SYNERGISTIC BIOACTIVE ENHANCEMENT OF PHYTOCHEMICAL-LINKED FOOD SAFETY RELEVANT AND HEALTH PROTECTIVE BENEFITS OF SELECT PLANT-BASED FOODS AND BY-PRODUCTS

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The Supervisory Committee certifies that this ***disquisition*** complies with North Dakota State University's regulations and meets the accepted standards for the degree of

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ABSTRACT

Under-utilized, ethnic, or traditional plant-based foods, food ingredients, or processed plant by-products have dual functional food safety and health protective benefits due to their rich phytochemical profile. Pre- and post-harvest strategies can enhance the phytochemical content in plant-based foods and by-products with an improvement in the overall food safety and health protective benefits. Based on this rationale, the food safety relevant antimicrobial properties of amla, kokum, clove, garlic, grape, flaxseed, emmer wheat and corn distillers dried grain with solubles (DDGS) were selectively screened for *in vitro* antimicrobial activity against common food borne pathogens *Salmonella*, *Listeria*, *Escherichia coli* and one human gut pathogen *Helicobacter pylori*. Additionally, the health protective benefits such as antioxidant, anti-hyperglycemic, anti-hypertensive, and prebiotic activity were also investigated using *in vitro* assay models. Pre-harvest (ozone treatment) and post-harvest (milling, fermentation, extraction, synergy) strategies were also utilized to improve the phytochemical-linked functionality of these plant-based foods and plant by-product. Amla, kokum, clove, and garlic displayed high antimicrobial activity against most of the *Salmonella*, *Listeria*, and *E. coli* serovars tested, and the antimicrobial activity was specific for the bacterial strain. Integration of amla and kokum in grape juice further enhanced the antimicrobial, anti-hyperglycemic, and anti-hypertensive activity of the grape juice. Pre-harvest ozone treatment of white and red grape cultivars resulted in improved TSP content and anti-hyperglycemic relevant α-amylase inhibitory activity. High phenolic-linked antioxidant activity was found in aqueous extracts of milled flaxseeds, while flax lignan had high anti-hypertensive property. Emmer wheat with hull had highest TSP content, antioxidant and antihyperglycemic activity (before and after milling) when compared to commercial wheat cultivars. Furthermore, extracts of Emmer fermented with lactic acid bacteria

(LAB) showed an altered phenolic profile with mild antimicrobial activity against *H. pylori*. Extracts of corn DDGS fermented with LAB showed an altered phenolic profile and the antimicrobial activity against *H. pylori* was attributed a culture isolated from corn DDGS which was later identified as *Bacillus amyloliquefaciens*. These results indicate that screening and pre- or post-harvest processing strategies are an effective approach to improve the phytochemical-linked food safety and human health protective benefits of underutilized and common plant-based foods and plant by-product.

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DEDICATION

I would like to dedicate this thesis to my late father D.S. Christopher and my mother Lumena Christopher, whose support and guidance ultimately led me down the path to pursuing my doctoral program. I would also like to dedicate this thesis to my sister, grandmothers, family members and friends, who have helped shape and mold me into the person that I am today. Finally, I would like to dedicate this thesis to my wife Ankaa, you are the lighthouse that guides me home and I'm happy and blessed to have you in my life.

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LIST OF ABBREVIATIONS

ABTS	2,2-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid).
ACE	Angiotensin I-converting enzyme.
DDGS	Distiller's dried grains with solubles.
DPPH	2,2-diphenyl-1-picrylhydrazyl.
MIC	Minimal inhibitory concentration.
NCD	Non-communicable chronic disease.
PCR	Polymerase chain reaction.
ROS	Reactive oxygen species.
T2D	Type 2 diabetes.
TSP	Total soluble phenolic

CHAPTER 1. INTRODUCTION

1.1. Global Food and Nutritional Insecurity Related Public Health Challenges

One of the major concerns of global food insecurity is the lack of access to or the under-utilization of phytochemical rich plant-based foods that have health-protective and food safety relevant benefits. This concern has significantly impacted the sustainable development mission of the United Nations to ensure holistic and health-targeted food security for the wider global population. Globally, more than 1 billion people are estimated to lack sufficient macronutrient availability for energy needs and at least 2 billion suffer from micronutrient deficiencies while concurrently in the United States alone, nearly 50 million people are affected by food and nutritional insecurities from these deficiencies (Barrett, 2010; Gundersen & Ziliak, 2015). Furthermore, rapid climate change has exacerbated the global food insecurity challenges due to added pressure on the food production systems, which in turn has disrupted the process to achieve food security solutions worldwide. Models based on climatic trends linked to the production of the largest global plant-based commodity crops (wheat, maize, rice, and soybean) from 1980 to 2008 has shown a 3.8 to 5.5% decline in the production of maize and wheat respectively, while the net impact on rice and soybean production was insignificant due to gains in production in some countries balancing out the losses in others (Lobell, Schlenker & Costa-Roberts, 2011).

Current strategies such as plant breeding through hybridization or the use of plant tissue culture techniques, mostly focus on improving the genotypic traits that code for desirable food production-related phenotypic characteristics such as high grain yield and grain size, improved photosynthetic efficiency, and high protein or carbohydrate content in different plant-based foods. However over time, the selective breeding and cultivation of a limited choice of food

crops has led to a genetic erosion of genotypic traits that code for other important phenotypic characteristics, such as resilience against biotic and abiotic stress, as well as superior quality or quantity of micronutrients and health-relevant phytochemicals that are generally present in diverse plant-based foods (Bijlsma & Loeschcke, 2012; van de Wouw, Kik, van Hintum, van Treuren & Visser, 2010; Renna, Serio, Signore & Santamaria, 2014). Another important factor linked to food insecurity is the rise in consumption of hyper processed foods that satisfy calorific requirements from soluble refined carbohydrates but provide poor nutrition and little or no health-protective benefits. Indeed, the consumption of hyper-processed foods can be directly linked to rise of NCDs such as type 2 diabetes, hypertension, dyslipidemia, and chronic obesity. In North America, food insecurity is consistently more prevalent among households with a person living with type 2 diabetes or other NCDs (Gucciardi, Vahabi, Norris, Del Monte & Farnum, 2014).

However, before examining the role of plant-based foods and the different strategies or solutions that can help address the food insecurity linked public health challenges, it is necessary to first understand the extent to which these challenges have placed a burden on our society at the global and national level. These global public health related challenges not only affect the morbidity or mortality rates of a country, but they can also cause economic damage due to other factors such as loss of productivity or earnings and increase in medical expenses during treatment. Indeed, lower to middle income nations often bear the brunt of the economic burden caused by poor nutrition and the associated food safety burdens from contaminated food related public health challenges.

1.1.1. Burden of foodborne illnesses caused by bacterial pathogens

An important coupled burden of improving food insecurity related food systems challenges is the need for concurrent improvement in food safety associated with microbial contamination. Despite significant attempts to address the burden of foodborne diseases, microbial contamination by bacterial pathogens can occur at any step in the food chain since these microbes are highly versatile enabling them to survive and adapt to different environmental conditions (Havelaar et al, 2010). Furthermore, the estimation of the global burden of foodborne diseases is a complex process due to the diversity of microbial hazards present in foods, the different health outcomes they cause, and the complex pathways of transmission for these hazards (Havelaar et al, 2013). In 2006, the World Health Organization (WHO) launched the 'Initiative to Estimate the Global Burden of Foodborne Diseases', and in 2007, the Foodborne Disease Burden Epidemiology Reference Group was established to help lead this initiative (World Health Organization, 2015). According to global estimates, 31 foodborne hazards causing 32 diseases were mentioned in the report which included 7 bacterial pathogens that were classified as diarrheal agents (*Campylobacter*, enteropathogenic *Escherichia coli*, enterotoxigenic *E. coli*, shiga toxin-producing *E. coli*, non-typhoidal *Salmonella enterica*, *Shigella* spp., and *Vibrio cholerae*), and 5 bacterial pathogens that were classified as invasive infectious agents (perinatal *Listeria monocytogenes*, acquired *L. monocytogenes*, *Mycobacterium bovis*, *S. paratyphi*, and *S. typhi*) (Devleesschauwer, Haagsma, Mangen, Lake & Havelaar, 2018; WHO, 2015). The most frequent causes of foodborne illnesses were attributed to diarrheal disease agents, particularly *Campylobacter* and norovirus (WHO, 2015). In the United States, the Foodborne Diseases Active Surveillance Network (Food-NET) under the Center of Disease Control (CDC) provides regular preliminary reports on the incidence and trends of infections

with pathogens transmitted commonly through diverse foods. Some of the microbial contamination risk factors of plant-based foods that exist in the farm-to-fork food chain include irrigation water, manure or compost used, livestock or wild animal fecal contamination, field and worker sanitation, cross-contamination during post-harvest preservation, storage, transportation, and distribution of food to the consumer (Olaimat & Holley, 2012; Riggio, Wang, Kniel & Gibson, 2019). The latest report by Food-NET based on preliminary data collected in 2019 indicated that the incidence of infections caused by *Listeria*, *Salmonella*, and *Shigella* remain unchanged when compared to the previous years (2016 to 2018), while infections caused by other pathogens (*Campylobacter*, shiga toxin-producing *E. coli* [STEC], *Vibrio*, and *Yersinia*) increased in 2019 (Tack et al 2020). The same report found that the overall incidence per 100,000 population was the highest for *Campylobacter* (19.5), followed by *Salmonella* (17.1), STEC (6.3), *Shigella* (4.8), *Yersinia* (1.4), *Vibrio* (0.9), and *Listeria* (0.3) (Tack et al, 2020). A study on the attribution of foodborne illnesses, hospitalizations, and deaths to food commodities using outbreak data from 1998 to 2008 in the U.S concluded that among the 17 food commodities analyzed, leafy vegetables were found to have the highest number of cases associated with foodborne illnesses (22%) followed by dairy (14%), fruits/nuts (12%), and poultry (10%) (Painter et al, 2013). These statistics indicate that the burden of foodborne diseases due to bacterial pathogens continues to exist on a global and national scale and these trends are likely to continue well into the foreseeable future. Solutions to foodborne pathogen burdens are essential as health-targeted food insecurity solutions that support the macronutrient and micronutrient balance and requires synergizing to improve the quality and safety of daily food intake for overall solutions.

1.1.2. Burden of non-communicable chronic diseases (NCDs) such as type 2 diabetes and hypertension

The global burden of NCDs such as heart disease, stroke, cancer, chronic respiratory diseases, and type 2 diabetes all account for the leading cause of mortality worldwide (WHO, 2021a). Several risk factors such as tobacco use, physical inactivity, unhealthy diet, and alcohol consumption can increase the risk of NCDs which are responsible for 41 million deaths each year, accounting for 71% of deaths of all deaths globally (WHO, 2021b, 2021c). Obesity, physical inactivity, diet, tobacco use, and genetic predisposition can all contribute to the onset of type 2 diabetes, and furthermore, type 2 diabetes is the major cause of blindness, kidney failure, heart attacks, stroke, and lower limb amputation (Bottalico, 2007; WHO, 2021c;). In the United States, the national diabetes statistics report showed an estimate of around 34.2 million people of all ages, or 10.5% of the U.S population who were affected by type 2 diabetes in 2018 (CDC, 2020). The national prevalence rate of diabetes increased from 9.5% to 12.5% from the year 2002 to 2016, and in 2017 diabetes was the seventh leading cause of death in the U.S (CDC, 2020). Apart from type 2 diabetes, hypertension is another disorder that plays a key role in worldwide mortality and morbidity from cardiovascular diseases that account for 15 million deaths (Mensah, 2002). According to recent estimates by WHO, around 1.28 billion adults aged 30-79 years worldwide have hypertension with low and middle-income countries sharing the burden of this disease (WHO, 2021d). These statistics indicate that burden of NCDs such as diabetes (type 1, type 2, and gestational diabetes) and hypertension will continue to persist on a global and national scale and will continue to cause significant cases of morbidity and mortality in the future. Poor diet based on increasing consumption of hyper-processed and calorie-dense food from macronutrients such as hyper processed soluble carbohydrates is one of the major

contributors to the increasing prevalence of type 2 diabetes and other NCDs globally. Therefore, advancing dietary strategies based on whole food diversity and balanced nutritional profile is critical to reduce the risks of type 2 diabetes and other NCDs and improving health and wellness of wider communities.

1.2. Role of Plant-based Foods to Help Address Food Insecurity Linked- Public Health Challenges

The current global food insecurity linked-public health challenges with rising NCD coupled with the associated persistence of foodborne diseases along the multi-layered food chain requires further screening of plant food diversity that have a wider phytochemical profile with NCD health-protective and food safety relevant antimicrobial functional qualities. The plant-based diet includes a synergy of a wide variety of cereals, oilseeds, vegetables, and fruits, as well as herbs and spices, and through regular dietary intake these compliment of synergized nutritional- and phytochemical-rich plant-based foods can be helpful in the mitigation and management of NCDs such as type 2 diabetes (T2D) and hypertension. Furthermore, these same phytochemical-rich plant-based foods can help in the improvement of food safety due to the potential antimicrobial activity of the bioactive phytochemicals present in these plant-based foods. In general, plants have a greater bioactive rich phytochemical content, especially phenolic compounds, in the protective tissues (pericarp, seed coat, hull and related reproductive and stress exposed tissues) when compared to the nutritional tissues such as germ and endosperm because of the protective functions of phenolics against abiotic (high temperature, UV radiation, etc.) and biotic stresses (insects, herbivores, etc.), while the germ and endosperm have a higher content of macronutrients (starch, protein, and lipids) that are needed for plant growth and energy needs (Wang, Li, Ge & Lin, 2020). Therefore, preserving and improving bioavailability and bioactivity

of phytochemicals in plant foods can have dual functional benefits of concurrently improving food safety qualities and health protective nutritional qualities. Enhancing the safety of phytochemical rich plant-based foods is a good strategy to address the public health challenges due to foodborne illnesses, and concurrently such foods can also be targeted and coupled as dietary strategies to help mitigate the prevalence of NCDs. In this context, the enhancement of the phytochemical-linked bioactivity of plant-based foods is gaining increasing interest in the food industry as well as among health-conscious consumers. Plants produce a diverse group of antimicrobial compounds such as phenolic phytochemicals as part of their natural protective and adaptive responses against biotic and abiotic stresses (Christopher, Sarkar & Shetty, 2021). These phenolic phytochemicals with antimicrobial potential also have nutritional and health-protective functional benefits, which can be targeted as a part of dietary strategies to prevent and manage NCDs such as early stages of type 2 diabetes, and its associated macro and microvascular complications (Sarkar & Shetty, 2014; Sarkar, Christopher & Shetty, 2021). Furthermore, plant-based foods having a rich phenolic profile along with dietary fibers can also have prebiotic properties that support the growth of beneficial gut bacteria in humans and animals when these foods are consumed as part of the diet. Therefore, enhancing the phenolic phytochemical-linked antimicrobial, antioxidant, prebiotic, anti-hyperglycemic, and antihypertensive properties of plant-based foods to address concurrently the food safety and food security-linked public health challenges has significant merit and global relevance.

1.3. Strategies to Improve Plant Phytochemical-linked Food Safety and Human Health Benefits

An obvious solution to the food insecurity risk posed by the lack of access to or the under-utilization of plant-based foods with health-protective benefits, would be to increase the

availability and diversity of such foods that usually have a high content of phytochemicals. These phytochemicals, specifically inducible phenolics have a dual functional role in providing resilience against biotic or abiotic stress, while also providing health-protective benefits when such phytochemically rich plant-based foods are consumed in the regular diet. In addition to improving the availability or diversity of plant-based foods to help address the food insecurity risk, the health-protective benefits of consuming such plant-based foods can also help to counter and manage foodborne illnesses parallel with the rise in NCDs. Apart from ease of access and diversification of plant-based foods with a good phytochemical profile and bioactivity, different pre- and post-harvest strategies can be advanced to improve bioactive phytochemical-linked food safety and health benefits of plant derived foods or animal feeds. The regular screening of plant-based foods or food by-products for their bioactivity, the synergistic applications of plant-based foods with other bioactive food ingredients, and the biotransformation of plant-based foods through microbial processing (fermentation) can provide useful strategies to help address the foodborne illnesses and concurrently improving NCD countering benefits. Therefore, the rationale and strategy for this disquisition and results presented are that bioactive phenolic phytochemicals are good targets which have dual function benefits for countering bacterial food safety challenges while concurrently having benefits for countering NCD challenges, which are both linked to global food insecurity.

CHAPTER 2. REVIEW OF LITERATURE

2.1. Integration of Plant Food Diversity with Superior Phenolic Profile to Address Food Safety and NCD-linked Public Health Challenges

Improving the diversity of plant-based foods in the daily human diet is essential to address the key concerns of poor nutrition-linked NCDs and to counter the associated risks of food contamination with foodborne pathogens. During the course of human evolution, diet has evolved from the earliest hominins mostly consuming gathered seeds and fruits to early *Homo sapiens* having an omnivorous diet, and later on a diet rich in meat that has been consumed over the last 800,000 years of human evolution (Andrews & Johnson, 2020). Domestication of different food plants and animals in last 10,000 years has further contributed to the evolution of modern human diet. Today, the food metabolome is highly heterogeneous and complex, comprising of different plant and animal-based foods with diverse profile of macro- and micro-nutrients, secondary bioactive metabolites, additives, and cooking-derived compounds (González-Domínguez et al, 2020; Scalbert et al, 2014). However, in several regions of the world, especially in developed and some underdeveloped countries, the diet is often composed of high calorific hyper-processed foods that are lacking in human health protective functional qualities. Furthermore, these foods are often at risk of contamination with bacterial pathogens. The diversity of plant-based foods in the diet can be improved by incorporating phytochemical- and protein-rich foods with a good fiber profile. These phytochemical-rich foods can include fruits, vegetables, herbs, and spices, as well as plant-based by-products. Some of these plant-based foods are often under-utilized in regular diet, and in addition, the cultivation and consumption of these foods may be indigenous to specific countries or regions. Hence, there is a lack of scientific knowledge or consensus on incorporating these ethnic and non-ethnic plant-

based foods into the human diet to help address the NCD-linked health challenges and food safety qualities that are linked to global food insecurity challenges. Therefore, screening and optimization of plant food diversity based on their wider phytochemical-linked functional qualities such as antimicrobial, antioxidant, anti-diabetic, anti-hypertension, and gut health benefits have significant coupled human health and food safety benefits relevance. There is growing empirical evidence that indicates the dual functional benefits of phytochemical rich plant foods and plant-based animal feed, and hence provides a scientific foundation to further screen and optimize diverse plant-based foods and animal feed for specific health-targeted and food safety relevant benefits.

2.1.1. Antimicrobial activity of plant-based foods

Plant secondary metabolites including phenolic phytochemicals found in various whole grains, legumes, medicinal plants, fruits, herbs, and vegetables, display potential antimicrobial activity against human gut-associated and foodborne bacterial pathogens such as *E. coli*, *Salmonella*, *Listeria Campylobacter*, *Vibrio*, *Shigella* & *Helicobacter pylori* (Christopher et al, 2021). The biosynthesis of these phenolic phytochemicals in plants occurs via the phenylpropanoid pathway and depends upon the availability of primary metabolites that are produced during primary metabolic activities which include the catabolic glycolytic pathway and the anabolic pentose phosphate and shikimic acid pathways (Dixon & Paiva, 1995; Kabera, Semana, Mussa & He, 2014; Fraser & Chapple, 2011). The mechanism of antimicrobial activity of phenolic phytochemicals is due to their ability to function as inhibitors in microbial biosynthetic pathways involving DNA, protein, and cell wall synthesis, or as chelating agents that reduce the availability of micronutrients for bacterial growth, or as hydrophobic molecules that destabilize the bacterial cell membrane, all of which can help inhibit the growth of bacterial

pathogens (Chibane, Degraeve, Ferhout, Bouajila & Oulahal, 2019; Cowan 1999; Daglia, 2012; Jayaraman, Sakharkar, Lim, Tang & Sakharkar, 2010a; Jayaraman, Sakharkar, Sing, Chow & Sakharkar, 2010b; Radulovic, Blagojevic, Stojanovic-Radic & Stojanovic, 2013). The antimicrobial activity of plants can vary due to different abiotic factors such as geography, ecology, and climate, which in turn can affect the plants' phytochemical profile and content (Mekinić, et al 2019). In general, gram-positive bacteria tend to show a greater susceptibility toward the antimicrobial activity of phenolic phytochemicals when compared to gram-negative bacteria (Mekinić et al 2014, 2019). This difference in susceptibility can be attributed to the morphological differences in the cell wall structure of gram-positive and gram-negative bacteria. The cell wall of gram-negative bacteria consists of a peptidoglycan layer enclosed by an outer membrane made up of lipopolysaccharides, proteins, and phospholipids while the cell wall of gram-positive bacteria consists of only a thick peptidoglycan layer. The presence of the outer membrane in the cell wall of gram-negative bacteria can potentially reduce the interaction of phenolic compounds with the bacterial plasma membrane and even slow down the uptake of phenolic compounds into the bacterial cell (Compean & Ynalvez, 2014; González-Lamothe et al, 2009; Mekinić et al, 2014; Munyendo, Orwa, Rukunga & Bii, 2011; Radulovic et al, 2013). Phenolic phytochemicals have the potential to act in synergy with antibiotic therapy to help improve the efficacy of treatment against multidrug-resistant bacteria. Additionally, phenolic phytochemicals can function as antibiotic potentiators through the inhibition of bacterial efflux pumps or by the destabilization of the bacterial cell membrane, as well as through the attenuation of virulence that can disrupt bacterial quorum sensing leading to loss of biofilm, or through modulation of the host's immune system leading to an immune response (González-Lamothe et al, 2009; Lewis & Ausubel, 2006) Therefore, plant-foods that are rich sources of phenolic

phytochemicals with antimicrobial properties can be targeted to counter microbial contamination of foods and associated foodborne diseases.

