROS may have triggered a higher SOD response. The changes in CAT activity over the storage period were treatment dependent. CAT activity had increased for DPA 1000 ppm at three months of storage. SOD and CAT activity are related to stimulation of pentose phosphate pathway in response to oxidative stress and it is possible that the

activity of these antioxidant enzymes may be more relevant when apples are stored at room temperatures when scald is in its developmental stages or measuring the activity after scald development may present a clearer picture in the involvement of these antioxidant enzymes. It is also possible that other antioxidant enzymes such as glutathione peroxidase or ascorbate peroxidase may be involved. Contradictory results have been reported in the literature on the effect of antioxidant enzymes on scald susceptibility. It has been reported that hydrogen peroxide concentration increased during storage (Zubini et al., 2007) and H_2O_2 concentration was higher in scald susceptible cultivars (Rao et al., 1998). Du and Bramlage (1995) reported no correlation between ROS, H_2O_2 and scald susceptibility. They also reported higher CAT and POX activity in scald resistant 'Empire' as compared to scald susceptible 'Cortland' and 'Delicious'. Du and Bramlage (1995) further reported that SOD activity in 'Empire' did not correlate to scald whereas Abbasi and Kushad (1995), reported correlation of scald and SOD but not POX. Rao et al., (1998), reported higher activity of CAT to lower H_2O_2 concentration and lower scald susceptibility. Overall relation of scald susceptibility to antioxidant enzyme has been inconclusive. However, a general theory of free radical induced α-farnesene oxidation and scald development and the role of antioxidant enzyme in lowering free radicals may explain the biochemical rationale.

Hyperglycemia an abnormal increase in post prandial blood glucose rise has been associated with the onset of type 2 diabetes (Kwon et al., 2008). α -Glucosidase and α-amylase are the key carbohydrate metabolizing enzyme and moderate inhibition of these enzymes can help slow the breakdown and absorption of glucose into the blood stream which may be an important strategy in management of early stage type 2

diabetes. Phenolic compounds naturally found in fruits have been reported to have inhibitory potential against these carbohydrate metabolizing enzymes (Matsui et al., 2001; Matsui et al., 2007; McCue and Shetty, 2004). Our aim was to investigate the changes in phenolic content and profile during storage and treatments and its effect on key enzymes for potential management of hyperglycemia. Phenolic profile and content in water and ethanolic extracts of peel and pulp changed during storage and these changes were time and treatment dependent. Similarly the changes in water and ethanolic extracts of peel and pulp α -glucosidase and α-amylase inhibitory activity were treatment dependent and it was difficult to draw a correlation between phenolic content and inhibitory activity against α -glucosidase and α-amylase. Barbosa et al., reported a sustained inhibitory activity profile in peel and pulp of long term stored apples. Similar results were reported by Adyanthaya et al., (2007) where inhibitory activity in Cortland apples were maintained over a three month storage time. The results obtained in our study may be slightly different because the apples in our study were treated with DPA and COS. This may influence the way phenolics are synthesized, polymerized which may have direct implication in the *in vitro* inhibition of these carbohydrate dugetsing enzymes.

Oxidation of α –farnesene leads to formation of conjugated triene and this oxidation product damages apple tissue eventually causing cell death and development of scald. DPA treatment had significantly lower CT content and scald development. However this did not correlate to malondialdehyde (MDA) content which reflects membrane breakdown. This indicates other oxidative species or process may be involved in the breakdown of cellular membranes which led to MDA content being not

significantly different between treatments. Similarly PPO content was not significantly different between treatments. PPO content may be more relevant depending on the extent of tissue breakdown and availability of phenolics for polymerization. Scald development is an oxidative process and coincides with the oxidation of α-farnesene. It has been suggested that α-farnesene oxidation products damage cellular membrane causing reaction of PPO with phenolic substrates leading to the typical browning observed in superficial scald (Abdallah et al., 1997). They further suggested that the loss in membrane fluidity of chloroplast due to a decrease in unsaturation index of glycolipids may decompartmentalize PPO. Ethylene production is known to stimulate α-farnesene accumulation and therefore treatments that modify ethylene production have an effect on α-farnesene accumulation. Lower production of α-farnesene leads to lower accumulation of its oxidation products consequently lowering the scald susceptibility of apples (Lurie and Watkins, 2012). Du and Bramlage (1993), further hypothesized that the ratio of CT258:CT281, which reflect the different oxidation production of α-farnesene is more important.

Different classes of phenolic compounds such as phenolic acids, hydroxycinnamic acid derivatives as well as flavonoids have been detected in apple peel (Ju et al., 1996). A rapid decline in dihydrochalcones, flavanols, chlorogenic acid and flavanol has been reported during the early developmental stages (Burda et al., 1990; Treutter, 2001). During storage, a general decline in polyphenol fraction including epicatechin, quercetin and unknown polyphenolic fraction has been reported (Piretti et al., 1994; Ju et al., 1996). Phenolic compounds in apple peel may have a dual role in superficial scald development. α-Farnesene oxidation may result into disintegration of

cellular membranes and increase the incidence of scald in apples. High levels of chlorogenic acid may support the oxidation process by acting as a cofactor for PPO, the decompartmentalisation of which results into acceleration of this browning reaction (Ju et al., 1996; Treutter, 2001). Ju et al., (1996) reported that different phenolics have different roles in scald development. They suggested that while lignins are stable and flavonoids are not suitable substrates for PPO, simple phenols are unstable and may be prone to oxidation by PPO which leads to browning of tissue. Abdallah et al., (1997) reported that only cinnamic acid derivatives serve as substrates for PPO oxidation whereas by contrast Ju et al., (1996) and Piretti et al., (1994) reported that both simple phenols and flavonols may also be involved.

7.1.6 Conclusion

The objective of this investigation was to understand how inducible phenolics and antioxidants coupled with proline linked pentose phosphate pathway may have positive consequences for scald prevention in apples. Understanding this link will help us identify key markers involved in the process of scalding which will help us select varieities with better scald prevention based on the activity of these markers. Although chitosan oligosaccharide treatments increased the activity of the pentose phosphate pathway, TCA cycle, antioxidant enzyme response and phenolic biosynthesis, as compared to DPA and control treatment, these factors did not relate to superficial scald

development. Change in phenolic profile was dependent on treatment and storage conditions. Significant difference was not observed between treatments in the inhibition of α-amylase and α-glucosidase but overall apple is a good source of inhibitory bioactives against these target enzymes. PPO and MDA content were similar in treatments. However, there was a significant difference between CT content. Although antioxidant enzyme response was stimulated during storage in chitosan oligosaccharide treatments it did not correlate to inhibition of oxidation of α-farnesene to CT. Other natural alternative treatments combination of chitosan oligosaccharide and DPA in different combinations may be investigated as a potential inhibitor of superficial scald in apples.

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