2.1.2. Antidiabetic activity of plant-based foods

Type 2 diabetes (T2D) is a metabolic disorder that is associated with insulin resistance, β cell dysfunction, abnormal glucose and lipid metabolism, inflammation, and oxidative stress (Bahadoran, Mirmiran & Azizi, 2013). Altered carbohydrate absorption, depletion of stored glycogen, increased gluconeogenesis, β cell dysfunction, insulin resistance in peripheral tissues, and altered insulin signaling pathways can lead to hyperglycemia (Bahadoran et al, 2013). Phenolic phytochemicals such as phenolic acids and flavonoids exhibit antihyperglycemic properties in the small intestine, liver, muscle/adipose, and pancreatic tissue, and the anti-hyperglycemic activity of these compounds is through the modulation of enzymes involved in glucose metabolism, as well as through the improvement of β cell function (insulin synthesis and secretion) (Bahadoran et al, 2013; Burton-Freeman et al, 2019). Additionally, the antioxidant and anti-inflammatory properties of these compounds can also help with associated complications linked to type 2 diabetes (Bahadoran et al, 2013; Burton-Freeman et al, 2019). Plant-based foods that are a rich source of phenolics such as cocoa, coffee, fruits, nuts, vegetables, spices, and wine have shown anti-hyperglycemic activity through different mechanisms of action, especially through the inhibition of key enzymes such as α-amylase, α-glucosidase, and aldose reductase that are involved in glucose metabolism (Xiao & Hogger, 2015). Through insulin-dependent pathways that involve modifying cellular redox status and cell signaling activity, phenolics can improve pancreatic β cell function (insulin synthesis and secretion) and peripheral insulin sensitivity in muscle, adipose, and other tissues (Burton-Freeman et al, 2019; Kang et al, 2020). Phenolics also display anti-hyperglycemic activity through insulin-independent pathways such as

activation of AMP-activated protein kinase (AMPK) in the liver, muscle, and adipose tissue, and through altered carbohydrate digestion and glucose absorption in the small intestine via inhibition of α-amylase and/or α-glucosidase activities (Burton-Freeman et al, 2019). *In vitro* analysis of extracts of clonal oregano lines have shown inhibitory activity towards porcine pancreatic amylase and this inhibitory activity was attributed to the presence of five disulfide bridges (oxidized cysteines) on the surface of the enzyme that is reduced due to the antioxidant activity of phenolic compounds, leading to a change in protein structure and potential inhibition of enzyme activity (McCue, Vattem & Shetty, 2004). Furthermore, modification of the chemical structure of phenolic compounds via hydroxylation, methylation, glycosylation, hydrogenation, or galloylation, can in turn increase or decrease the associated inhibitory activity against α-amylase and α-glucosidase enzymes (Xiao & Hogger, 2015). The effect of phenolic compounds on these pathways can be correlated with improved β cell function and insulin sensitivity, reduced inflammation and lipotoxicity, and reduced hepatic glucose output and glucose absorption, all of which can help maintain normal glucose homeostasis (Kang et al, 2020). The molecular mechanisms by which phenolic compounds display activity against T2D can be broadly classified into four main areas that include transcriptional modulation, translational modulation, modulation of enzyme activity, and epigenetic regulation (Hoda, Hemaiswarya & Doble, 2019). Experimental models using gene-knockout rats and mice including Wistar rats, Sprague-Dawley rats, Goto-Kakizaki rats, and Otsuka Long-Evans Tokushima Fatty rats, as well as cell culture lines isolated from *Homo sapiens, Mus musculus*, and *Rattus norvegicus* that include myoblasts, lipocytes, fibroblasts, and pancreatic cells, have all been useful in understanding the molecular mechanism of action of several phenolic compounds that display activity against T2D (Hoda et al, 2019; Kang et al, 2020). Therefore, phenolic enriched plant

foods are effective dietary targets to counter metabolic disorders and health risks associated with type 2 diabetes.

2.1.3. Antihypertension activity of plant-based foods

Several plant foods including plant phenolics and plant protein hydrolysates or peptides have been utilized or have the potential to be utilized in the treatment and management of hypertension (Parvez, Rahman, Faysal, Munna & Faruk, 2020). One of the strategies aimed at reducing hypertension is through the inhibition of angiotensin converting enzyme (ACE), an enzyme that is well known for its role in converting inactive angiotensin I to active angiotensin II, which is a potent vasoconstrictor and stimulator for the synthesis and release of aldosterone, which subsequently increases blood pressure by promoting sodium retention in the distal tubules, thereby playing an important role in the control of blood pressure (Amirdivani & Baba, 2011; Wong, 2021). Using *in vitro* assays, inhibitory activity against ACE-1 isolated from rabbit lung was observed in water and ethanolic extracts of fruits, vegetables, and medicinal plants, with the percentage of ACE-1 inhibitory activity ranging from 0% to 90% (Cheplick, Kwon, Bhowmik & Shetty, 2007, 2010; Kwon, Vattem & Shetty, 2006; Kwon, Apostolidis & Shetty, 2008). Individual phenolic compounds as well as other food extracts have shown ACE-1 inhibitory activity, which was attributed to the interaction of phenolic compounds with the enzyme (Ademiluyi & Oboh 2013; McCue, Kwon & Shetty, 2005; Kwon et al, 2006). Pure standards of coumaric acid, hydroxybenzoic acid and resveratrol at a concentration of 1000 µg/ml were found to have mild ACE inhibitory activity at 24%, 19%, and 2% respectively (Kwon et al, 2006). Free and bound phenolic extracts of soybean showed ACE inhibitory activity at IC_{50} values of 159 µg/ml and 143 µg/ml respectively and the higher ACE inhibitory activity of the bound phenolics was attributed to the hydrophilic nature of bound phenolics which mostly exist as glycosides

(Ademiluyi & Oboh, 2013). Since ACE enzyme operates in the aqueous phase, the bound phenolics being more hydrophilic than free phenolics would have greater interaction with the enzyme leading to higher inhibitory activity (Ademiluyi & Oboh, 2013). Plant-derived flavonoid compounds including anthocyanins, flavones, flavonols, flavan-3-ols, and chalcones have shown inhibitory activity against ACE with IC_{50} values ranging from 8 µM to 730 µM, and the activity was dependent upon the compound tested (Parvez et al, 2020). Herbal yogurt containing extracts of peppermint (*Mentha piperita*), dill (*Anethum graveolens*), and basil (*Ocimum basilicum*) showed higher inhibitory activity against ACE present in rabbit lung extracts when compared to plain yogurt (Amirdivani & Baba, 2011). The ACE isolated from rabbit lung is homologous to the human ACE in which the enzyme structure contains four cysteine groups of which three groups are close to the surface of the enzyme and form disulfide bridges. Phenolic compounds that display antioxidant activity can potentially reduce the disulfide bridges containing oxidized cysteines, which can in turn alter the structure of ACE leading to inhibition of enzyme activity (Ademiluyi & Oboh 2013; McCue et al, 2005). The ACE inhibitory and additional anti-hypertensive benefits of phenolic phytochemicals need to be examined in detail for its effective integration into a healthy diet targeting wider anti-hypertensive benefits.

2.1.4. Antioxidant activity of plant-based foods

NCDs are often linked to an imbalance in cellular reduction-oxidation processes that can lead to elevated levels of reactive oxygen species (ROS) and the generation of oxidative stress, which ultimately affects normal cellular function. Phenolic compounds display antioxidant activity and can help ameliorate oxidative stress associated with NCDs. Therefore, the screening of phenolic-rich plant-based foods for dietary intake is an important step in the management and treatment of chronic oxidative stress and associated NCDs (Sarkar & Shetty, 2014). Several

plant-based foods including cereal grains, fruits, vegetables, herbs, spices, and other food ingredients display antioxidant activity due to their wider phytochemical profile and content. Among the cereal grains analyzed for phytochemical content and antioxidant activity, corn was found to have the highest total phenolic content and antioxidant activity followed by wheat, oats, and rice, and most of the phytochemical content in these grains existed in the bound form and was a major contributor to the antioxidant activity (Adom & Liu, 2002). The antioxidant activity of 28 plant products including sunflower seeds, flaxseeds, wheat germ, buckwheat, several fruits, vegetables, and medicinal plants, was found to range from 53.7 % to 99.1%, and a strong positive correlation and statistically significant relationship ($p<0.001$) was observed between total phenolic content and antioxidant activity of the flaxseed ($R^2=0.963$) and cereal ($R^2=0.905$) products (Velioglu, Mazza, Gao & Oomah, 1998). Spices and culinary herbs such as cloves, basil, parsley, rosemary, and sage display antioxidant activity due to the presence of phenolic acids (e.g., rosmarinic acid, gallic acid, and caffeic acid), flavonoids (e.g., catechin, quercetin, and rutin), and essential oils (e.g., eugenol and isoeugenol) (Yashin, Yashin, Xia & Nemzer, 2017). The antioxidant activity of these biologically active compounds is due to their free radical scavenging activity and metal chelating properties, as well as their ability to modulate cell signaling processes such as the mitogen-activated protein kinase (MAPK) pathway, which leads to changes in expression in the target genes involved in response to oxidative stress (Soobrattee, Neergheen, Luximon-Ramma, Aruoma & Bahorun, 2005). Due to such high antioxidant potentials, phenolic rich foods are gaining increasing attention in novel food design and in dietary and therapeutic approaches to counter chronic oxidative stress and associated health risks.

2.1.5. Gut health-relevant properties of plant-based foods

Plant-based foods and various food ingredients that contain a wide range phenolic profiles (e.g., phenolic acids), complex carbohydrates (e.g., dietary fiber), and probiotics (e.g., lactic acid bacteria), are considered as good dietary sources to improve human gut health. These fiber and phenolic rich foods contribute to microbial fermentation in the gut to yield phenolic (e.g., aglycones) and carbohydrate (e.g., short chain fatty acids) metabolites, accompanied with changes in gut microflora and microbial activity, which can cause local and systemic physiological effects, which in turn help improve overall gut health (Puupponen-Pimiä et al, 2002). It is estimated that 90–95% of total phenolic compounds consumed in the diet are not absorbed in the small intestine but accumulate in the large intestinal lumen where gut microbiota can convert these phenolic compounds into active metabolites which are responsible for the observed health effects (Calderón-Pérez et al, 2021; Selma, Espin & Tomas-Barberan, 2009). In general, *in vitro,* and *in vivo* studies have shown that phenolic-rich foods can improve the gut health by reducing the population of potential pathogens such as *Clostridium perfringens, C. histolyticum*, and *Bacteroides* spp., while also enhancing the growth of other beneficial gut bacteria such as *Clostridia*, *Bifidobacteria,* and *Lactobacilli* (Dueñas et al, 2015). The release of short-chain fatty acids (SCFAs) such as acetate and propionate during fermentation of fiber with gut microbiota have been linked to lower blood pressure levels in experimental models of hypertension (Marques, Mackay & Kaye, 2018). Propionate is often the second most predominant SCFA and is known for its role in the reduction of lipogenesis, inhibition of cholesterol synthesis, activation of G protein-coupled receptors, and release of satiety hormones, along with other metabolic and anti-inflammatory effects (Verbeke et al, 2015). Plant-derived dietary phenolic compounds were found to be associated with discriminant gut bacterial taxa and

fecal metabolites in hypertensive and healthy subjects, thus indicating the interplay between phenolic compounds, the human gut microbiome and hypertension (Calderón-Pérez et al, 2021).

2.2. Pre- and Post-harvest Strategies to Enhance Phenolic Phytochemical-linked Functional Qualities in Plant-Based Foods and Animal Feeds

To address the global challenges of food insecurity and safety in the face of the rising prevalence of NCDs and the continuing persistence of foodborne illnesses, it is necessary to continue to adopt safe and nutritionally balanced dietary strategies that integrate phenolic phytochemical rich foods and plant-based animal feeds. Additionally, advancing pre-harvest and post-harvest strategies to enhance bioactivity of inducible phenolics in diverse plant derived human foods and animal feeds have significant commercial relevance. Some of the strategies to improve and optimize phenolic phytochemical-linked functional benefits include regular screening of plant food diversity for phenolic-linked functional qualities, optimization of pre- or post-harvest bio-processing strategies, synergizing of plant-based foods with other bioactive food ingredients, and biotransformation of plant-based foods via microbial fermentation. These strategies can help identify or improve the inducible phenolic-linked human health and food-safety-relevant benefits of plant-based foods and food ingredients in terms of their antimicrobial, antidiabetic, antihypertensive, antioxidant, and gut health-related properties.

2.2.1. Screening of plant-based foods for phenolic-linked functional qualities

The rapid and *in vitro* assay model-based screening of plant-based such as cereals, fruits, vegetables, and spices for their bioactive-linked functional qualities is an important strategy to help address the global concern of rising cases of NCDs and the associated persistence of foodborne illness in fresh and processed foods. Plant-based foods that display good phytochemical-linked functional qualities in terms of antioxidant, antihyperglycemic, prebiotic

and antimicrobial properties can be incorporated into dietary strategies to improve overall human health and food safety. Plant phytochemicals such as anthocyanins are colored, water-soluble pigments belonging to the phenolic group, and several scientific studies including cell culture studies, animal models, and human clinical trials have shown that anthocyanidins and anthocyanins possess antioxidative and antimicrobial activities which can help improve visual and neurological health, as well as provide protection against various non-communicable diseases. (Khoo, Azlan, Tang & Lim, 2017). However, the plant phytochemical content and the associated bioactivity can vary among cultivars or varieties and depends on other factors including the crop year, geographical or climatic conditions, and biotic or abiotic stress factors that affect plant growth before and after harvest (Brandolini, Castoldi, Plizzari & Hidalgo, 2013; Mekinić et al, 2019; Reddivari, Hale & Miller, 2007). In this regard, regular screening of plant-based foods for their phytochemical-linked health and food safety benefits will help design specific dietary strategies to address the issue of food safety and poor diet-linked NCDs.

2.2.2. Extraction and processing of plant-based foods

The basic steps in the extraction of phytochemical components from plant-based foods include pre-washing of plant material, drying and grinding of plant material to obtain a homogenous sample, followed by extraction using a solvent system (Sasidharan, Chen, Saravanan, Sundram & Latha, 2011). However, the phytochemicals present in the plant materials and their bioactivity may be altered or lost during the extraction process and there is also the issue of additional clean-up after solvent extraction. Some of the modern techniques used in extraction include solid-phase micro-extraction, supercritical-fluid extraction, pressurized-liquid extraction, microwave-assisted extraction, solid-phase extraction, and surfactant-mediated techniques, and these techniques can help reduce the use of organic solvents and the loss of

phytochemical-linked bioactivity that occurs during extraction (Sasidharan et al, 2011). In this regard, it is important to optimize the extraction procedures used in the screening of plant-based foods for their potential health benefits. A study done on using the pressurized solvent extraction technique for different fruits and vegetables, followed by the analysis of the antioxidant activity and total phenolic content, has shown that the type of solvent and extraction temperature used can affect the bioactivity of different plant extracts (Wibisono, Zhang, Saleh, Stevenson, & Joyce, 2009). The study found that extraction using 95% methanol as solvent at 100°C gave a moderately higher phenolic content for some fruit and vegetable extracts but appeared to reduce the antioxidant activity, especially for turnip leaf, elderberry, and sour cherry extracts, while extraction at 40°C gave a higher antioxidant activity for apple puree and elderberry extracts. (Wibisono et al, 2009). Different pre- and post-harvest processing techniques of plant-based foods can offer exciting strategies to optimize phytochemical-linked benefits that are relevant to addressing the rising prevalence of NCDs and any associated foodborne illnesses. The post-harvest processing of plant-based foods can affect the profile and quantity of their phenolic content. Frying, grilling, boiling, and steaming can be detrimental to phenolic acid content in vegetables, while vacuum processing, freezing, and storage can help retain a higher phenolic acid content (Rashmi & Negi, 2020). Therefore, optimization of extraction processes based on food grade formulation is important for improving the bioavailability and bioactivity of phenolic phytochemicals in plant-based foods, for targeting wider health and food safety benefits.

2.2.3. Synergies of plant-based foods

The underlying concept of food synergy is that the action of the food matrix (the composite of naturally occurring food components) on human biological systems is greater than or different from the corresponding actions of the individual food components (Jacobs, Gross &

Tapsell, 2009). In a recent study, *Ogi*, a fermented cereal-based food of Nigeria and Sub-Saharan Africa was synergized with tigernut (*Cyperus esculentus*) and sesame (*Sesamum indicum* L.) to make up for the loss of nutritional and health-protective benefits during the processing of Ogi from grains to slurries (Banwo, Oyeyipo, Mishra, Sarkar & Shetty, 2022). The study concluded that synergy of Ogi with sesame seeds and tigernut improved the *in vitro* type 2 diabetes-relevant α-glucosidase inhibitory activity (Banwo et al, 2022). Hence in this regard, the concept of food synergy can lead to new thinking in nutritional science and can also help to develop rational nutrition policymaking for future nutrition research strategies (Jacobs & Tapsell, 2013).

2.2.4. Fermentation of plant-based foods

Several foods have been traditionally utilized in fermentation that include fruits, vegetables, legumes, and cereal products, as well as milk, meat, and fish, (Şanlier, Gökcen & Sezgin, 2019). Fermentation can enhance or alter the nutritive and health-modulating properties of foods, and many of the bacterial species found in fermented foods are phylogenetically related to probiotic strains (Marco et al, 2017). Fermentation imparts unique aroma, flavor, and texture to food, as well as improves the digestibility of the food, degrades anti-nutritional factors including toxins and allergens, converts phytochemicals into more bioactive or bioavailable forms, and enriches the overall nutritional quality of food (Terefe & Augustin, 2020). In fact, the phytochemicals present in plant-based foods that are consumed in the diet must first be rendered biologically available, often through deglycation and hydrolysis by the gut microbiota before being absorbed by the host, and the microbial metabolic transformation can have an impact on the overall biological activity of plant phenolics (Verbeke et al, 2015). Several phenolic compounds including flavonoids (isoflavones, flavonols, flavanones, flavan-3-ols, and anthocyanins) and non-flavonoids (hydrolyzable tannins, lignans, hydroxycinnamates, stilbenes,

and benzoic acids), can undergo biotransformation via hydrolysis, reduction, dihydroxylation, demethylation, decarboxylation, isomerization or fission reactions, which are catalyzed by various microbial enzymes including β-glucuronidase, β-glucosidase, esterase, hydrogenase, dehydroxylase, demethylase, decarboxylase, and isomerase (Selma et al, 2009). Some of the biologically active compounds produced during fermentation that display human health-relevant benefits include conjugated linoleic acids that have a blood pressure lowering effect, exopolysaccharides that exhibit prebiotic properties, bacteriocins that show anti-microbial effects, and sphingolipids that have anti-carcinogenic and anti-microbial properties (Şanlier et al., 2019).

Apart from enhancing the nutritive and health modulating properties of foods, fermentation can also improve the shelf-life qualities in terms of reducing spoilage by bacteria or fungi. In recent a study, palm kernel cake was fermented with *Lactobacillus casei* and several cationic peptides released during fermentation showed strong antifungal activity against *Aspergillus flavus* (69.15%), *A. niger* (88.08%), *Fusarium* sp. (87.14%), and *Penicillium* sp. (71.84%) (Asri, Muhialdin, Zarei & Saari, 2020). Furthermore, in the same study, the cationic peptide mixture was added to bread at a concentration of 2g/kg, and it was found that the peptide mixture delayed fungal growth and extended the shelf life of the bread for up to 10 days (Asri et al, 2020). In another recent study, blanched mealworm pastes were treated with chemical preservatives (sodium nitrite and sodium lactate) or fermented with lactic acid bacteria (LAB) (starter culture Bactoferm® F-LC and *Lactobacillus farciminis*) and then stored at 4°C, and it was found that mealworm paste fermented with LAB had a stabilized microbial population due to the acidic pH (pH < 4.5) generated during fermentation, allowing it to be consumed for up to 8 weeks of storage when compared to the mealworm paste treated with chemical preservatives

(Borremans, Smets & van Campenhout, 2020). Furthermore, in the same study, the bacterial pathogens *L. monocytogenes*, *Salmonella*, and *B. cereus* were not detected in any of the fermented samples and lipid oxidation of the samples was minimal, indicating that lactic acid fermentation can be used successfully to inhibit microbial growth, maintain chemical quality, and extend the shelf life of mealworm pastes stored at 4°C (Borremans et al, 2020). Therefore, traditional, or controlled fermentation using beneficial bacteria is an effective approach to improve shelf-life and human health targeted-benefits of plant foods relevant for NCD-linked health and food safety solutions.

2.3. Antimicrobial and NCD-Countering Properties of Select Plant –Based Food Sources

2.3.1. Amla

Amla (*Emblica officinalis*) is an important underutilized fruit that has been used in ethnic food preparation and in traditional therapeutic approaches across South Asian countries like India, Sri Lanka, Pakistan, Uzbekistan, China, Malaysia, and other southeast Asian countires (Khan, 2009). Amla fruit is a rich source of phytochemicals and vitamins and is known to possess a wide range of bioactive properties that include antioxidant, antihyperglycemic, antimicrobial, antipyretic, analgesic, cardioprotective, gastroprotective, antihyperglycemic, antidiarrheal, anti-atherosclerotic, hepatoprotective, nephroprotective, neuroprotective and wound healing properties (Bhandari & Kamdod, 2012; Khan, 2009). *In vitro* studies have shown amla to possess antimicrobial activity against different gram-positive (*Staphylococcus*, *Micrococcus*, and *Bacillus*) and gram-negative (*E. coli* and *Salmonella*) bacterial pathogens (Gautam & Shukla, 2017; Saeed & Tariq, 2007; Singh, Moses & David, 2019; Tyagi & Singh 2014; Vijayalakshmi et al, 2007). Amla also has good antioxidant activity due to its high ascorbic acid and total phenolic content (Khopde et al, 2001; Kumar, Nayaka, Dharmesh &

Salimath, 2006; Sonkar, Rajoriya, Chetana & Murthy, 2020). Aqueous extracts of amla were found to inhibit gamma radiation-induced lipid peroxidation in rat liver microsomes as well as prevent damage to superoxide dismutase in rat liver mitochondria (Khopde et al, 2001). In another study, free and bound phenolics of amla displayed free radical scavenging activity and the higher level of antioxidant activity was attributed to the phenolic content (12.9%, w/w, correlation coefficient $R = 0.74$), of which gallic acid and tannic acid were the major compounds responsible for antioxidant activity (Kumar et al, 2006). Furthermore, the same study showed amla to protect DNA against damage from oxidative stress, based on the migration pattern of DNA during agarose gel electrophoresis (Kumar et al, 2006). Amla can also play an important role as a dietary ingredient in the management of type 2 diabetes due to its anti-hyperglycemic properties. A review of scientific literature has shown that amla can ameliorate alloxan, streptozotocin (STZ), and high-fat-diet-fed/low-dose STZ-induced diabetes in rats (D'souza et al, 2014). Normal and diabetic human volunteers given 1, 2 or 3 g of amla powder per day showed a significant decrease in fasting and 2-hour postprandial blood glucose levels ($p<0.05$), while 2 or 3 g of amla powder significantly improved high-density lipoprotein-cholesterol and lowered low-density lipoprotein-cholesterol levels (Akhtar, Ramzan, Ali & Ahmad, 2011). Therefore, due to its high bioactivity, amla has the potential to be utilized as a functional food ingredient in different food formulations for the improvement of human health and potentially to counter microbial spoilage of foods. Studies have been done on incorporating amla into mixed fruit beverages, pan bread, and chicken feed (Alkandari, Sarfraz & Sidhu, 2019a, 2019b; Bhalerao, Mahale, Dhar & Chakraborty, 2020; Patel et al, 2016). A combination of pomegranate, amla, and muskmelon juice at 57, 5, and 38 mL respectively, was optimized based on sensory analysis, and heat treatment of the beverage at 95°C for 5 minutes gave a 5-log cycle reduction in bacteria,

yeasts, and molds, as well as a 90% reduction in the activity of the spoilage enzymes- polyphenol oxidase and peroxidase (Bhalero et al, 2020). Amla-supplemented pan bread with its superior nutritional and sensory qualities can be used to improve consumer nutrition due to its high vitamin C and total phenolic content, as well as high antioxidant activity (Alkandari et al, 2019a). Different amla powders (sun-dried, oven-dried, and freeze-dried) were found to have no effect on the mixing characteristics of pan bread, while the color characteristics of the bread were affected by the type of powder used (Alkandari et al, 2019b). Furthermore, amla powder in low quantity gave a satisfactory loaf volume and dough strength and hence can serve as a potential replacement to the artificial additive potassium bromate which is believed to have adverse health effects (Alkandari et al, 2019b).

2.3.2. Kokum

Kokum (*Garcinia indica*) is a fruit belonging to the Clusiaceae family (mangosteen family) that has many phytopharmacological properties due to high phytochemical profile, which in turn have relevant food, pharmaceutical, and industrial applications (Ajjakana & Nayak, 2021; Jagtap, Bhise & Prakya, 2015; Lim, Lee, Lee & Choi, 2021; Swami, Thakor & Patil, 2014; Waghmare, Shukla & Kaur, 2019). Kokum is an indigenous crop in India that is commonly found in the Western Ghats including the coastal regions of Maharashtra, Kerala, Goa, and Karnataka (Swami et al, 2014, Waghmare et al, 2019). The major phytochemicals present in kokum that are responsible for the bioactivity include garcinol, hydroxycitric acid (HCA), anthocyanins, flavonoids, and phenolic acids (Jagtap et al, 2015; Lim et al, 2021; Singh et al, 2022; Swami et al., 2014). In a recent study, a total of 38 anthocyanins, 18 phenolic acids, and 12 flavonoids were detected in the rinds of 6 different kokum accessions, indicating that Kokum is a rich source of phytochemicals (Singh et al, 2022). Garcinol, a yellow fat-soluble pigment found

in the rinds of kokum at a 2 to 3% concentration, was found to reduce elevated levels of blood glucose, glycosyated hemoglobin, and lipids in streptozotocin-induced diabetic rats (Mali, Dias, Havaldar & Yadav, 2017). Several *in vitro* and *in vivo* studies have shown Kokum to display pharmacological activities that include antioxidant, anti-obesity, anti-arthritic, anti-inflammatory, antibacterial, hepatoprotective, cardioprotective, and antidepressant activity (Barve, 2021; Dhamija, Parle & Kumar, 2017; Lim et al, 2021; Panda, Ashar & Srinath, 2012; Patel et al., 2015; Tung et al, 2021). Furthermore, extracts of kokum were also found to have antibacterial activity against *Micrococcus aureus*, *Bacillus megaterium*, *Micrococcus luteus*, *Salmonella typhimurium*, *Pseudomonas aeruginosa*, *E. coli*, *Bacillus subtilis*, *Enterobacter aerogenes*, and *Staphylococcus aureus* (Sutar, Mane & Ghosh, 2012; Varalakshmi, Sangeetha, Shabeena, Sunitha & Vapika, 2010). These studies indicate that kokum has the potential to help in the management and prevention of metabolic disorders associated with NCDs, as well as to help improve food safety by reducing the risk of contamination with foodborne and gut-associated bacterial pathogens. Furthermore, since kokum rind, fruit, and fat are used in the production of value-added consumer products such as kokum syrup, juice concentrate, and butter (Ananthakrishna & Rameshkumar, 2016), this makes kokum an attractive candidate to be utilized in different dietary synergistic strategies to improve health protective functional qualities as well as to help improve the food safety of diverse value-added products.

2.3.3. Clove

Clove (*Syzygium aromaticum*), a native of the Maluku Islands in Indonesia, is one of the most valuable spices that has been used for centuries as a food preservative and for other medicinal purposes due to its high antioxidant and antimicrobial activity, and the spice is currently grown in several countries including Indonesia, India, Malaysia, Sri Lanka,

Madagascar, Tanzania, and Brazil (Diego & Wanderely, 2014). The phenolic phytochemicals found in clove include flavonoids such as kaempferol, quercetin, and their derivates, as well as phenolic acids such as gallic acid, caffeic acid, ferulic acid, ellagic acid, and salicylic acid (Diego & Wanderely, 2014). In addition to phenolic acids, clove also contains essential oils (EOs) such as eugenol which is the major essential oil accounting for 89% of the total oil content (Diego & Wanderely, 2014). Clove (flower buds) and clove essential oils have potent antimicrobial activity against different gram-positive (*Staphylococcus, Listeria*, and *Bacillus*) and gram-negative (*Enterobacter*, *Shigella*, *E. coli*, *Salmonella, Klebsiella*, and *Pseudomonas*) bacterial pathogens, thereby making them valuable natural preservatives with potential for food safety applications (Arora & Kaur, 1999; Ayoola et al, 2008; Cava, Nowak, Taboada & Marin-Iniesta, 2007; Fu et al, 2007; Mytle, Anderson, Doyle & Smith, 2006). Eugenol (4-Allyl-2-methoxyphenol) is an amphipathic hydroxyphenyl propene present in a variety of plants that belong to the families Myrtaceae (e.g., *Syzygium aromaticum*), Lamiaceae (e.g., *Ocimum basilicum*), Myristicaceae (e.g., *Myristica fragrans* Houtt) and Lauraceae (e.g., *Cinnamomum* spp.) (Marchese et al, 2017). Eugenol has been accepted as a food preservative in the United States, European Union, China, and other countries (Hu, Zhou, & Wei, 2018). The mechanism of antimicrobial activity of eugenol has been attributed to the presence of free hydroxyl groups which can bind to proteins thereby potentially inhibiting the action of bacterial enzymes (Marchese et al, 2017). In addition to enzyme inhibition, the hydrophobicity of eugenol especially at a low pH, can enhance the binding of eugenol to the bacterial cell membrane leading to an alteration of membrane fatty acids, an increase in membrane permeability, production of intracellular ROS, and disruption of ion and ATP transport across the membrane (Marchese et al, 2017). Eugenol can affect migration, adhesion, biofilm formation and other

virulence factors associated with *E. coli*, *S. enteriditis*, and *L. monocytogenes*, and can also display cytotoxicity against fibroblasts and other mammalian cells *in vivo* (Hu et al, 2018). In a study, chicken frankfurters treated with clove EOs (1% or 2% v/w, respectively) showed a reduction in growth of seven strains of *L. monocytogenes* (H9666, Scott A, H7550, G3982, 12443, H7776, and 101M) that were inoculated onto the frankfurters, when compared to the control group (no clove oil treatment) (Mytle et al, 2006). In another study, clove EOs was found to have a minimal inhibitory concentration (MIC) of 3,000 ppm against *L. monocytogenes* Scott A that was inoculated in semi-skimmed milk and an increase in fat content of the milk resulted in lower antimicrobial activity of the clove EOs (Cava et al, 2007). Furthermore, both these studies have also shown that the food (frankfurters or skimmed milk) stored at lower temperatures (5°C or 7°C) can drastically improve the antimicrobial activity of clove oil and reduce the growth of *L. monocytogenes* when compared to the food stored at higher temperatures (15°C or 35°C) (Cava et al, 2007; Mytle et al, 2006;). The phospholipid composition of the cytoplasmic membrane of the bacterial cell at lower temperatures can have a higher degree of unsaturation when compared to cells grown at higher temperatures, hence lower temperatures can lead to an increase in the fluidity of the cell membrane, enabling the hydrophobic EOs to dissolve more easily into the lipid bilayer of the bacterial cell, thereby increasing the antimicrobial activity of the EOs (Cava et al, 2007). Apart from antimicrobial activity, clove oil present in the flower, stem and leaves also display *in vitro* antioxidant activity via 2,2-diphenyl-β-picryl-hydrazyl (DPPH) and 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) radical scavenging activity, which is due to the flavonoid (e.g. quercetin) and essential oil (e.g. eugenol) content (Alfikri, Pujiarti, Wibisono & Hardiyanto, 2020; Gülçin, Vahabi, Norris, Del Monte & Farnum, 2012; Nassar et al 2007). Clove also has potential anti-hyperglycemic properties that can be

useful in the management of type 2 diabetes. Clove bud powder was found to gradually reduce blood glucose levels in type 2 diabetes-induced rat model (high-fat diet and low-dose streptozotocin) compared to diabetic rats with no clove bud powder (control group) (Adefegha, Oboh, Adefegha, Boligon, Athayde, 2014). Furthermore, the same study showed that clove bud powder inhibited the activity of type 2 diabetes relevant enzyme, α-glucosidase, while also elevating the levels of glutathione, ascorbic acid, superoxide dismutase, and catalase, which are involved in antioxidant activity (Adefegha et al, 2014). In an earlier study, the free and bound phenolics of clove bud were found to display antioxidant activity via DPPH and ABTS radical scavenging activity, as well as anti-hyperglycemic activity via inhibition of α-glucosidase and α-amylase activity (Adefegha & Oboh, 2012).

2.3.4. Garlic

Garlic (*Allium sativum*) is another example of a widely used botanical with phyto-pharmacological properties that include antioxidant, antihypertension, antidiabetic, anticancer, and antimicrobial activity (Ansary et al, 2020; Matsutomo, 2020; Nasir, Fatma, Neshat & Ahmad, 2020; Parham et al, 2020; Reiter, Hübbers, Albrecht, Leichert & Slusarenko, 2020; Ried, 2020). The bioactive compounds found in garlic include phenolic acids, flavonoids, tannins, saponins, polysaccharides, and sulfur containing compounds, including enzymes and other proteins (Ansary et al, 2020; Zhang et al, 2020). The bioactivity of garlic is mostly due to the presence of organosulfur compounds such as allicin and S-allylcysteine which are present at a concentration of around 2.3 % (Nakamoto, Kunimura, Suzuki & Kodera, 2020; Zhang et al, 2020). Allyl methyl sulfide, an organosulfur compound present in garlic, was found to restore circulatory antioxidant status, as well as attenuate the glycoprotein components in sstreptozotocin-induceddiabetic rats (Sujithra, Srinivasan, Indumathi & Vinothkumar, 2019).

Garlic can maintain glucose homeostasis by improving insulin sensitivity, as well as by inhibiting intestinal glucosidase activity, and by reducing levels of cholesterol, triacylglycerol, and LDL-cholesterol (Ota & Ulrih, 2017; Ríos, Francini & Schinella, 2015; Shabani, Sayemiri & Mohammadpour, 2019; Wang, Zhang, Lan & Wang, 2017). Aged garlic extracts prepared by prolonged extraction of fresh garlic with ethanol is considered to have good bioactive properties such as antioxidant, anticancer, anti-neuroinflammatory, and anti-neurodegeneration activity, due to the presence of bioactive compounds such as S-allylcysteine and allicin which are present in good quantities these extracts (Ansary et al, 2020; Song et al, 2020; Zhang et al, 2020). Garlic can also alter the gut microbiota by displaying prebiotic activity towards beneficial gut bacteria such as *Lactobacillales*, *Bifidobacterium*, *Clostridium*, and *Prevotella* (Ribeiro et al, 2021). Apart from prebiotic activity, garlic displays antimicrobial activity against bacterial pathogens and can also inhibit or prevent biofilm formation, and this activity is mostly due to the presence of biologically active sulfur containing compounds such as allicin, vinyldithiins, and ajoenes (Borlinghaus, Albrecht, Gruhlke, Nwachukwu & Slusarenko, 2014; Nakamoto et al., 2020). Allicin, a reactive sulfur species, shows potent antimicrobial activity due to its ability to oxidize protein cysteines and glutathione, which in turn can lead to an increase in oxidative stress in bacteria along with inhibition of bacterial enzyme activity due change in protein configuration (Reiter et al., 2020). The health-relevant benefits of garlic can be used in dietary strategies that can help in mitigating health risks associated with NCDs. Furthermore, the antimicrobial properties of garlic can potentially improve the food safety properties of the food matrix and can be targeted to counter food spoilage and bacterial contaminations.

2.3.5. Grape

Grapes are a rich source of bioactive compounds that include phenolic acids, flavonoids, anthocyanins, stilbenes, and lipids, and these compounds display antioxidant, antimicrobial, anti-inflammatory, and anti-carcinogenic activities and have wide applications in the food and nutraceutical industry (Sabra, Netticadan & Wijekoon, 2021; Yang & Xiao, 2013). Grape seed and processed grape by-products including grape pomace (skin, seed, stem, and pulp) are also rich in plant phytochemicals such as proanthocyanidins, and have many health-relevant bioactivity including anti-inflammatory, antioxidant, antidiabetic, anticancer, antimicrobial, and wound healing activity (Averilla, Oh, Kim, Kim & Kim, 2019; Gupta et al, 2020; Ma & Zhang, 2017). Grape and grape by-products also display inhibitory activity against type 2 diabetes-relevant α-amylase and α-glucosidase enzymes (Christopher, Orwat, Sarkar, Hatterman-Valenti & Shetty, 2018a; Hogan et al, 2010; Lavelli, Sri Harsha & Fiori, 2015; Zhang et al, 2011). Grape skin extract was shown to inhibit mammalian intestinal α-glucosidase activity and suppress postprandial glycemic response in streptozocin-treated mice (Zhang et al, 2011). Extracts of eight grape cultivars showed high α-amylase and α-glucosidase inhibitory activity, and the white grape cultivar, Vignoles, was found to have high enzyme inhibitory activity even at $1/5^{th}$ dilutions of the grape extract (Christopher et al, 2018a). In another study, grape skins of seven white grape varieties displayed inhibitory activity against α-amylase and α-glucosidase, and the grape skin was also used to fortify tomato puree and unleavened flat bread with the goal of creating fortified foods (Lavelli, Harsha, Ferranti, Scarafoni & Iametti, 2016). Several phenolic compounds present in grape and grape by-products including catechin, epicatechin, catechin 3-gallate, epigallocatechin gallate, and other procyanidins are responsible for the α-amylase and α-glucosidase inhibitory activity (Kato-Schwartz et al, 2020; Yilmazer-Musa, Griffith, Michels,

Schneider & Frei, 2012). Grape and grape by-products such as grape pomace, a by-product of wine making, also display antimicrobial activity against gram-positive and gram-negative bacterial pathogens, with gram-positive bacteria being more susceptible than gram-negative bacteria (Caponio et al, 2022; Leal et al, 2020; Silva et al, 2020; Žiarovská, et al, 2020). Grape is a widely popular fresh fruit and source of different alcoholic (wine) and non-alcoholic beverages. Therefore, the screening and improvement of human health-protective and food safety-relevant benefits of grape and grape-derived food products have significant commercial relevance.

2.3.6. Flaxseed

Flax (*Linum usitatissimum* L) is utilized as an oilseed and fiber crop and is known for its role as a functional food and food ingredient source to improve human health (Rabetafika, Van Remoortel, Danthine, Paquot & Blecker, 2011; Toure and Xueming 2010). Canada is the largest producer of flax at 38% of total global production, followed by China, United States, and India (Kasote, 2013; Toure & Xueming, 2010). Flaxseed has a good nutritional profile due to its high content of alpha-linolenic acid and dietary fiber as well as high quality of protein content (Rabetafika et al, 2011). Flax seed contains 40 to 45% oil, 20-25% fiber, 20-25% protein, and 1% lignan (Rabetafika et al, 2011). Flaxseed also has a good health-relevant functional bioactive profile due to its high phenolic acid content as well as other bioactive compounds that include lignans, flavonoids, and tannins (Kasote, 2013). The total phenolic content of flaxseed samples from different cultivars, growing locations, and growing seasons was analyzed and was found to contain 8 to 10 g/kg of total phenolic acid content, of which 5 g/kg were esterified phenolic acids and 3 to 5 g/kg were etherified phenolic acids (Oomah, Kenaschuk & Mazza, 1995). The total phenolic content of flaxseed varied between different cultivar, crop year, and growing location,

with the cultivar× location× year interactions (Oomah et al, 1995). Phenolic acids present in defatted flour of flaxseed include p-hydroxybenzoic acid, trans-p-coumaric acid, trans-ferulic acid and trans-caffeic acids, with trans-ferulic acid having the highest concentration at 37.6 mg/100g (Dabrowski & Sosulski, 1984). Flaxseeds are a rich source of plant lignans, especially secoisolariciresinol diglycoside (SDG), and these lignans act as natural antioxidants due to their hydroxyl and peroxyl radical scavenging activity as well as their ability to inhibit lipid peroxidation (Kasote, 2013; Toure & Xueming, 2010). SDG is converted to secoisolariciresinol (SECO) and the mammalian lignans, enterodiol (ED) and enterolactone (EL), by gut bacteria found in the colon of humans and animals (Kasote, 2013; Prasad, 2000; Toure & Xueming, 2010). Flaxseeds are a rich source of omega-3 fatty acid, alpha-linolenic acid, and SDG, and these compounds are responsible for the anti-inflammatory, antioxidant, and lipid modulating properties of flaxseed (Parikh et al, 2019). The antioxidant activity of SDG, SECO, ED, EL, and vitamin E were analyzed based on the chemiluminescence of zymosan-activated polymorphonuclear leukocytes, and SECO, ED, and EL were found to have greater antioxidant potency than SDG and vitamin E (Prasad, 2000). In another study, a statistically significant correlation coefficient was observed between total phenolics and antioxidant activity in flaxseed products (Velioglu et al, 1998). Flaxseed also has potential antihyperglycemic activity which can be useful in the management of type 2 diabetes. Flaxseed extracts at two doses (200 mg/kg and 400 mg/kg) were found to significantly reduce ($p< 0.001$) fasting blood sugar levels in diabetes-induced rats when compared to the control group (no flaxseed) (Qureshi et al, 2019). In a randomized, placebo-controlled clinical trial, type 2 diabetic patients given flaxseed-containing cookies showed a significant decrease in fasting plasma glucose levels when compared to type 2 diabetic patients that received the placebo cookie (Soltanian & Janghorbani, 2018). In another

clinical study, diabetic participants were given 10 g of flaxseed powder daily for one month and supplementation of flaxseed powder in the diet was found to reduce fasting blood glucose and HbA1c levels when compared to the control group (no flaxseed) (Mani, Mani, Biswas & Kumar, 2011). Apart from antioxidant and antihyperglycemic activity, flaxseed also has potent antimicrobial activity against human bacterial pathogens (Fadzir, Darnis, Mustafa & Mokhtar, 2018). *In vitro* studies have shown that flaxseed extracts can display antimicrobial activity against different gram-negative (*E. coli, Salmonella, Klebsiella, Pseudomonas, Shigella*) and gram-positive (*Enterococcus*, *Staphylococcus*, and *Bacillus*) bacterial pathogens, and the antimicrobial activity can be attributed to the phenylpropanoid compounds (phenolic acids, flavonoids, and lignans) as well as proteins present in flaxseed (Barbary, El-Sohaimy, El-Saadani & Zeitoun, 2010; Czemplik, Zuk, Kulma, Kuc & Szopa, 2011; Gaafar, Salama, Askar, El-Hariri, & Bakry, 2013; Narender et al, 2016; Tehrani, Batal, Kamalinejad & Mahbubi, 2014). Flaxseed lignans can bind to calcium and magnesium ions present in the lipopolysaccharide of the outer bacterial cell membrane leading to potential destabilization of the membrane (Gaafar et al, 2013; Narender et al, 2016). Therefore, due to the high phenolic-linked antioxidant potential and associated antimicrobial activity, flaxseeds can be targeted as value-added functional foods or functional ingredients in dietary strategies to prevent and manage chronic oxidative stress associated with common NCDs such as type 2 diabetes and hypertension. Furthermore, flax is also a rich source of dietary fibers and oligosaccharides and many health-relevant bioactive compounds such as phenolics of flax can be found in bound forms or as complex conjugates.

2.3.7. Whole grains

There is increasing evidence that consumption of whole grain foods can improve overall human health and help in the control and management of NCDs through regular dietary intake.

An analysis of twenty-three studies (systematic reviews and meta-analysis) has shown evidence of an inverse association between whole grain consumption and risk of type-2 diabetes, along with possible evidence of decreased risk of colon cancer and cardiovascular mortality (Tieri et al, 2020). In a randomized, double-blinded crossover trial, participants consumed wheat-based products (bread, pasta, crackers, and biscuits) that were made from ancient Khorasan wheat or modern wheat, and the study concluded that a replacement diet with Khorasan wheat led to a reduction of LDL cholesterol, insulin, and blood glucose levels, as well as a reduction in ROS and other inflammatory risk factors associated with type 2 diabetes (Whittaker et al, 2017). However, in another randomized double-blinded trial, participants with type 2 diabetes were given wheat germ for consumption and the study concluded that wheat germ intake had no significant effect on glycemic status, blood pressure, triglyceride, and low-density lipoprotein levels (Mohammedi, Karimifar, Heidari, Zare & Amani, 2020). Black-grained wheat was analyzed for its health benefits in a randomized controlled trial and the study found that the intake of black-grained wheat was associated with lower levels of glycated albumins and the inflammatory markers, TNF-α and IL-6, that are commonly found in type 2 diabetes patients (Liu, Qiu, Yue, Li &Ren, 2018). However, the same study found no significant differences in blood glucose, glycated hemoglobin or insulin levels between the treatment and control groups (Liu et al., 2018). The effect of whole grain processing such as milling on glycemic control in adults with type 2 diabetes was analyzed in a randomized crossover trial, and the study concluded that consumption of less processed whole grain foods over a two-week period improved glycemic control when compared to consumption of whole grain foods that were finely milled (Åberg, Mann, Neumann, Ross & Reynolds, 2020). The use of food grains that have a high nutritional and anthocyanin content can be helpful in creating products that have enhanced

nutritional value and health protective bioactivity. In a study, the bran and flour from a purple grain spring wheat line containing the anthocyanin biosynthesis gene *Pp3/TaMyc1* were used in the production of biscuits, and it was found that the anthocyanin content in biscuits made from purple wheat was 2.5 to 2.6 times higher when compared to biscuits made from a red wheat line (Usenko et al, 2018).Results of these previous studies indicated that traditional or underutilized whole grains are good source of health protective phytochemicals and can be integrated in dietary strategies to counter human metabolic disorders and associated disease risks.

2.3.7.1. Ancient Emmer wheat

Emmer (*Triticum dicoccum*) is a tetraploid wheat (AABB, 2n=4x=28) belonging to the group of wild ancient cereal grains that were cultivated in the Near East region, 10,000 to 19,000 years before the present, and this wheat formed an important component of cereal grains utilized in Old World agriculture (Nevo, 2014; Peng, Sun & Nevo, 2011; Tanno & Wilcox, 2006). Wild emmer (*T. dicoccoides*) is characterized by a long brittle rachis with indehiscent tough glumes and years of cultivation has genetically transformed this wheat into a free threshing modern day variety characterized by a short non-brittle rachis with dehiscent glumes (Nevo, 2014; Peng et al, 2011). The origin of wild emmer, *T. dicoccoides* (Schrank) can be traced back to Israel and parts of Iran, Iraq and Turkey, where this wheat developed due to hybridization between diploid wheat, *T. urartu* (AA, 2n=2x=14), and diploid goat grass, *Aegilops speltoides* (BB, 2n=2x=14), that occurred 300,000 to 400,000 years ago (Luo, Yang, You, Kawahara & Waines, 2007; Nevo, 2014; Peng et al, 2011). Wild emmer wheat is mostly self-pollinating and is inter fertile with *T. turgidum*, *T. durum* and other tetraploid and diploid wheat varieties (Nevo, 2014). The cultivation of wild emmer began around 10,000 years before present and over time cultivated emmer *T. dicoccum* (AABB, 2n=4x=28) hybridized with the diploid goat grass, *A. tauschii* (DD,

2n=2x=14) resulting in spelt wheat (*T. spelta*, AABBDD, 2n=6x=42), and after years of cultivation, tetraploid emmer and hexaploid spelt have further evolved into the free threshing durum wheat (*T. durum*, AABB, 2n=4x=28) and bread wheat (*T. aestivum* (AABBDD, 2n=6x=42) respectively (Peng et al, 2011; Nevo, 2014). The cultivation of other domesticated wheat cultivars such as einkorn (*T. monococcum* Linn) and emmer (*T. dicoccum* Schübi) dates to 7500 B.C with the latter emmer variety *T. dicoccum* Schübi predominantly grown in Southern India (Kajale, 1974). Emmer is now cultivated in over 17 countries including Ethiopia where it is grown as a major crop, while in other countries like India emmer is grown as a minor crop (Stallknecht, Gilbertson, & Ranney, 1996; Zaharieva, Ayana, Hakimi, Misra & Monneveux, 2010) In the United States, thousands of acres of land in the Midwest and Western regions were utilized for the cultivation of emmer wheat during the early 1900's, which was followed by a steady decline in emmer cultivation over time, with the states of North Dakota and Montana producing only a small number of the two commercially available varieties, Cenex emmer and common emmer (Stallknecht et al, 1996; Zaharieva et al, 2010). A possible explanation for the decline in emmer cultivation could be attributed to the farmer's preference for free threshing wheat varieties since the non-free threshing varieties such as emmer have intact glumes or hulls even after threshing (Peng et al, 2011; Zaharieva et al, 2010). Wild emmer has high allelic genetic diversity, which enables them to adapt to ecological changes in the environment, thereby helping the plant to develop resistance against abiotic stresses like drought and salinity (Nevo, 2014). This genetic diversity is found among emmer populations and is apparent at the local and regional levels, and is affected by the geological, edaphic, climatic, and biotic conditions during plant growth (Nevo, 2014). Wild emmer can play an important role in maintaining the nutritional status of cereal grains due of its higher levels of protein content and micronutrient concentration

(zinc and iron) in the grain when compared to modern wheat cultivars, and these levels positively correlate with each other and vary widely among different emmer accessions (Zaharieva et al, 2010). A study of the phytochemical content and total antioxidant activity of emmer, einkorn and bread varieties have shown some of the emmer varieties to have a significantly higher total antioxidant activity, total phenolic, ferulic acid and flavonoid content than the einkorn and bread wheat varieties ($p<0.05$) (Serpen, Gökmen, Karagöz & Köksel, 2008). In general, secondary metabolites like phenolic acids, carotenoids, flavonoids, tocopherols, and tocotrienols are largely responsible for protection against oxidative stress via their antioxidant activity, and such compounds are commonly found at higher levels in ancient wheat varieties compared to modern day cultivars (Serpen et al, 2008). Due to the phenolic-linked antioxidant potential and associated benefits, emmer can be targeted as a value-added functional food or food ingredient, especially in dietary strategies to prevent and manage chronic oxidative stress and other metabolic complications commonly associated with common NCDs such as type 2 diabetes and hypertension. Furthermore, fermentation of emmer with LAB can be an effective strategy to enhance the phenolic content and associated bioactivity, which can be targeted as a dietary strategy (e.g., fermented beverages) to help address the current public health challenges and to design and develop functional food ingredients or health-targeted beverages.

2.3.8. Plant by-products

Plant by-products are a good source of polyphenolic compounds that include bound or non-extractible polyphenols such as proanthocyanidins, hydrolysable tannins, and complex tannins as well as flavonoids and phenolic acids that are present in the free or bound state (Dzah, Duan, Zhang, Boateng & Ma, 2020). Furthermore, plant by-products are also a good source of bioactive peptides that display antimicrobial, antioxidant, antidiabetic, and antihypertensive

activity (Görgüç, Gençdağ & Yılmaz, 2020). Plant by-products can be utilized as sources of animal feed in terms of meeting the calorific needs along with providing animal-relevant health-protective benefits. In the United States, plant by-products that are used in animal feed include processed grain by-products such as distillers' products, brewers dried grains, corn gluten, sorghum germ cake and meal, peanut skins, and wheat bran; fruit by-products such as dried citrus pulp, apple pomace, and pectin pulp; and other miscellaneous plant-based by-products such as almond hulls and ground shells, buckwheat hulls, and legume by-products (Sapkota, Lefferts, McKenzie & Walker, 2007). Studies have shown that grape-pomace, grape-seed extracts, and tamarind seeds stimulate beneficial gut bacteria, while green tea and pomegranate by-products can enhance the health status of farm animals including poultry, pigs, cattle, sheep, goats, and fish (Guil-Guerrero et al, 2016).

2.3.8.1. Corn distillers dried grains with solubles

Distillers dried grains with solubles (DDGS) is a corn bioenergy by-product from the dry grind ethanol process. In 2009, over 10 billion gallons of ethanol were produced from corn in the United States with 25 million metric tons of DDGS being generated in the process (Winkler-Moser & Breyer, 2011). DDGS is commonly used as a livestock feed in the dairy and beef cattle industry and has potential applications as a livestock feed for poultry, swine, sheep, goat, and horses, as well as in aquaculture diets for fish and shrimp (U.S. grains council, 2018). Egg laying hens fed on a diet supplemented with corn DDGS resulted in the egg yolk having a lower proportion of saturated fatty acids and a higher proportion of unsaturated fatty acids, while DDGS supplemented up to 10% in the diet had no adverse effect on egg laying performance (Jiang, Zhang & Shan, 2013). Broiler chicken feed rations supplemented with corn DDGS was found to alter the intestinal microbiota of the broiler chickens and DDGS was negatively

correlated with the genera *Faecalibacterium* and *Streptococcus*, while *Turicibacter* was positively associated with corn DDGS supplemented broiler feed (Abudabos et al, 2017). Commercial pork production operations in the U.S. have reduced feed costs by $3 to $7/head by adding corn DDGS to the diet and because of favorable economics, many pork producers have been feeding diets containing as much as 30% DDGS and have achieved excellent growth performance and carcass lean % (Shurson, 2011). However, because of the high unsaturated oil content of DDGS (~60% linoleic acid), belly firmness is reduced, and pork fat becomes softer when DDGS is included in the diet (Shurson, 2011). The nutrient composition and digestibility of DDGS vary among corn sources with the ash, fiber, fat, lysine, tryptophan, and phosphorus content having high variation among the different DDGS sources (U.S. grains council, 2018). Similarly, the chemical profile and mineral content (sulfur, calcium, and phosphorus) vary among corn and wheat DDGS and in corn-wheat DDGS blends (Nuez Ortín & Yu, 2009). The phenolic acid content and antioxidant activity in corn DDGS was found to be 3.4 and 2.6-fold higher respectively when compared to corn and this is due to the depletion of starch that occurs during the fermentation of corn in the dry grind process for biofuel production (Luthria, Liu & Memon, 2012). Among phenolic acids, vanillic, p-coumaric, caffeic, ferulic, and sinapic acids were predominant in both corn and corn DDGS, and furthermore, there was a significant variation in total phenolic acid content and antioxidant activity among corn and corn DDGS samples obtained from different industrial plants involved in biofuel production (Luthria et al, 2012). A study was done to optimize the parameters of anthocyanin extraction from purple and dark corn DDGS based on three independent variables- ethanol concentration (0, 12.5, and 25%), liquid-to-solid ratio (30:1, 40:1, 50:1 mL/g), and extraction temperature (4, 22, and 40 °C), and the study concluded that anthocyanin extraction from colored corn DDGS was optimized using

12.5% ethanol, at a liquid-to-solid ratio of 40:1 mL/g, and at a temperature of 22 °C (Dia et al, 2015). Protein hydrolysates of corn DDGS were found to have potential use as naturally derived antioxidants in food, pet food, and feed systems with good protection efficiency against lipid oxidation to improve product storage stability (Hu et al, 2020). Rheological and sensory analysis of corn DDGS blends (10%, 15%, 20%, and 25%) with wheat flour has shown the resulting dough to have higher water absorption, along with increased hardness and adherence in the bread, and corn DDGS substituted at 15% was not found to be detrimental to dough functionality, texture, and taste. (Li, Wang & Krishnan, 2020). Corn DDGS collected from different ethanol production plants in the US were analyzed for their phytochemical content and antioxidant activity and it was found that corn DDGS had a higher content of tocopherols, tocotrienols, lutein, and ferulic acid (free and bound) when compared to yellow corn, and furthermore, the antioxidant activity varied greatly among the corn DDGS samples, and the antioxidant activity was higher when compared to yellow corn (Shin, Shurson & Gallaher, 2018). Therefore, it is important to screen and analyze bioactive enriched corn DDGS from different sources for their phenolic-linked antimicrobial and antioxidant properties prior to targeting them in strategies for designing value-added antimicrobial feed or as a source of functional food ingredients for animal feed.

Based on the above evidence and scientific rationale, the overarching aim of this disquisition was to screen and optimize plant-derived foods and plant byproducts for their phenolic-linked food safety and health protective functional qualities. Specific objectives of this disquisition were derived from this overarching theme and aligned with appropriate experimental design to achieve key deliverables.

CHAPTER 3. OBJECTIVES

Phenolic phytochemicals of plant foods and plant-based by-products have dual functional benefits of improving human health protective functional qualities in foods as well as to counter microbial spoilage due to antimicrobial properties. However, concentration and distribution of phenolic phytochemicals and their functional qualities in plant foods and plant-based products vary widely due to different pre-harvest (cultivar/variety, growing climate, agricultural practices etc.) and post-harvest (storage, transportation, processing, cooking) conditions. Therefore, the primary objective of this dissertation was to screen the phenolic-linked antimicrobial, antioxidant, anti-hyperglycemic and anti-hypertensive benefits relevant for food safety and human health protective functional qualities using *in vitro* assay models. The secondary objective of this dissertation was to advance food processing, formulation, and fermentation strategies to improve and optimize phenolic-linked food safety and NCD countering properties in select plant foods and plant-derived by products. The thematic description of the overarching theme and specific objectives are presented in Figure 3.1.

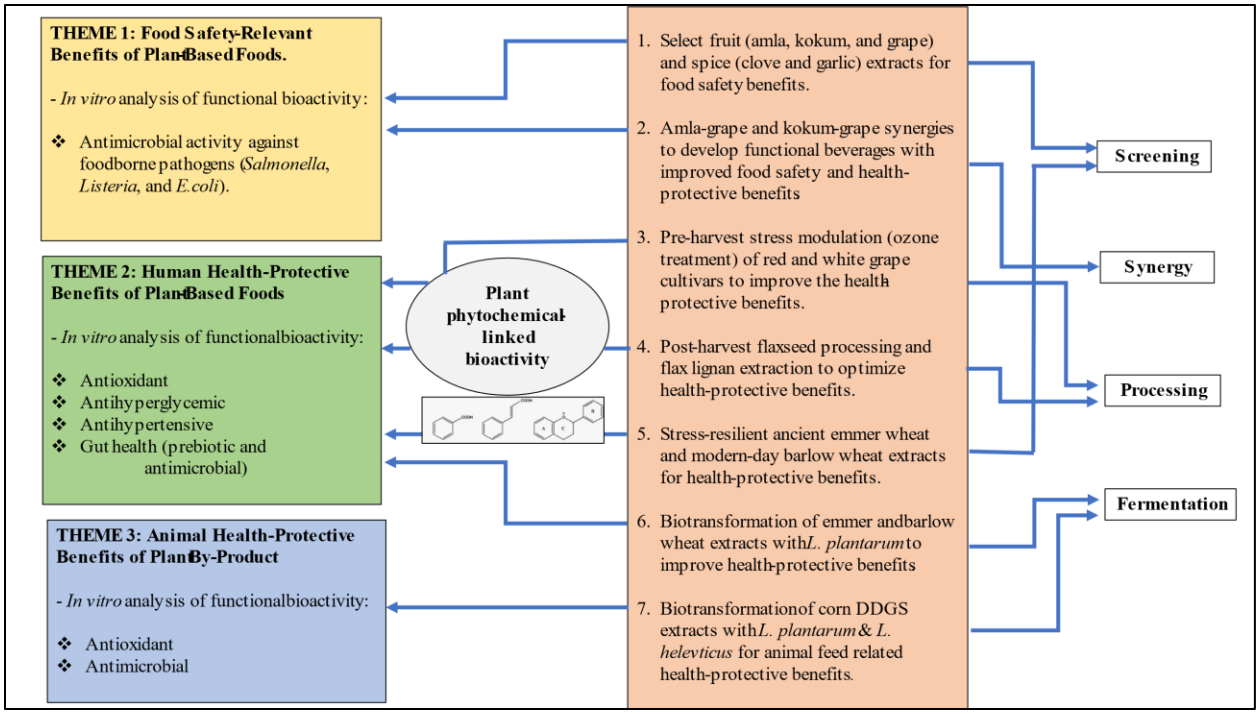


Figure 3.1. Description of the overarching themes and specific objectives of the study

CHAPTER 4. PHENOLIC PHYTOCHEMICAL-LINKED FOOD SAFETY AND HUMAN HEALTH PROTECTIVE BENEFITS OF SELECT BOTANICALS

4.1. Abstract

Botanicals that are good sources of phenolic phytochemicals can be utilized for dual functional benefits of improving food safety and human health relevant qualities of ethnic and non-traditional foods. Due to their antimicrobial and antioxidant relevant biological activities, phenolic enriched botanicals and foods are good dietary and therapeutic targets to counter microbial spoilage and food contamination, while also addressing diet-linked non-communicable chronic diseases (NCDs). Therefore, screening of select botanicals for their phenolic phytochemical-linked antimicrobial, antioxidant, anti-hyperglycemic and anti-hypertensive functional qualities has wider food and ingredient application relevance. Based on the above rationale, hot water extracts of amla (*Phyllanthus emblica*), clove (*Syzygium aromaticum*), kokum (*Garcinia indica*), and garlic (*Allium sativum*) were screened for their total soluble phenolic (TSP) content, phenolic profile, as well as antioxidant, anti-hyperglycemic and anti-hypertensive properties using *in vitro* assay models. Additionally, the antimicrobial properties of the extracts against strains of *Salmonella Enteritidis*, *Listeria monocytogenes*, and *Escherichia coli* that are commonly associated with food-borne illnesses were also determined. High baseline TSP content and antioxidant and anti-hyperglycemic properties were observed among all botanical extracts. The major phenolic phytochemicals detected in the extracts were gallic, cinnamic, ellagic, benzoic, dihydroxybenzoic, protocatechuic, and *p*-coumaric acids along with catechin and rutin. All extracts displayed significant antimicrobial activity against most of the bacterial strains that were tested, and the antimicrobial activity was specific for each strain targeted in this study. Therefore, these phenolic-enriched botanicals and plant foods can be

integrated in food preservation and dietary support strategies to counter food borne illnesses and to reduce common health risks associated with NCDs.

Keywords: Antioxidant, Antihyperglycemic, Antimicrobial, Botanicals, Food Borne Pathogen, Phenolics Phytochemicals

4.2. Introduction

The screening of botanicals including traditional and widely consumed food plants with rich antimicrobial phytochemical profile is important to address foodborne diseases, which commonly occur due to microbial contamination of foods with bacterial pathogens. These phytochemical rich botanicals are also relevant for integrating in dietary support strategies aimed at the prevention or management of diet-linked non-communicable chronic diseases (NCDs). Amla (*Emblica officinalis* Gaertn or *Phyllanthus emblica* Linn), commonly known as Indian gooseberry, is an important traditional fruit and medicinal plant in the Indian Subcontinent and other parts of the South-East Asia. This underutilized fruit is widely found in India, Pakistan, Uzbekistan, Sri Lanka, China, and Malaysia (Khan, 2009). Amla is known to possess a wide range of bioactive properties including antioxidant, antidiabetic, and antimicrobial activity (Akhtar et al, 2011; Baliga et al, 2019; Bhandari & Kamdod, 2012; D'souza et al, 2014; Khan, 2009; Khopde et al, 2001; Kumar et al, 2006; Sonkar et al, 2020). *In vitro* studies have shown amla to possess antimicrobial activity against gram-positive (*Staphylococcus*, *Micrococcus*, and *Bacillus*) and gram-negative (*E. coli* and *Salmonella*) bacterial pathogens (Gautam & Shukla, 2017; Saeed & Tariq, 2007; Singh et al, 2019; Tyagi & Singh, 2014; Vijayalakshmi et al, 2007). Due to its high bioactivity, amla has the potential to be utilized as a functional food ingredient in different food formulations for the improvement of food safety and human health benefits. Previous research has targeted incorporation of amla into mixed fruit beverages, pan bread, and

chicken feed to improve functional properties (Alkandari et al, 2019a, 2019b; Bhalerao et al, 2020; Patel et al, 2016; Zafar, Allafi, Alkandari & Al-Othman, 2021).

Like amla, kokum (*Garcinia indica*) from *Clusiaceae* family (mangosteen) is an indigenous fruit commonly found in India and has many phyto-pharmacological properties with relevance in wider food, pharmaceutical, and industrial applications (Ajjakana & Nayak 2021; Jagtap et al, 2015; Lim et al, 2021; Swami et al., 2014; Waghmare et al., 2019). The major phytochemicals present in kokum that are responsible for their wider bioactivity include garcinol, hydroxycitric acid (HCA), cyanidin-3-sambubioside, and cyanidin-3-glucoside (Jagtap et al., 2015; Lim et al., 2021; Singh et al, 2022; Swami et al, 2014). Extracts of kokum were found to have antimicrobial activity against *Micrococcus aureus, Bacillus megaterium*, *Micrococcus luteus, Salmonella typhimurium, Pseudomonas aeruginosa*, *E. coli*, *Bacillus subtilis*, *Enterobacter aerogenes*, and *Staphylococcus aureus* (Sutar et al, 2012; Varalakshmi et al, 2010).

Clove (*Syzygium aromaticum*), a native of the Maluku Islands in Indonesia, is one of the most valuable spices that has been used for centuries as a food preservative and other medicinal purposes. This spice is currently grown in several countries including Indonesia, India, Malaysia, Sri Lanka, Madagascar, Tanzania, and Brazil (Cortés-Rojas, de Souza & Oliveira, 2014). Clove has high antioxidant activity which is mostly due to its flavonoid (e.g., quercetin) and essential oil (e.g., eugenol) content (Alfikri et al, 2020; Gülçin et al, 2012; Nassar et al, 2007). Clove also has potential antihyperglycemic properties through inhibition of carbohydrate digestive enzymes such as α-amylase and α-glucosidase, which is a common therapeutic target to manage chronic hyperglycemia (Adefegha et al, 2014; Adefegha & Oboh, 2012). Clove (flower buds) and clove essential oils (e.g., eugenol) have shown antimicrobial activity against different gram-positive

(*Staphylococcus*, *Listeria*, and *Bacillus*) and gram-negative (*Enterobacter*, *Shigella*, *E. coli*, *Salmonella*, *Klebsiella*, and *Pseudomonas*) bacterial pathogens, thereby making them valuable natural preservatives with potential for food safety applications (Arora & Kaur, 1999; Ayoola et al, 2008; Cava et al, 2007; Fu et al, 2007; Mytle et al, 2006).

Similarly, garlic (*Allium sativum*) is another example of a botanical with phytopharmacological properties that include antioxidant, antihypertension, antidiabetic, and antimicrobial activity (Ansary et al, 2020; Matsutomo, 2020; Nasir et al, 2020; Parham et al, 2020; Reiter et al, 2020; Ried, 2020). The bioactivity of garlic is mostly due to the presence of organosulfur compounds such as allicin and S-allylcysteine which are present at a concentration of around 2.3 % (Nakamoto et al, 2020; Song et al, 2020; Zhang et al, 2020). Additionally, garlic is also good source of phytochemicals with high antimicrobial and antioxidant properties.

Based on these wider antimicrobial and health protective functional properties of above botanicals, the goal of this study was to screen different forms (powder, slice or pickle) of these select botanicals for their antimicrobial activity against outbreak strains of bacterial pathogens (*E. coli*, *Listeria*, and *Salmonella*) that are associated with foodborne illnesses. Additionally, total soluble phenolic content, phenolic profile, and antioxidant activity, as well as inhibitory activity against type 2 diabetes-relevant α-amylase and α-glucosidase enzymes and hypertensive-relevant angiotensin-I-converting enzyme (ACE) of these botanicals were also investigated. The results of this study will help determine the food safety-relevant and human health protective functional benefits of these plant-based foods which can be utilized in future dietary strategies such as synergies, to counter foodborne illnesses caused due to microbial contamination and to help address the emerging prevalence of type 2 diabetes and chronic hypertension.

4.3. Materials and Methods

4.3.1. Chemicals used

Other than mentioned, all chemicals and enzymes were purchased from Sigma Chemical Co (St. Louis, MO).

4.3.2. Samples used

Clove (flower buds), amla (powder, slices, and pickle), kokum (dried slices), and garlic (slices and pickle) were purchased from a local Indian grocery store of Fargo (North Dakota, USA). The clove and kokum slices were ground using a coffee blender to obtain a coarse powder while the amla, kokum, and garlic samples (slices and pickle) were chopped into smaller pieces before extraction.

4.3.3. Preparation of extracts

The extraction of all samples was done using hot water. For the clove, amla, and kokum samples (powder and pickle), 10 g of the sample was used in the hot water extraction protocol, while for the rest of the samples- amla, kokum, and garlic (slices), 25 g of the sample was used. For the hot water extraction protocol, 10 g or 25 g of the respective samples were added to 50 mL of boiling water (100 °C) and boiled for 15 min after which the samples were cooled down to room temperature and centrifuged at 8,500 rpm for 15 min. The supernatant was collected and re-centrifuged at 8,500 rpm for 15 min and the extracts were stored at -20 °C. For the antimicrobial assay, the frozen extracts were thawed at room temperature and filter-sterilized using 0.22 µm syringe filters (Millipore Corp, MA, USA) prior to the assay. The amla slice, amla pickle, garlic slice and garlic pickle extracts were analyzed on a fresh weight (FW) basis while the clove, amla powder, kokum slice and kokum powder extracts were analyzed on a dry weight (DW) basis.

4.3.4. Bacterial strains used

The *Salmonella* strains tested in this study were *Salmonella enterica* subsp. *enterica* serovar Enteritidis (ATCC BAA-1045), *S. enterica* subsp. *enterica* serovar Typhimurium (FSL R8-0865), *S. enterica* subsp. *enterica* serovar Montevideo (FSL R8-3417), *S. enterica* subsp. *enterica* serovar Stanley (FSL R8-3511), and *S. enterica* subsp. *enterica* serovar Saintpaul (FSL R8-3582). The *Listeria* strains used in this study were *Listeria monocytogenes* serovar 1/2a (10403S), *L. monocytogenes* serovar 1/2b (FSL J1-0194), *L. monocytogenes* serovar 1/2a (FSL F2-0515), and *L. monocytogenes* serovar 4b (H7858). The *E. coli* strains used in this study were *E. coli* serovar O157:H7 (EDL-933) and *E. coli* serovar O26:H11 (TW07936). The *Salmonella* and *Listeria* stocks were stored at -8 0°C in brain heart infusion broth (Oxoid, Basingstoke, UK) containing 25 % glycerol. The *E. coli* stocks were stored at -80 °C in LB broth, Miller (Luria-Bertani) (Difco, Becton, Dickinson and Company, Sparks, MD) containing 25 % glycerol. The *Salmonella* and *Listeria* strains were obtained from the Food Safety Laboratory at Cornell University, except for *Salmonella enterica* subsp. *enterica* serovar Enteritidis (ATCC BAA-1045) which was obtained from the American Type Culture Collection (Manassas, VA). The *E. coli* strains were obtained from the Thomas S. Whittam STEC Center at Michigan State University.

4.3.5. Total soluble phenolic (TSP) content

The TSP content of the extracts was determined using the Folin-Ciocalteu method based on a protocol as described previously (Shetty et al, 1995). For this assay, the extracts were diluted in water at 1:20 dilution and 0.5 mL aliquots of the diluted extracts were taken into respective glass tubes after which 1 mL of 95% ethanol, 0.5 mL of 50 % (v/v) Folin-Ciocalteu reagent, and 1 mL of 5 % sodium carbonate were added sequentially to each tube. Due to higher

ascorbic acid content of the amla, which interferes with the Folin-Ciocalteu reagent, we have used multiple dilutions of the samples to avoid overestimation of total soluble phenolic content. The tubes were then mixed using a vortex machine and incubated for 60 min under dark conditions. The absorbance values in each tube were measured at 725 nm with a UV-visible spectrophotometer (Genesys 10S UV-VIS spectrophotometer, Thermo Scientific, NY, USA). Using a standard curve of different concentrations of gallic acid in 95 % ethanol, the absorbance values of the extracts were converted, and the TSP content was expressed in milligram gallic acid equivalents per gram fresh weight or dry weight (mg GAE/g FW or DW).

4.3.6. Phenolic profile

The profile of the phenolic compounds was determined using the high-performance liquid chromatography (HPLC) assay method. The extracts were centrifuged at 13,500 rpm for 5 min after which 5 µL of the supernatant were injected using an Agilent ALS 1200 auto-extractor into an Agilent 1260 series (Agilent Technologies, Palo Alto, CA) HPLC equipped with a D1100 CE diode array detector. The solvents used for gradient elution were 10 mM phosphoric acid (pH 2.5) and 100 % methanol. The methanol concentration was increased to 60 % for the first 8 min, then to 100 % over the next 7 min, then decreased to 0 % for the next 3 min and was maintained for 7 min with a total run time of 25 min per injected sample run. 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 0.7 mL/min at room temperature. The absorbance values were recorded at 214 nm, 230 nm, 260 nm, and 306 nm and the chromatogram was integrated using Agilent Chem station enhanced integrator. Pure standards of benzoic acid, gallic acid, protocatechuic acid, ellagic acid, cinnamic acid, dihydroxybenzoic acid, p-coumaric acid, rutin, and catechin in 100 % methanol were used to calibrate the respective standard curves and retention times. The phenolic

compounds detected in the extracts were expressed in microgram per gram fresh weight or dry weight (µg/g FW or DW).

4.3.7. Antioxidant activity

The antioxidant activity of the extracts was measured by their scavenging activity against the free radicals 2, 2-Dipheny-1-Picryl hydrazyl (DPPH) (D9132-5G, Sigma-Aldrich), and 2, 2-Azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) (A1888-5G, Sigma-Aldrich) respectively. The DPPH scavenging assay was based on a protocol as described earlier (Kwon et al, 2006) in which 0.25 mL of the extracts were added to 1.25 mL of 60 mM DPPH prepared in 95% ethanol while the controls had 0.25 mL of 95 % ethanol instead of the extract. After 5 min of incubation, the extracts and their corresponding controls were centrifuged at 13,000 rpm for 1 min and the absorbance values of the supernatants were measured at 517 nm using a UV-visible spectrophotometer (Genesys 10S UV-VIS spectrophotometer, Thermo Scientific, NY). The ABTS scavenging assay was based on a protocol as described earlier (Re et al, 1999) in which 0.05 mL of the extracts was added to 1 mL of ABTS prepared in 95 % ethanol while the controls had 0.05 mL of 95 % ethanol instead of the extract. After 2 min of incubation, the extracts and their controls were centrifuged at 13,000 rpm for 1 min and the absorbance values of the supernatant were measured at 734 nm with a UV-visible spectrophotometer (Genesys 10S UV-VIS spectrophotometer, Thermo Scientific, NY). The absorbance values from the DPPH and ABTS radical scavenging assays were used to calculate the percentage of antioxidant activity for each extract using the following formula:

$$\% \text{ Antioxidant activity} = \frac{\text{Control}^{\text{absorbance}} - \text{Extract}^{\text{absorbance}}}{\text{Control}^{\text{absorbance}}} \times 100$$

Using a standard curve of different concentrations of Trolox in 95 % ethanol, the percentages of inhibitory activity obtained from the DPPH and ABTS radical scavenging assays were expressed as millimolar equivalents of trolox (mM TE).

4.3.8. Alpha-amylase enzyme inhibitory activity

The α-amylase enzyme inhibitory activity of the extracts was measured based on a protocol as described earlier (Kwon et al, 2006) and the activity was measured in a dose-dependent manner at undiluted, half, and one-fifth dilutions of the extracts. The extracts were diluted using distilled water and 500 µL of undiluted and diluted extracts were added to respective glass tubes, while the control tubes had 500 µL of 0.1M sodium phosphate buffer (containing 0.006M sodium chloride at pH 6.9) instead of the extract. Additionally, each extract had a corresponding blank tube containing 500 µL of the extract and 500 µL of the buffer instead of the enzyme. Then 500 µL of porcine pancreatic α-amylase (0.5 mg/mL buffer) (EC 3.2.1.1, purchased from Sigma Chemical Co, St Louis, MO) was added to the extract and control tubes and incubated for 10 min at 25 °C. After incubation 500 µL of the substrate (1% starch in buffer) was added to all the tubes and incubated again for 10 min at 25 °C. Then 1 mL of 3, 5 dinitro salicylic acid was added and the tubes were incubated in a boiling water bath for 10 min to stop the reaction. After removing from the water bath and cooling down to room temperature, 10 mL of distilled water was added to all the tubes to ensure that the absorbance values in the control tubes ranged between 1.0 and 1.2, and the absorbance of all the tubes was measured at 540 nm using a UV-visible spectrophotometer (Genesys 10S UV-VIS spectrophotometer, Thermo Scientific, NY). The absorbance values were then used to calculate the percentage of enzyme inhibitory activity of the extracts using the following formula:

$$\% \text{ Inhibition} = \frac{\text{Control}^{\text{absorbance}} - (\text{Extract}^{\text{absorbance}} - \text{Extract blank}^{\text{absorbance}})}{\text{Control}^{\text{absorbance}}} \times 100$$

Using a standard curve of different concentrations of acarbose in distilled water, the percentages of α-amylase inhibitory activity obtained from the enzyme inhibition assay were expressed as millimolar equivalents of acarbose (mM AE).

4.3.9. Alpha-glucosidase enzyme inhibitory activity

The α-glucosidase enzyme inhibitory activity of the extracts was measured based on a protocol as described earlier (Kwon et al, 2006) and the activity was measured in a dose-dependent manner at undiluted, half and one-fifth dilutions of the extracts. The extracts were diluted with 0.1M potassium phosphate buffer (pH 6.9) in 96 well microtiter plates in which 50 µL, 25 µL, and 10 µL of each extract were pipetted and the final volume made up to 50 µL by the addition of potassium phosphate buffer. Each extract had a corresponding control of 50 µL buffer instead of the sample and the volume in all the wells was made up to a final volume of 100 µL by the addition of 50 µL of the buffer. Then 100 µL of buffer containing yeast α-glucosidase enzyme (1 U/mL) (EC 3.2.1.20, purchased from Sigma Chemical Co, St. Louis, MO) was added to each well and incubated for 10 min at 25 °C, after which 50 µL of the substrate, 5 mM p-nitrophenyl-α-D- glucopyranoside solution (prepared in buffer) was added to each well followed by 5-min incubation at 25 °C. The absorbance of all the wells was measured at 405 nm using a microplate reader (Thermomax, Molecular device Co., VA) at the 0- and 5-min time points of the 5-min incubation period, and the absorbance values were used to calculate the percentage of enzyme inhibitory activity using the following formula:

$$\% \text{ Inhibition} = \frac{(\text{Control}^{\text{abs}}\ 5 \text{ min} - \text{Control}^{\text{abs}}\ 0 \text{ min}) - (\text{Extract}^{\text{abs}}\ 5 \text{ min} - \text{Extract}^{\text{abs}}\ 0 \text{ min})}{(\text{Control}^{\text{abs}}\ 5 \text{ min} - \text{Control}^{\text{abs}}\ 0 \text{ min})} \times 100$$

Using a standard curve of different concentrations of Acarbose in distilled water, the percentages of α-glucosidase inhibitory activity obtained from the enzyme inhibition assay were expressed as millimolar equivalents of acarbose (mM AE).

4.3.10. Angiotensin-I-converting enzyme (ACE) inhibitory activity

The ACE inhibitory activity of the extracts was measured based on a protocol as described earlier (Kwon et al, 2006) and the activity was measured in a dose-dependent manner at undiluted, half, and one-fifth dilutions of the extracts. The extracts were diluted using distilled water. The substrate used was hippuryl-histidyl-leucine (HHL) and the enzyme ACE was obtained from rabbit lung (EC 3.4.15.1). To 50 µL of the extracts, 200 µL of 1M NaCl-borate buffer (pH 8.3) containing 2mU of ACE was added and then incubated at room temperature for 10 minutes. For the blank tubes, 50 µL of distilled water and 200 µL of buffer were used instead of the extract and enzyme. After this, 100 µL of 5mM HHL substrate (prepared in buffer) was added to all the tubes and incubated for one hour at 37 °C. The reaction was stopped by the addition of 150 µL of 0.5N HCl. The hippuric acid formed due to ACE activity was detected using the HPLC method for which, 5 µL of the reaction mixtures were injected using Agilent ALS 1200 autosampler into an Agilent 1260 series (Agilent Technologies, Palo Alto, CA) HPLC equipped with a D1100 CE diode array detector. The solvents used for the gradient were a combination of 10mM phosphoric acid (pH2.5) and 100% methanol. For the first 8 min, the methanol concentration was increased to 60% then to 100% for 5 min, and finally to 0% for the next 5 min and the total run time was 18 min for each sample. 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 0.7 mL/min at room temperature. The absorbance was measured at 228 nm and the chromatogram was integrated using Agilent Chemstation (Agilent Technologies) enhanced

integrator for detection of hippuric acid. A hippuric acid standard was used to calibrate the standard curve and retention time and the percentage of ACE inhibition was calculated using the formula:

$$\% \text{ inhibition} = \frac{(\text{Control}^{\text{absorbance}} - \text{Extract}^{\text{absorbance}})}{(\text{Control}^{\text{absorbance}} - \text{Blank}^{\text{absorbance}})} \times 100$$

4.3.11. Antimicrobial activity

The antimicrobial activity of the extracts was measured using the broth microdilution method based on the protocol as described by the Clinical and Laboratory Standards Institute (CLSI, 2012). A single colony of each bacterial strain was inoculated into 15 mL centrifuge tubes containing 10 mL Mueller-Hinton (MH) broth and the tubes were incubated overnight at 37 °C. The overnight culture was centrifuged, and the bacterial pellet was resuspended in 10 mL phosphate-buffered saline (PBS) (VWR). The turbidity of each culture was adjusted with PBS to a 0.5 McFarland standard with the help of a 0.5 McFarland standard reference solution (Remel, Thermo Fisher Scientific, Waltham, MA) and the turbidity was confirmed using a UV-visible spectrophotometer (SmartSpec3000, Bio-Rad, Hercules, CA). The adjusted cultures were then diluted in PBS at 1:20 dilution and used as the inoculum in the broth microdilution assay. A two-fold serial dilution of each filter-sterilized extract was done in microtiter plates using MH broth as the dilutant. For the control wells, water was used instead of the extract. Around 10 µL of the adjusted bacterial inoculum were added to the respective wells and the microtiter plates were sealed with a plastic film and incubated for 16 h at 37 °C in a microplate reader (Bio Tek Instruments, Agilent Technologies). At every 15-min interval the microtiter plate was shaken for 5 seconds followed by a measurement of the absorbance of the wells at 600 nm. The absorbance

values were plotted on a graph to get the growth curves and the minimal inhibitory concentration (MIC) of the extracts was expressed in mg GAE/g FW or DW.

4.3.12. Statistical analysis

The complete *in vitro* analysis was repeated four times. Analysis at every time point from each experiment was carried out in triplicates except for the antimicrobial assay which was done in duplicate. Means, standard errors, and standard deviations were calculated from replicates within the experiments and analyses were done using Microsoft Excel XP. The data was analyzed with analysis of variance (ANOVA) using Statistical Analytical Software (SAS version 9.4; SAS Institute, Cary, NC) and significant differences among extracts were determined by the Tukey's least mean square test at the 0.05 probability level.

4.4. Results and Discussion

4.4.1. Total soluble phenolic content and phenolic profile

The TSP content of different forms of select botanicals ranged from 0.7 to 132.6 mg GAE/g FW or DW and significant differences in TSP content were observed among the extracts ($p<0.05$) (Figure 4.1). Amla powder had significantly higher TSP content at 132.6 mg GAE/g DW when compared to the rest of the extracts ($p<0.05$) while garlic slice had the lowest TSP content at 0.7 mg GAE/g FW (Figure 4.1). The TSP content of amla can vary depending upon the cultivar as well as the physical condition of the fruit (slices, juice, or powder) (Kaushal & Sharma, 2012; Mishra, Srivastava, Verma, Chauhan & Rai, 2009; Tyagi & Pal, 2015). In the current study, although the TSP content of amla powder was significantly higher when compared to the other extracts ($p<0.05$), the content was still lower than what was reported in earlier studies.

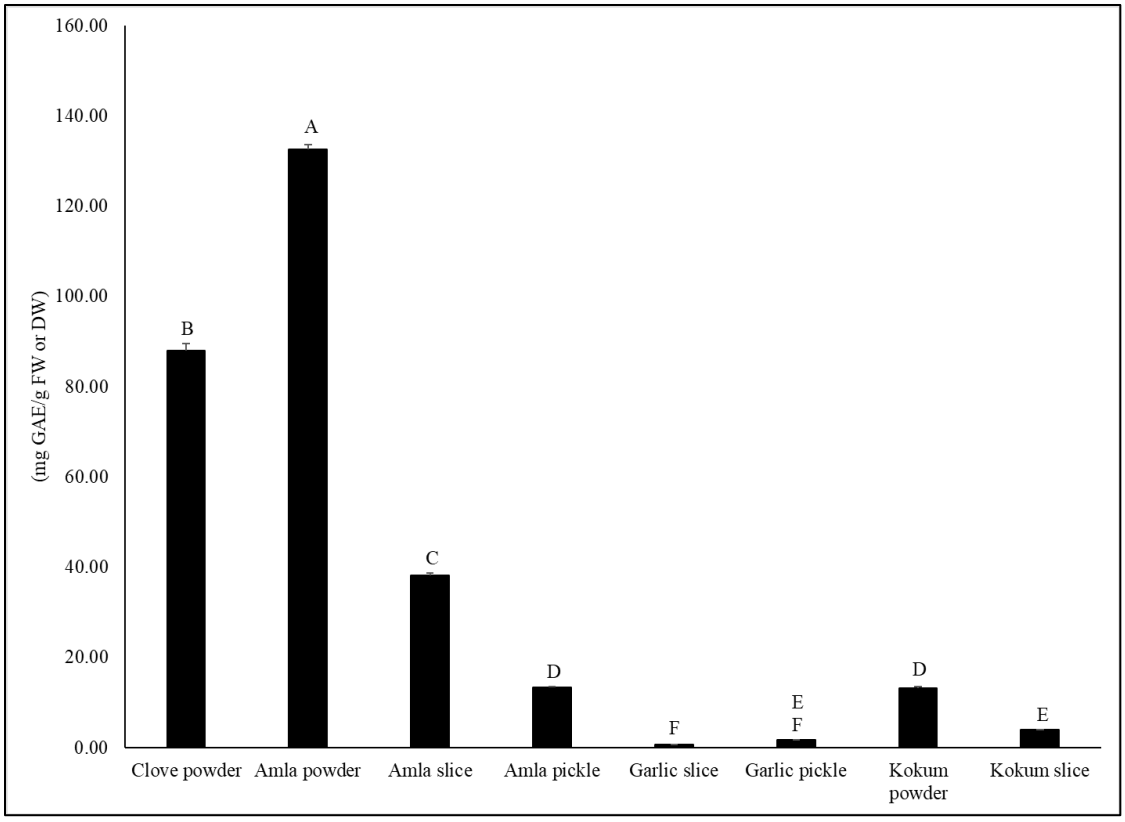


Figure 4.1. Total soluble phenolic (TSP) content (mg GAE/g FW or DW) of select botanical extracts. Different indicate significant differences in TSP content among the extracts (p<0.05). The TSP content of the amla slice, amla pickle, garlic slice and garlic pickle extracts were analyzed on a fresh weight (FW) basis while the clove, amla powder, kokum slice and kokum powder extracts were analyzed on a dry weight (DW) basis.

The Folin-Ciocalteu (FC) reagent used in the estimation of TSP content can be affected by other compounds such as ascorbic acid, sugars and organic acids that may be present in plant-based food matrix (Lester, Lewers, Medina & Saftner, 2012). Therefore, amla having a high ascorbic acid content, can potentially interfere with the accurate estimation of TSP content. We have used serial dilution to avoid overestimation of TSP content of amla. Overall, TSP result of this study indicated that select botanicals are good sources of phenolic phytochemical and can be integrated for dual functional benefits of improving food safety and human health protective qualities of plant foods.

The phenolic profile and content of the extracts varied greatly, and statistical differences in the concentration of phenolic compounds detected were observed among the select botanical extracts ($p<0.05$) (Table 4.1). The phenolic compounds detected were gallic acid (0.54 to 6.56 µg/g FW or DW), ellagic acid (6.27 to 133.38 µg/g FW or DW), cinnamic acid (0.40 to 41.11 µg/g FW or DW), dihydroxybenzoic acid (2.24 to 4.04 µg/g FW or DW), benzoic acid (0.15 µg/g FW or DW), p-coumaric acid (1.78 µg/g FW or DW), protocatechuic acid (1.29 to 2.44 µg/g FW or DW), rutin (2.24 µg/g FW or DW), and catechin (1.35 to 24.01 µg/g FW or DW). The garlic slice, amla powder, clove, and kokum powder extracts were found to have significantly higher concentrations of gallic acid, ellagic acid, cinnamic acid, and catechin, respectively ($p<0.05$) (Table 4.1). Benzoic acid, p-coumaric acid, and protocatechuic acid were detected only in the respective amla slice, garlic pickle, and kokum (powder and slice) extracts (Table 4.1). These results indicate that the TSP content and phenolic profile varies greatly among botanicals. Furthermore, phenolic-rich fruits such as amla or kokum can be incorporated in dietary strategies as fresh slices, powder, or pickles, due to the potential phytochemical-linked health protective benefits, that can be beneficial to improve health targeted food qualities.

Table 4.1. Phenolic profile and content of select botanicals expressed in microgram per gram fresh weight or dry weight (µg/g FW or DW).

Extracts	**Gallic acid[1,2,3]**	**Ellagic acid[1,2,3]**	**Cinnamic acid[1,2,3]**	**Dihydroxy benzoic acid[1,2,3]**	**Benzoic acid[1,2,3]**	**p-coumaric acid[1,2,3]**	**Protocatechuic acid[1,2,3]**	**Rutin[1,2,3]**	**Catechin[1,2,3]**
Clove powder	4.38 ± 0.6b	10.16 ± 1.3c	41.11 ± 1.2a	ND	ND	ND	ND	2.24 ± 0.2	13.67 ± 1.4b
Amla powder	4.51 ± 0.1b	133.38 ± 6.6a	5.94 ± 0.6b	ND	ND	ND	ND	ND	1.88 ± 0.0d
Amla slice	1.05 ± 0.1de	30.93 ± 1.6b	1.96 ± 0.1b	ND	0.15 ± 0.0	ND	ND	ND	1.53 ± 0.1d
Amla pickle	2.76 ± 0.2c	6.27 ± 0.3c	0.40 ± 0.0b	ND	ND	ND	ND	ND	1.35 ± 0.0d
Garlic slice	6.56 ± 0.1a	ND	ND	ND	ND	ND	ND	ND	3.42 ± 0.1d
Garlic pickle	1.67 ± 0.1d	ND	ND	2.42 ± 0.2c	ND	1.78 ± 0.1	ND	ND	2.93 ± 0.1d
Kokum powder	0.83 ± 0.0de	ND	ND	4.04 ± 0.0a	ND	ND	2.44 ± 0.0a	ND	24.01 ± 0.3a
Kokum slice	0.54 ± 0.0e	ND	ND	3.10 ± 0.0b	ND	ND	1.29 ± 0.0b	ND	10.18 ± 0.0c

ND- Not Detected

[1] Mean ± standard error

[2] Different letters in each column indicate significant differences among extracts (p<0.05).

[3] The phenolic concentration of the amla slice, amla pickle, garlic slice and garlic pickle extracts were analyzed on a fresh weight (FW) basis while the clove, amla powder, kokum slice and kokum powder extracts were analyzed on a dry weight (DW) basis.

4.4.2. Antioxidant activity

The antioxidant activity of the extracts was measured via their ABTS and DPPH radical scavenging activity, and the activity was expressed in millimolar trolox equivalents (mM TE). The ABTS and DPPH scavenging activity ranged from 0.49 to 0.52 mM TE/mL and 0.09 to 0.39 mM TE, respectively, and statistical differences in radical scavenging activity were observed among the extracts ($p<0.05$) (Figure 4.2). Amla slice and amla pickle extracts had higher ABTS scavenging activity at 0.52 mM TE ($p<0.05$), while garlic slice had the lowest ABTS scavenging at 0.49 mM TE. Similarly, amla slice extract had higher DPPH scavenging activity at 0.39 mM TE when compared to the rest of the botanical extracts ($p<0.05$), while garlic slice had the lowest DPPH scavenging activity at 0.09 mM TE (Figure 4.2). In general, the extracts had higher ABTS scavenging activity when compared to DPPH scavenging activity, which could be due to the difference in the chemical nature of these synthetic radicals, in which DPPH being more stable than ABTS, requires stronger antioxidant activity. In an earlier study, amla extracts showed high DPPH radical scavenging activity at 92.1% (Majeed et al, 2020). In another study, ice containing kokum was found to improve the oxidative stability and shelf life of chilled Indian mackerel (*Rastrelliger kanagurta*) (Apang et al., 2020). These results indicate that amla and kokum fruits can be incorporated in dietary strategies as slices, powder, or pickles, due to their phytochemical-linked antioxidant activity which can offer chronic oxidative stress protective benefits, relevant for management of common NCDs.

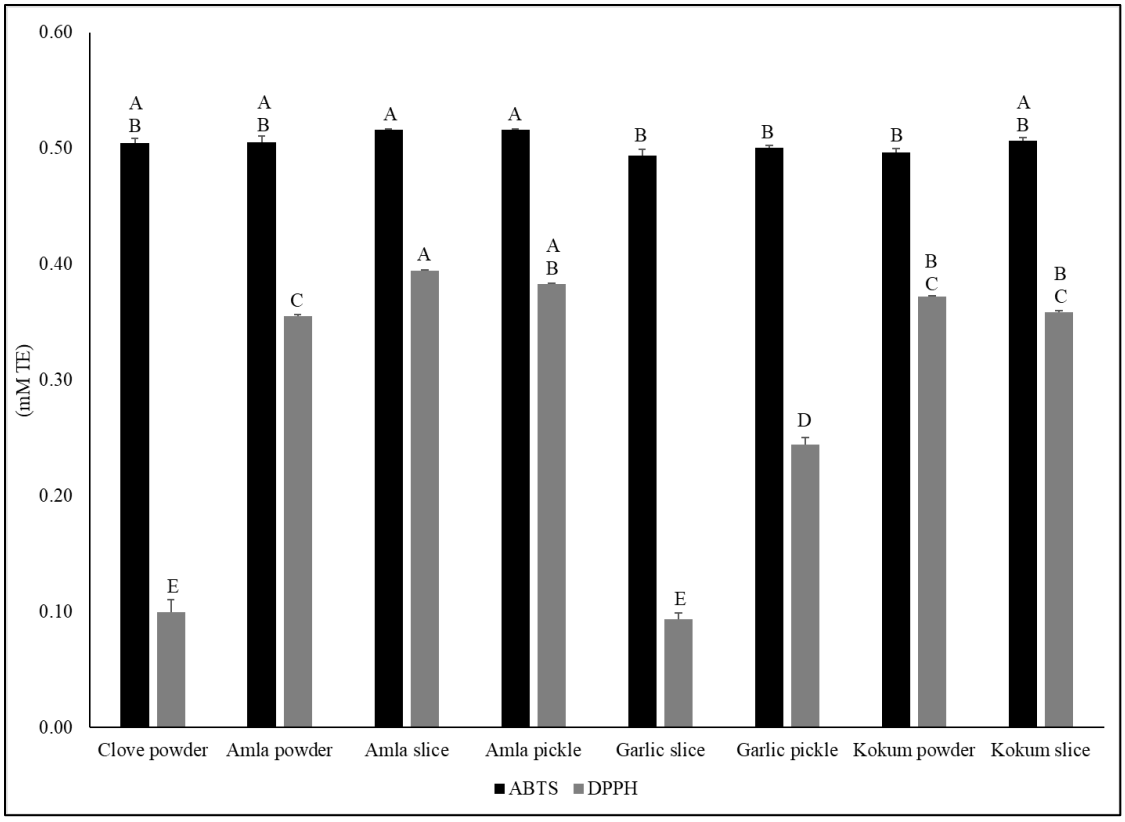


Figure 4.2. Antioxidant activity (mM TE) of select botanical extracts. Different letters indicate significant differences in ABTS and DPPH-based scavenging activity among the extracts ($p<0.05$).

4.4.3. Alpha-amylase and α-glucosidase enzyme inhibitory activity

The α-amylase and α-glucosidase inhibitory activity of the extracts was measured in a dose-dependent manner using undiluted, half-, and one-fifth dilutions of the extracts, and the inhibitory activity was expressed in millimolar acarbose equivalents (mM AE). The α-amylase inhibitory activity of the undiluted, half-, and one-fifth diluted extracts ranged from 0.03 to 0.19 mM AE, 0.005 to 0.19 mM AE, and 0.00 to 0.18 mM AE, respectively, and statistical differences in α-amylase inhibitory activity were observed among the undiluted, half-, and one-fifth dilutions of the extracts ($p<0.05$) (Table 4.2). Among the undiluted extracts, the clove, kokum and amla extracts (slice and powder) had higher α-amylase inhibitory activity ranging from 0.18 to 0.19 mM AE ($p<0.05$), while garlic slice had the lowest α-amylase inhibitory activity at 0.03 mM AE. The

same trend was observed for the half-diluted and one-fifth-diluted extracts (Table 4.2). The α-glucosidase inhibitory activity of the undiluted, half-, and one-fifth diluted extracts ranged from 0.24 to 1.62 mM AE, 0.04 to 1.62 mM AE, and 0.00 to 1.61 mM AE, respectively, and statistical differences in α-glucosidase inhibitory activity were observed among the undiluted, half-, and one-fifth-diluted extracts ($p<0.05$) (Table 4.2). Among the undiluted extracts, the clove, kokum and amla extracts (slice and powder) had higher α-glucosidase inhibitory activity ranging from 1.59 to 1.62 mM AE ($p<0.05$), while garlic slice had the lowest α-glucosidase inhibitory activity at 0.24 mM AE. A similar trend was observed for the half-and one-fifth dilutions of the extracts. Interestingly, extracts of amla and kokum (slices and powder) had the same level of α-amylase and α-glucosidase inhibitory activity, even at one-fifth dilutions of the extracts.

In an earlier study, phenolic-rich fractions of kokum were found to have α-amylase inhibitory activity with IC_{50} values ranging from 349.7 to 980.0 µg/mL (Munjal et al, 2020). In another study, extracts of amla at the highest concentration were found to have α-amylase and α-glucosidase inhibitory activity at 84.15% and 93.9% respectively (Majeed et al, 2020). Catechin, a flavonoid compound, was detected in all the extracts anlyzed in the current study (Table 4.1). In an earlier study, grape seed and tea extracts were found to have potential α-amylase and α-glucosidase inhibitory activity and the activity was attributed to the catechin content (Yilmazer-Musa et al, 2012). The results of the current *in vitro* assay model-based screening study indicate that amla and kokum (slice or powder) with high carbohydrate digestive enzyme inhibitory potential can be incorporated into dietary strategies aimed at the management of chronic hyperglycemia, a common risk factor associated with type 2 diabetes. However, future clinical studies are required to further validate the anti-diabetic potential of amla and kokum and for its value-added integration in health targeted dietary and therapeutic applications.

Table 4.2. Alpha-amylase and α-glucosidase enzyme inhibitory activity of the botanical extracts expressed in millimolar acarbose equivalents (mM AE).

Extracts	**α-amylase inhibitory activity[1,2]**			**α-glucosidase inhibitory activity[1,2]**		
	Undiluted	Half-diluted	One-fifth-diluted	Undiluted	Half-diluted	One-fifth-diluted
Clove powder	0.18 ± 0.01ab	0.17 ± 0.00ab	0.06 ± 0.00c	1.61 ± 0.01a	1.61 ± 0.01a	1.52 ± 0.02b
Amla powder	0.19 ± 0.00a	0.18 ± 0.01ab	0.17 ± 0.00a	1.62 ± 0.02a	1.61 ± 0.00a	1.59 ± 0.00a
Amla slice	0.17 ± 0.01ab	0.17 ± 0.00ab	0.16 ± 0.00b	1.62 ± 0.00a	1.62 ± 0.00a	1.54 ± 0.01b
Amla pickle	0.15 ± 0.01c	0.14 ± 0.01c	0.03 ± 0.00d	1.44 ± 0.03b	1.22 ± 0.02b	1.20 ± 0.01c
Garlic slice	0.03 ± 0.00d	0.005 ± 0.00d	NA	0.24 ± 0.01d	0.04 ± 0.03d	NA
Garlic pickle	0.16 ± 0.01bc	0.12 ± 0.01c	0.03 ± 0.00d	1.17 ± 0.01c	0.80 ± 0.01c	0.35 ± 0.01d
Kokum powder	0.19 ± 0.00a	0.19 ± 0.00a	0.18 ± 0.00a	1.61 ± 0.00a	1.61 ± 0.01a	1.61 ± 0.00a
Kokum slice	0.19 ± 0.00a	0.19 ± 0.00a	0.18 ± 0.00a	1.59 ± 0.01a	1.59 ± 0.02a	1.52 ± 0.01b

NA- No Activity.
[1] Mean ± standard error.
[2] Different letters in each column indicate significant differences among extracts (p<0.05).

4.4.4. Angiotensin-I-converting enzyme (ACE) inhibitory activity

The ACE inhibitory activity was measured in a dose-dependent manner using undiluted, half-, and one-fifth dilutions of the extracts, and the inhibitory activity was expressed in percentages (%). The ACE inhibitory activity of the undiluted, half-diluted, and one-fifth diluted extracts ranged from 90.1 to 100 %, 45.9 to 100 %, and 0 to 100 %, respectively, and statistical differences in ACE inhibitory activity were observed among the undiluted, half-, and one-fifth-diluted extracts (p<0.05) (Figure 4.3). Among the undiluted extracts, amla and kokum extracts (powder and slice) had significantly higher ACE inhibitory activity at 100% (p<0.05), while extracts of clove, amla pickle, and garlic (slice and pickle) had lower baseline ACE inhibitory

activity ranging from 90.1 to 93.3%. (Figure 4.3). A similar trend was observed for the half-, and one-fifth diluted extracts.

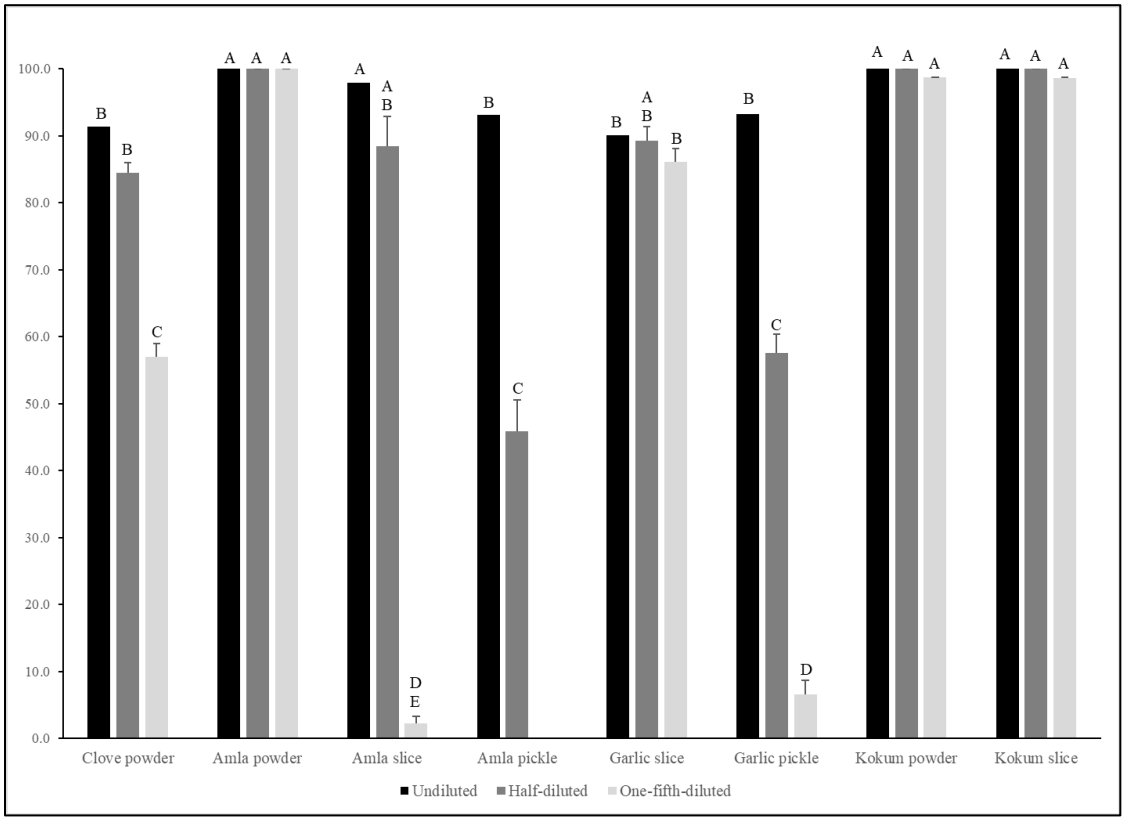


Figure 4.3. Angiotensin-I-converting enzyme inhibitory activity of the undiluted, half-, and one-fifth diluted botanical extracts expressed as percentages (%). Different uppercase letters represent significant differences among the extracts (p<0.05).

Interestingly the ACE inhibitory activity of the amla powder and kokum (powder and slice) extracts was still high even at one-fifth dilutions of the extracts. In a clinical study, an eight-week combination therapy of amla with antihypertensive drugs was found to significantly reduce the systolic blood pressure and diastolic blood pressure in patients with hypertension when compared to control (placebo) group (Ghaffari et al, 2020). In the current study the *in vitro* ACE inhibition result indicates that amla, kokum and other select botanicals can be incorporated in dietary support strategies as slices or powder to counter chronic hypertension commonly associated with type 2 diabetes and other NCDs.

4.4.5. Antimicrobial activity

The antimicrobial activity of the botanical extracts was measured against serovars of *Salmonella*, *Listeria* and *E. coli* and the minimal inhibitory concentration (MIC) was expressed in mg GAE/g FW or DW. The MIC of the extracts against *Salmonella*, *Listeria*, and *E. coli* serovars ranged from 0.25 to 22.00 mg GAE/g FW or DW, 0.12 to 11.00 mg GAE/g FW or DW, and 0.25 to 22.00 mg GAE/g FW or DW, respectively, and statistical differences in MIC was observed among all the botanical extracts for all the microorganisms that were tested ($p<0.05$) (Table 4.3). Among the *Salmonella* serovars (Enteritidis, Typhimurium, Montevideo, Stanley, and Saintpaul), extracts of kokum slice had lower MIC at 0.25 and 0.50 mg GAE/g DW ($p<0.05$), while extracts of clove had the highest MIC at 22.00 and 44.00 mg GAE/g DW (Table 4.3). No antimicrobial activity was detected for the garlic extracts (slice and pickle) against the *Salmonella* serovars Montevideo, Stanley, and Saintpaul. Instead, the garlic extracts enhanced the growth of these serovars when compared to the control (data not shown). Among the *Listeria* serovars (1/2a, 1/2b, and 4b), extracts of kokum slice had lower MIC at 0.12 mg GAE/g DW ($p<0.05$), while extracts of clove had the highest MIC at 11.00 mg GAE/g DW (Table 4.3). No antimicrobial activity was detected for the clove and garlic (slice and pickle) extracts against the *Listeria* serovars 1/2a, 1/2b, and 4b. Among the *E. coli* serovars (O157:H7 and O26:H11), extracts of kokum slice had lower MIC at 0.25 mg GAE/g DW ($p<0.05$), while extracts of clove had the highest MIC at 22.00 mg GAE/g DW (Table 4.3). No antimicrobial activity was detected from garlic slice extracts against the *E. coli* serovars. Overall, the *Listeria* serovars were more susceptible towards the antimicrobial activity of the extracts as evident by the lower MIC values when compared to *Salmonella* or *E. coli*. The mechanism of antimicrobial activity of phenolic phytochemicals is due to their ability to permeablize the microbial cell membrane, inhibit DNA

or protein synthesis, inhibit microbial metabolic activity, as well as chelate compounds required for microbial growth (Christopher et al, 2021; Chibane et al, 2019; Jayaraman et al, 2010; Radulovic et al, 2013). The antimicrobial activity observed in the current study clearly suggests that the select botanicals with high baseline phenolic content are good dietary and therapeutic targets to counter bacterial contamination of foods and to address foodborne illnesses.

Table 4.3. Minimal inhibitory concentration (MIC) of botanical extracts expressed in milligram gallic acid equivalents per gram fresh weight or dry weight (mg GAE/g FW or DW)

Extracts	**Minimal inhibitory concentration[1,2,3]**										
	Salmonella					*Listeria*				*E. coli*	
	S. enterica subsp. *enterica* serovar Enteritidis	*S. enterica* subsp. *enterica* serovar Typhimurium	*S. enterica* subsp. *enterica* serovar Montevideo	*S. enterica* subsp. *enterica* serovar Stanley	*S. enterica* subsp. *enterica* serovar Saintpaul	*L. monocytogenes* serovar 1/2a	*L. monocytogenes* serovar 1/2b	*L. monocytogenes* serovar 1/2a	*L. monocytogenes* serovar 4b	*E. coli* serovar O157:H7	*E. coli* serovar O26:H11
Clove powder	22.00 ± 0.0a	22.00 ± 0.0a	22.00 ± 0.0a	44.00 ± 0.0a	44.00 ± 0.0a	11.00 ± 0.0a	NA	NA	NA	22.00 ± 0.0a	22.00 ± 0.0a
Amla powder	8.29 ± 0.0c	8.29 ± 0.0c	8.29 ± 0.0b	33.16 ± 0.0b	16.58 ± 0.0c	4.15 ± 0.0c	4.15 ± 0.0b	4.15 ± 0.0b	4.15 ± 0.0b	8.29 ± 0.0c	8.29 ± 0.0c
Amla slice	9.54 ± 0.0b	9.54 ± 0.0b	7.16 ± 2.4b	19.09 ± 0.0c	19.09 ± 0.0b	4.77 ± 0.0b	4.77 ± 0.0a	4.77 ± 0.0a	9.54 ± 0.0a	9.54 ± 0.0b	9.54 ± 0.0b
Amla pickle	6.67 ± 0.0d	6.67 ± 0.0d	6.67 ± 0.0b	6.67 ± 0.0d	6.67 ± 0.0d	3.33 ± 0.0d	3.33 ± 0.0c	3.33 ± 0.0c	3.33 ± 0.0c	6.67 ± 0.0d	6.67 ± 0.0d
Garlic slice	0.36 ± 0.0g	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Garlic pickle	0.85 ± 0.0f	0.85 ± 0.0f	NA	NA	NA	NA	NA	NA	NA	0.85 ± 0.0e	0.85 ± 0.0e
Kokum powder	1.65 ± 0.0e	1.65 ± 0.0e	3.30 ± 0.0bc	3.30 ± 0.0e	3.30 ± 0.0e	0.82 ± 0.0e	0.41 ± 0.0d	0.41 ± 0.0d	0.41 ± 0.0d	0.82 ± 0.0f	0.82 ± 0.0f
Kokum slice	0.25 ± 0.0h	0.25 ± 0.0g	0.50 ± 0.0c	0.50 ± 0.0f	0.50 ± 0.0f	0.12 ± 0.0f	0.12 ± 0.0e	0.12 ± 0.0e	0.12 ± 0.0e	0.25 ± 0.0g	0.25 ± 0.0g

NA- No Activity

[1]Mean ± standard error

[2] Different lowercase letters in each column indicate significant differences in MIC among the extracts ($p < 0.05$).

[3] The amla slice, amla pickle, garlic slice and garlic pickle extracts were analyzed on a fresh weight (FW) basis while the clove, amla powder, kokum slice and kokum powder extracts were analyzed on a dry weight (DW) basis.

4.5. Conclusion

The screening of underutilized botanicals and plant-based foods including fruits, spices and other food ingredients is an important first step necessary for the selection and utilization these phytochemical-rich botanicals with associated food safety-relevant and health protective benefits. Under-utilized or indigenous fruits such as amla or kokum in different forms (slice, powder, or pickle) display health protective properties via their antioxidant, antihyperglycemic, and antihypertensive activity, and hence can be incorporated in dietary strategies to counter chronic inflammation, hyperglycemia, and hypertension. In addition to their health protective benefits, these phytochemical-rich fruits and botanicals with high antimicrobial property also have food safety-relevant benefits, specifically to counter foodborne illness related bacterial pathogens such as *Salmonella*, *Listeria*, and *E. coli*. The formulation of different forms of amla and kokum (slice, powder, or pickle) with other bioactive plant-based foods can potentially reduce the risk of microbial contamination with these bacterial pathogens and help manage the burden of foodborne illnesses. Future studies can focus on formulation or synergy strategies involving amla, kokum, and clove with other bioactive plant-based foods to improve phenolic phytochemical-linked functional qualities targeting wider food safety and human health benefits.

CHAPTER 5. AMLA AND KOKUM SYNERGIES WITH GRAPE JUICE TO IMPROVE THE FOOD SAFETY AND HUMAN HEALTH PROTECTIVE FUNCTIONAL QUALITIES

5.1. Abstract

Phenolic-rich fruits have relevance in wider food applications and can be utilized to improve food safety and human health protective functional benefits of foods and beverages. Previously, we observed high antimicrobial, antioxidant, anti-hyperglycemic and anti-hypertensive properties in amla and kokum, which are two underutilized fruits from Southeast Asia. Based on these promising findings, phenolic rich amla and kokum were combined with widely popular grape juice to improve food safety and non-communicable chronic disease (NCDs) related benefits. Different combinations (25:75, 50:50) of amla-grape and kokum-grape along with 100 % extracts of these fruits were analyzed for their antimicrobial activity against select serovars of *Salmonella*, *Listeria*, and *E. coli*. Additionally, the total soluble phenolic (TSP) content, phenolic profile, antioxidant activity (ABTS and DPPH-based), and anti-hyperglycemic relevant α-amylase and α-glucosidase and anti-hypertension relevant angiotensin-I-converting enzymes (ACE) of all fruit synergies were analyzed using *in vitro* assay models. The amla, kokum, and amla-grape and kokum-grape combinations showed antimicrobial activity against the *Salmonella*, *Listeria*, and *E. coli* serovars, while no antimicrobial activity was detected in the 100 % grape juice. Integration of amla and kokum in grape juice improved the TSP content, antimicrobial, anti-hyperglycemic, and anti-hypertensive relevant functional qualities of the juice. Results of this study indicated that incorporation of phenolic-rich fruits such as amla and kokum with commonly consumed fruit-based beverage like grape juice is an effective strategy to design novel functional beverages with food safety and NCD relevant health benefits.

5.2. Introduction

Phenolic-rich fruits such as amla (*Emblica officinalis* Gaertn) and kokum (*Garcinia indica*) are commonly used in traditional or ethnic food systems in Southeast Asia but are often underutilized in wider global food and dietary applications. These fruits with rich phenolic phytochemical profiles have health protective properties that can be potentially utilized in dietary strategies aimed at the prevention and management of non-communicable chronic diseases (NCDs). Some of the functional qualities of phenolic-rich fruits are antioxidant, anti-hyperglycemic, and anti-hypertensive properties, which are relevant to counter chronic health risk factors associated with NCDs like type 2 diabetes and hypertension. Additionally, the phenolic-rich amla and kokum also display food safety-relevant antimicrobial properties that can potentially help to reduce the risk of contamination of foods or beverages by common foodborne pathogens.

The high phytochemical-linked functional qualities of amla and kokum make them ideal candidates as food ingredient sources that can be synergized in widely used food and beverage matrices. Food synergy is a concept involving the non-random mixture of food ingredients that can provide stronger health benefits when compared to the individual foods or food ingredients (Jacobs et al, 2009; Jacobs & Tapsell, 2013). Earlier studies have been done on incorporating amla with mixed fruits beverages, pan bread, and chicken feed with the goal of improving the functional qualities of the resulting food matrix (Alkandari et al, 2019a, 2019b; Bhalerao et al, 2020; Patel et al, 2016; Purewal et al, 2022; Zafar et al, 2021). Likewise, other studies have also been done on incorporating kokum with different juices and food ingredients, with the goal of improving the overall health protective benefits of the food (Agte et al, 2018; Byanna & Gowda, 2013; Hegde et al, 2018; Nevase, Pawar, Khanvilkar, Kadam & Kasture, 2021; Siddharth &

Sharma, 2013). *In vitro* studies have shown amla to possess antimicrobial activity against gram-positive (*Staphylococcus, Micrococcus,* and *Bacillus*) and gram-negative (*E. coli* and *Salmonella*) bacterial pathogens (Gautam & Shukla, 2017; Saeed & Tariq, 2007; Singh et al, 2019; Tyagi & Singh, 2014; Vijayalakshmi et al, 2007). Likewise, other *in vitro* studies have shown extracts of kokum to have antimicrobial activity against *Micrococcus aureus, Bacillus megaterium, Micrococcus luteus, Salmonella typhimurium, Pseudomonas aeruginosa, E. coli, Bacillus subtilis, Enterobacter aerogenes,* and *Staphylococcus aureus* (Sutar et al, 2012; Varalakshmi et al, 2010). In our previous study (Chapter 4), high phenolic phytochemical-linked antimicrobial, antioxidant, anti-hyperglycemic, and anti-hypertensive properties were observed in hot water extracts of amla and kokum powder. Therefore, integration of these phenolic rich fruit extracts in widely consumed fruit like grape has relevance in designing novel grape-based functional beverages targeting wider food safety and human health benefits.

Grape and grape-based non-alcoholic beverages are widely consumed and gaining increasing interest in health-targeted beverage market worldwide. Previously, we observed high phenolic-linked antioxidant and anti-hyperglycemic functional properties in ozone treated grapes (Christopher et al. 2018; Chapter 6). However, it is important to improve antimicrobial qualities of grape-based beverages to improve their shelf-life and food safety related qualities. Additionally, integration of phenolic-rich underutilized fruits in grape is also relevant to widen the phytochemical profile and subsequently improving human health protective functional qualities. Based on these wider antimicrobial and health protective functional properties of amla and kokum (Chapter 4), the goal of this study was to integrate phenolic rich amla and kokum in liquid grape extracts to develop fruit synergies with high antimicrobial and NCD-countering functional qualities. Different combinations of amla-grape (v/v) and kokum-grape (v/v) blends

were analyzed for total soluble phenolic (TSP) content, phenolic profile, antioxidant, anti-hyperglycemic, anti-hypertensive, and antimicrobial activity using *in vitro* assay models.

5.3. Materials and Methods

5.3.1. Chemicals used

Other than mentioned, all chemicals and enzymes were purchased from Sigma Chemical Co (St. Louis, MO).

5.3.2. Samples used

The amla powder and kokum slices (dried) were purchased from a local Indian grocery store in Fargo (Fargo Fresh, North Dakota, USA). The kokum slices were ground using a coffee blender to obtain a coarse powder. The grape variety King of North was obtained from the North Dakota State University grape breeding program (Fargo, North Dakota).

5.3.3. Preparation of samples

The extraction of amla and kokum powder was done using hot water in which 10 g of the respective powders were added to 50 mL of boiling water (100 °C) and boiled for 15 min. The samples were then cooled down to room temperature and centrifuged at 8,500 rpm for 15 min after which the supernatant was collected and re-centrifuged at 8,500 rpm for 15 min. The extraction of grape was done using cold water in which 200 g of the grape berries were added to 200 mL of water followed by homogenization using a Waring blender set at low speed for 5 min. The extract was then centrifuged at 8,500 rpm for 15 min and the supernatant was collected and re-centrifuged at 8,500 rpm for 15 min. The extracts of amla and kokum powder were blended with grape extract in the following ratios- 25 mL amla with 75 mL grape (25/75 A/G), 50 mL amla with 50 mL grape (50/50 A/G), 25 mL kokum with 75 mL grape (25/75 K/G), and 50 mL kokum with 50 mL grape (50/50 K/G), followed by mixing using a vortex machine. The samples

(grape, amla, and kokum extracts and the amla/ kokum-grape blends) were stored at 4 °C for analysis in the *in vitro* assays. Prior to the *in vitro* antimicrobial assay, the samples were filter-sterilized using 0.22 µm syringe filters (Millipore Corp, MA, USA) under aseptic conditions.

5.3.4. Bacterial strains used

The *Salmonella* strains tested in this study were *Salmonella enterica* subsp. *enterica* serovar Enteritidis (ATCC BAA-1045) and *S. enterica* subsp. *enterica* serovar Typhimurium (FSL R8-0865). The *Listeria* strains used in this study were *Listeria monocytogenes* serovar 1/2a (10403S) and *L. monocytogenes* serovar 1/2b (FSL J1-0194). The *E. coli* strains used in this study were *E. coli* serovar O157:H7 (EDL-933) and *E. coli* serovar O26:H11 (TW07936). The *Salmonella* and *Listeria* strains were obtained from the Food Safety Laboratory at Cornell University, except for *Salmonella enterica* subsp. *enterica* serovar Enteritidis (ATCC BAA-1045) which was obtained from the American Type Culture Collection (Manassas, VA). The *E. coli* strains were obtained from the Thomas S. Whittam STEC Center at Michigan State University.

5.3.5. Total soluble phenolic (TSP) content

The TSP content of the fruit synergy samples was determined using the Folin-Ciocalteu method based on a protocol as described previously (Shetty et al, 1995). For this assay, the samples were diluted in water at 1:20 dilution and 0.5 mL aliquots of the diluted samples were taken into respective glass tubes after which 1 mL of 95 % ethanol, 0.5 mL of 50 % (v/v) Folin-Ciocalteu reagent, and 1 mL of 5 % sodium carbonate were added sequentially to each tube. Due to higher ascorbic acid content of the amla, which interferes with the Folin-Ciocalteu reagent, we have used multiple dilutions of the samples to avoid overestimation of total soluble phenolic content. The tubes were then mixed using a vortex machine and incubated for 60 min under dark

conditions. The absorbance values in each tube were measured at 725 nm with a UV-visible spectrophotometer (Genesys 10S UV-VIS spectrophotometer, Thermo Scientific, NY, USA). Using a standard curve of different concentrations of gallic acid in 95 % ethanol, the absorbance values of the samples were converted, and the TSP content was expressed in milligram gallic acid equivalent per milliliter (mg GAE/mL).

5.3.6. Phenolic profile

The profile of the phenolic compounds of the samples was determined using the high-performance liquid chromatography (HPLC) assay method. The samples were centrifuged at 13,500 rpm for 5 min after which 5 µL of the supernatant were injected using an Agilent ALS 1200 auto-extractor into an Agilent 1260 series (Agilent Technologies, Palo Alto, CA) HPLC equipped with a D1100 CE diode array detector. The solvents used for gradient elution were 10 mM phosphoric acid (pH 2.5) and 100 % methanol. The methanol concentration was increased to 60 % for the first 8 min, then to 100 % over the next 7 min, then decreased to 0 % for the next 3 min and was maintained for 7 min with a total run time of 25 min per injected sample run. 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 0.7 mL/min at room temperature. The absorbance values were recorded at 214 nm, 230 nm, 260 nm, and 306 nm and the chromatogram was integrated using Agilent Chem station enhanced integrator. Pure standards of benzoic acid, gallic acid, protocatechuic acid, ellagic acid, cinnamic acid, dihydroxybenzoic acid, p-coumaric acid, rutin, and catechin in 100 % methanol were used to calibrate the respective standard curves and retention times. The phenolic compounds detected in the extracts were expressed in microgram per milliliter (µg/mL).

5.3.7. Antioxidant activity

The antioxidant activity of the fruit synergy samples was measured by their scavenging activity against the free radicals 2, 2-Dipheny-1-Picryl hydrazyl (DPPH) (D9132-5G, Sigma-Aldrich), and 2, 2-Azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) (A1888-5G, Sigma-Aldrich) respectively. The DPPH scavenging assay was based on a protocol as described earlier (Kwon et al, 2006) in which 0.25 mL of the samples were added to 1.25 mL of 60 mM DPPH prepared in 95% ethanol while the controls had 0.25 mL of 95 % ethanol instead of the extract. After 5 min of incubation, the samples and their corresponding controls were centrifuged at 13,000 rpm for 1 min and the absorbance values of the supernatants were measured at 517 nm using a UV-visible spectrophotometer (Genesys 10S UV-VIS spectrophotometer, Thermo Scientific, NY). The ABTS scavenging assay was based on a protocol as described earlier (Re et al, 1999) in which 0.05 mL of the samples was added to 1 mL of ABTS prepared in 95 % ethanol while the controls had 0.05 mL of 95 % ethanol instead of the sample. After 2 min of incubation, the samples and their controls were centrifuged at 13,000 rpm for 1 min and the absorbance values of the supernatant were measured at 734 nm with a UV-visible spectrophotometer (Genesys 10S UV-VIS spectrophotometer, Thermo Scientific, NY). The absorbance values from the DPPH and ABTS radical scavenging assays were used to calculate the percentage of antioxidant activity for each sample using the following formula:

$$\% \text{ Antioxidant activity} = \frac{\text{Control}^{\text{absorbance}} - \text{Extract}^{\text{absorbance}}}{\text{Control}^{\text{absorbance}}} \times 100$$

Using a standard curve of different concentrations of Trolox in 95 % ethanol, the percentages of inhibitory activity obtained from the DPPH and ABTS radical scavenging assays were expressed as millimolar equivalents of Trolox (mM TE).

5.3.8. Alpha-amylase enzyme inhibitory activity

The α-amylase enzyme inhibitory activity of the samples was measured based on a protocol as described in a previous study (Kwon et al, 2006) and the activity was measured in a dose-dependent manner at undiluted, half, and one-fifth dilutions of the samples. The samples were diluted using distilled water and 500 µL of undiluted and diluted samples were added to respective glass tubes, while the control tubes had 500 µL of 0.1M sodium phosphate buffer (containing 0.006M sodium chloride at pH 6.9) instead of the extract. Additionally, each sample had a corresponding blank tube containing 500 µL of the extract and 500 µL of the buffer instead of the enzyme. Then 500 µL of porcine pancreatic α-amylase (0.5 mg/mL buffer) (EC 3.2.1.1, purchased from Sigma Chemical Co, St Louis, MO) was added to the sample and control tubes and incubated for 10 min at 25 °C. After incubation 500 µL of the substrate (1% starch in buffer) was added to all the tubes and incubated again for 10 min at 25 °C. Then 1 mL of 3, 5 dinitro salicylic acid was added and the tubes were incubated in a boiling water bath for 10 min to stop the reaction. After removing from the water bath and cooling down to room temperature, 10 mL of distilled water was added to all the tubes to ensure that the absorbance values in the control tubes ranged between 1.0 and 1.2, and the absorbance of all the tubes was measured at 540 nm using a UV-visible spectrophotometer (Genesys 10S UV-VIS spectrophotometer, Thermo Scientific, NY). The absorbance values were then used to calculate the percentage of enzyme inhibitory activity of the samples using the following formula:

$$\% \text{ Inhibition} = \frac{\text{Control}^{\text{absorbance}} - (\text{Extract}^{\text{absorbance}} - \text{Extract blank}^{\text{absorbance}})}{\text{Control}^{\text{absorbance}}} \times 100$$

Using a standard curve of different concentrations of Acarbose in distilled water, the percentages of α-amylase inhibitory activity obtained from the enzyme inhibition assay were expressed as millimolar equivalents of Acarbose (mM AE).

5.3.9. Alpha-glucosidase enzyme inhibitory activity

The α-glucosidase enzyme inhibitory activity of the samples was measured based on a protocol as described in a previous study (Kwon et al, 2006) and the activity was measured in a dose-dependent manner at undiluted, half and one-fifth dilutions of the samples. The samples were diluted with 0.1M potassium phosphate buffer (pH 6.9) in 96 well microtiter plates in which 50 µL, 25 µL, and 10 µL of each sample were pipetted and the final volume made up to 50 µL by the addition of potassium phosphate buffer. Each sample had a corresponding control of 50 µL buffer instead of the sample and the volume in all the wells was made up to a final volume of 100 µL by the addition of 50 µL of the buffer. Then 100 µL of buffer containing yeast α-glucosidase enzyme (1 U/mL) (EC 3.2.1.20, purchased from Sigma Chemical Co, St. Louis, MO) was added to each well and incubated for 10 min at 25 °C, after which 50 µL of the substrate, 5 mM p-nitrophenyl-α-D- glucopyranoside solution (prepared in buffer) was added to each well followed by 5-min incubation at 25 °C. The absorbance of all the wells was measured at 405 nm using a microplate reader (Thermomax, Molecular device Co., VA) at the 0- and 5-min time points of the 5-min incubation period, and the absorbance values were used to calculate the percentage of enzyme inhibitory activity using the following formula:

$$\% \text{ Inhibition} = \frac{(\text{Control}^{\text{abs}}\ 5\text{ min} - \text{Control}^{\text{abs}}\ 0\text{ min}) - (\text{Extract}^{\text{abs}}\ 5\text{ min} - \text{Extract}^{\text{abs}}\ 0\text{ min})}{(\text{Control}^{\text{abs}}\ 5\text{ min} - \text{Control}^{\text{abs}}\ 0\text{ min})} \times 100$$

Using a standard curve of different concentrations of Acarbose in distilled water, the percentages of α-glucosidase inhibitory activity obtained from the enzyme inhibition assay were expressed as millimolar equivalents of Acarbose (mM AE).

5.3.10. Angiotensin-I-converting enzyme (ACE) inhibitory activity

The ACE inhibitory activity of the samples was measured based on a protocol as described in a previous study (Kwon et al, 2006) and the activity was measured in a dose-dependent manner at undiluted, half, and one-fifth dilutions of the samples. The samples were diluted using distilled water. The substrate used was hippuryl-histidyl-leucine (HHL) and the enzyme ACE was obtained from rabbit lung (EC 3.4.15.1). To 50 µL of the samples, 200 µL of 1M NaCl-borate buffer (pH 8.3) containing 2 mU of ACE was added and then incubated at room temperature for 10 minutes. For the blank tubes, 50 µL of distilled water and 200 µL of buffer were used instead of the sample and enzyme. After this, 100 µL of 5mM HHL substrate (prepared in buffer) was added to all the tubes and incubated for one hour at 37 °C. The reaction was stopped by the addition of 150 µL of 0.5N HCl. The hippuric acid formed due to ACE activity was detected using the HPLC method for which, 5 µL of the reaction mixtures were injected using Agilent ALS 1200 autosampler into an Agilent 1260 series (Agilent Technologies, Palo Alto, CA) HPLC equipped with a D1100 CE diode array detector. The solvents used for the gradient were a combination of 10 mM phosphoric acid (pH2.5) and 100% methanol. For the first 8 min, the methanol concentration was increased to 60% then to 100% for 5 min, and finally to 0% for the next 5 min and the total run time was 18 min for each sample. 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 0.7 mL/min at room temperature. The absorbance was measured at 228 nm and the chromatogram was integrated using Agilent Chemstation (Agilent Technologies)

enhanced integrator for detection of hippuric acid. A hippuric acid standard was used to calibrate the standard curve and retention time and the percentage of ACE inhibition was calculated using the formula:

$$\% \text{ inhibition} = \frac{(\text{Control}^{\text{absorbance}} - \text{Extract}^{\text{absorbance}})}{(\text{Control}^{\text{absorbance}} - \text{Blank}^{\text{absorbance}})} \times 100$$

5.3.11. Antimicrobial activity

The antimicrobial activity of the samples was measured using the broth microdilution method based on the protocol as described by the Clinical and Laboratory Standards Institute (CLSI, 2012). A single colony of each bacterial strain was inoculated into 15 mL centrifuge tubes containing 10 mL Mueller-Hinton (MH) broth and the tubes were incubated overnight at 37 °C. The overnight culture was centrifuged, and the bacterial pellet was resuspended in 10 mL phosphate-buffered saline (PBS) (VWR). The turbidity of each culture was adjusted with PBS to a 0.5 McFarland standard with the help of a 0.5 McFarland standard reference solution (Remel, Thermo Fisher Scientific, Waltham, MA) and the turbidity was confirmed using a UV-visible spectrophotometer (SmartSpec3000, Bio-Rad, Hercules, CA). The adjusted cultures were then diluted in PBS at 1:20 dilution and used as the inoculum in the broth microdilution assay. A two-fold serial dilution of each filter-sterilized sample was done in microtiter plates using MH broth as the dilutant. For the control wells, water was used instead of the sample. Around 10 µL of the adjusted bacterial inoculum were added to the respective wells and the microtiter plates were sealed with a plastic film and incubated for 16 h at 37 °C in a microplate reader (Bio Tek Instruments, Agilent Technologies). At every 15-min interval the microtiter plate was shaken for 5 seconds followed by a measurement of the absorbance of the wells at 600 nm. The absorbance

values were plotted on a graph to get the growth curves and the minimal inhibitory concentration (MIC) of the samples was expressed in mg GAE/mL.

5.3.12. Statistical analysis

The complete *in vitro* analysis was repeated four times. Analysis at every time point from each experiment was carried out in triplicates except for the antimicrobial assay which was done in duplicate. Means, standard errors, and standard deviations were calculated from replicates within the experiments and analyses were done using Microsoft Excel XP. The data was analyzed with analysis of variance (ANOVA) using Statistical Analytical Software (SAS version 9.4; SAS Institute, Cary, NC) and statistical differences among the samples were determined by the Tukey's least mean square test at the 0.05 probability level.

5.4. Results and Discussion

5.4.1. Total soluble phenolic content and phenolic profile

The TSP content of the samples ranged from 0.95 mg GAE/mL to 26.45 mg GAE/mL and statistical differences in TSP content were observed among the samples ($p<0.05$) (Figure 5.1). Amla powder extracts (100 %) had a higher TSP content at 26.45 mg GAE/mL ($p<0.05$) while grape had the lowest TSP content at 0.95 mg GAE/mL (Figure 5.1). The amla-grape and kokum-grape blends (25/75 A/G, 50/50 A/G, 25/75 K/G, and 50/50 K/G) had lower TSP content when compared to the respective amla and kokum extracts ($p<0.05$), however, the TSP content of the blends was still higher when compared to the 100 % grape extract ($p<0.05$). These results indicated that integration of amla and kokum is a promising approach to improve TSP content in grape-based fruit synergies. Though the TSP content of amla was the highest among the samples, the content was still lower when compared to previous studies. This could be due to the higher dilutions used in this study to avoid overestimation of TSP content due to interference of Folin-

Ciocalteu (FC) reagent with ascorbic acid content that is present in the amla sample (Lester, Lewers, Medina & Saftner, 2012). In a previous study, the total phenolic content of orange-raspberry-acerola and pineapple-plum-blackcurrant juice blends were found to be at 3085.1 and 309.2 mg caffeic acid equivalents per liter of the respective blends (Mendiola et al, 2008).

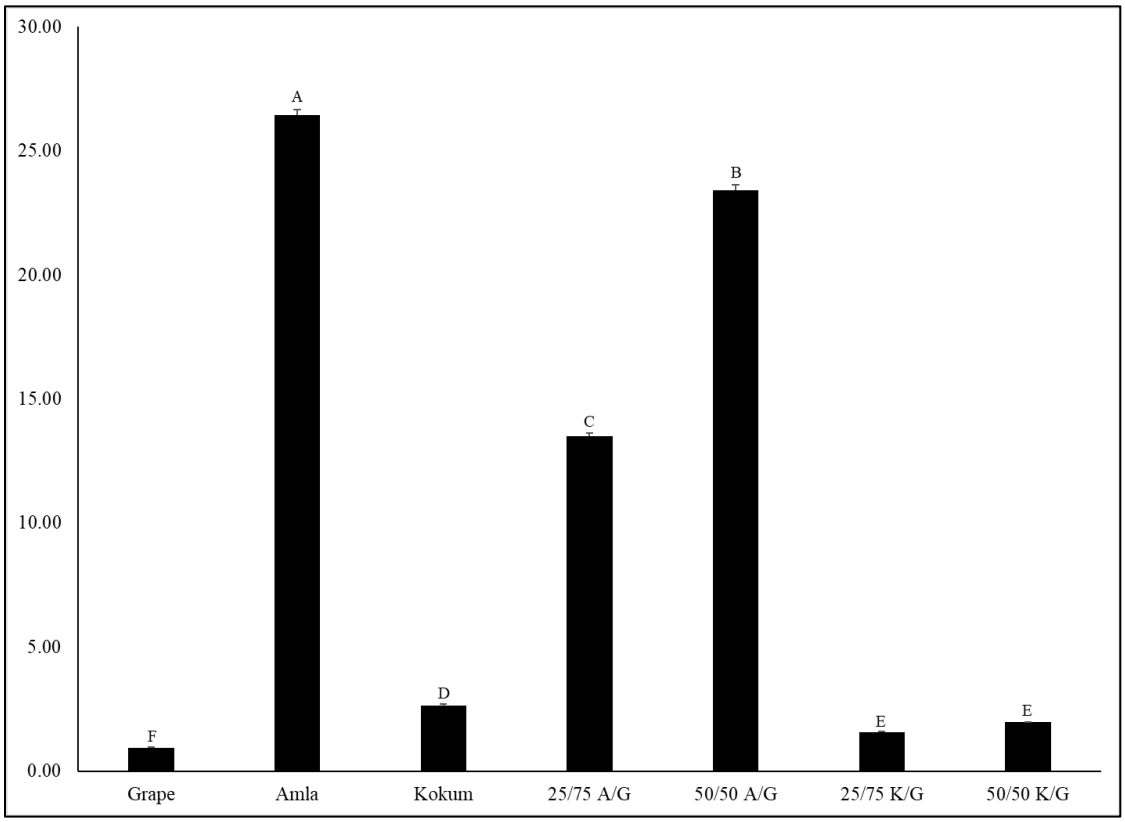


Figure 5.1. Total soluble phenolic (TSP) content (mg GAE/mL) of the samples. Different letters indicate significant differences in TSP content among the samples (p<0.05). The blends used were 25 mL amla with 75 mL grape (25/75 A/G), 50 mL amla with 50 mL grape (50/50 A/G), 25 mL kokum with 75 mL grape (25/75 K/G), and 50 mL kokum with 50 mL grape (50/50 K/G).

The phenolic compounds detected in the samples were *p*-coumaric acid, dihydroxybenzoic acid, gallic acid, cinnamic acid, ellagic acid, protocatechuic acid, and catechin, and the concentration of the compounds was expressed in µg/mL (Table 5.1). *p*-Coumaric and dihydroxybenzoic acid was detected only in the grape extract while protocatechuic acid was detected only in the kokum extract. Cinnamic and ellagic acid were detected only in the

amla extract and the amla-grape synergies (25/75 A/G and 50/50 A/G). Gallic acid and catechin was detected only in the grape, kokum, and kokum-grape blends (25/75 K/G and 50/50 K/G). In an earlier study, two juice blends (orange-raspberry-acerola and pineapple-plum-blackcurrant) were found to contain cinnamic acid and flavonoids, in addition to a high ascorbic acid content (Mendiola et al, 2008). In the current study, the disappearance of some of the phenolic acids in fruit synergies might be due to the formation of chemical complex or conjugates, which affected the detection of individual compounds in this study. Future strategies using different organic solvent-based extractions, purification, or different integration of chromatograms are required to identify and quantify wider phenolic compounds in grape-based fruit synergies.

Table 5.1. Phenolic profile and content of the samples expressed in microgram per milliliter (µg/mL).

Samples	***p*- coumaric acid[1,2]**	**Dihydroxybenzoic acid[1,2]**	**Gallic acid[1,2]**	**Cinnamic acid[1,2]**	**Ellagic acid[1,2]**	**Protocatechuic acid[1,2]**	**Catechin[1,2]**
Grape	0.10 ± 0.0	0.19 ± 0.0	2.81 ± 0.0	ND	ND	ND	2.42 ± 0.0
Amla	ND	ND	ND	6.63 ± 0.0	23.11 ± 0.0	ND	ND
Kokum	ND	ND	0.08 ± 0.0	ND	ND	0.59 ± 0.0	3.30 ± 0.0
25/75 A/G	ND	ND	ND	1.56 ± 0.0	21.39 ± 0.0	ND	ND
50/50 A/G	ND	ND	ND	3.27 ± 0.0	15.87 ± 0.1	ND	ND
27/75 K/G	ND	ND	2.12 ± 0.3	ND	ND	ND	3.40 ± 0.0
50/50 K/G	ND	ND	2.07 ± 0.0	ND	ND	ND	1.92 ± 0.0

ND- not detected

[1] Mean value ± standard error

[2] The blends used were 25 mL amla with 75 mL grape (25/75 A/G), 50 mL amla with 50 mL grape (50/50 A/G), 25 mL kokum with 75 mL grape (25/75 K/G), and 50 mL kokum with 50 mL grape (50/50 K/G).

5.4.2. Antioxidant activity

The antioxidant activity of the samples was measured via their ABTS and DPPH free radical scavenging activity, and the activity was expressed in millimolar Trolox equivalents (mM TE). The ABTS and DPPH scavenging activity ranged from 0.50 mM TE to 0.52 mM TE and 0.35 to 0.37 mM TE respectively, and statistical differences in antioxidant activity was observed among the fruit combination and individual samples ($p<0.05$) (Figure 5.2). In the case of ABTS-based antioxidant activity, the amla-grape combinations (25/75 A/G and 50/50 A/G) had higher ABTS-based antioxidant activity when compared to the amla extract, but it was at par with the 100 % grape extract. In the case of DPPH-based antioxidant activity, kokum extract had higher DPPH-based antioxidant activity at 0.37 mM TE ($p<0.05$), while grape extract and kokum-grape blends (25/75 K/G and 50/50 K/G) had the lowest DPPH-based antioxidant activity at 0.35 mM TE. These results indicated that combining amla and kokum extract with grape extract had no synergistic or additive effect on the ABTS and DPPH-based antioxidant activity of the amla-grape and kokum-grape blends. However, the high baseline antioxidant activity found in all combinations along with 100 % fruit extracts has relevance to target them to counter chronic oxidative stress, which is commonly associated with the pathogenesis of major NCDs. A juice formulation consisting of pomegranate with guava and roselle extract was found to have DPPH and ABTS scavenging activity at 88.9% and 0.47 mM TE/mL respectively (Malek, Haron, Mustapha & Shahar, 2017). In a clinical study, amla-grape and kokum-grape blends with other herbs and fruits were found to improve antioxidant status of 48 volunteers (Agte et al, 2018). *In vitro* antioxidant assays are sensitive to different food matrices, specifically due to differences in hydrophilic and lipophilic antioxidants. Hence, future studies with other *in vitro* and *in vivo*

assay methods are required to understand the differences in antioxidant capacities between these fruit synergies and their potential application in dietary designs against oxidative stress.

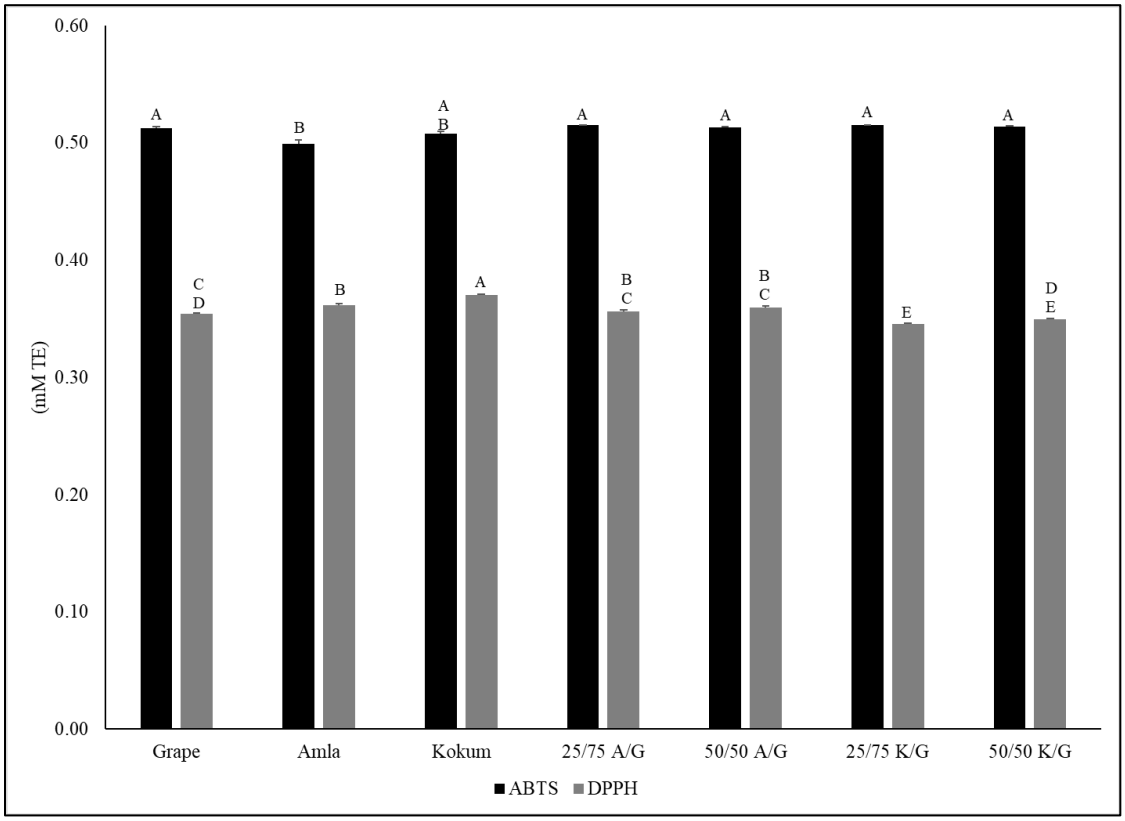


Figure 5.2. Antioxidant activity (mM TE) of the samples. Different letters indicate significant differences in TSP content among the samples (p<0.05). The blends used were 25 mL amla with 75 mL grape (25/75 A/G), 50 mL amla with 50 mL grape (50/50 A/G), 25 mL kokum with 75 mL grape (25/75 K/G), and 50 mL kokum with 50 mL grape (50/50).

5.4.3. Alpha-amylase and α-glucosidase enzyme inhibitory activity

The α-amylase and α-glucosidase enzyme inhibitory activities of the extracts was measured in a dose-dependent manner using undiluted, half-, and one-fifth dilutions of the samples, and the inhibitory activity was expressed in millimolar acarbose equivalents (mM AE). The α-amylase inhibitory activity of the undiluted, half-, and one-fifth diluted samples ranged from 0.16 to 0.19 mM AE, 0.18 to 0.19 mM AE, and 0.12 to 0.09 mM AE respectively, and

statistical differences in α-amylase inhibitory activity were observed among the undiluted and one-fifth-diluted samples ($p<0.05$) (Table 5.2). Among the undiluted samples, the grape extract and amla-grape combination (25/75 A/G) had higher α-amylase inhibitory activity at 0.19 mM AE ($p<0.05$), while the amla extract had the lowest α-amylase inhibitory activity at 0.16 mM AE. Among the one-fifth diluted samples, the amla, kokum, and amla-grape and kokum-grape combinations (50/50 A/G and 50/50 K/G) had higher α-amylase inhibitory activity ranging from 0.17 to 0.18 mM AE ($p<0.05$), when compared to the grape extract which had the lowest α-amylase inhibitory activity at 0.09 mM AE.

The α-glucosidase enzyme inhibitory activity of the undiluted, half-, and one-fifth diluted samples ranged from 1.57 to 1.62 mM AE, 1.55 to 1.62 mM AE, and 1.41 to 1.60 mM AE respectively, and statistical differences in α-glucosidase inhibitory activity were observed among the undiluted, half-, and one-fifth diluted samples ($p<0.05$) (Table 5.2). Among the undiluted samples, grape and kokum-grape combination (25/75 K/G) had higher α-glucosidase inhibitory activity at 1.62 mM AE ($p<0.05$), while amla had the lowest α-glucosidase inhibitory activity at 1.56 mM AE. Among the half-diluted samples, the kokum, grape and amla-grape and kokum-grape combinations (25/75 A/G, 50/50 A/G, 25/75 K/G, and 50/50 K/G) had higher α-glucosidase inhibitory activity ranging from 1.60 to 1.62 mM AE ($p<0.05$), while amla had the lowest α-glucosidase inhibitory activity at 1.55 mM AE. Among the one-fifth diluted samples, the amla, kokum, and amla-grape and kokum-grape blends (50/50 A/G) had higher α-glucosidase inhibitory activity ranging from 1.48 to 1.60 mM AE, when compared to grape extract which had the lowest α-glucosidase inhibitory activity at 1.41 mM AE ($p<0.05$). In a previous study, α-amylase inhibitory activity was not detected in whey while in jamun juice the activity was at 71% and the whey-jamun formulation had α-amylase inhibitory activity at 60%, along with an

improvement in TSP content and antioxidant activity of the whey-jamun juice formulations (Jan et al, 2021).

Table 5.2. Alpha-amylase and α-glucosidase inhibitory activity of the samples expressed in millimolar acarbose equivalents per milliliter (mM AE).

Sample extracts	**α-amylase inhibitory activity[1,2,3]**			**α-glucosidase inhibitory activity[1,2,3]**		
	Undiluted	Half-diluted	One-fifth-diluted	Undiluted	Half-diluted	One-fifth-diluted
Grape	0.19 ± 0.0a	0.19 ± 0.0	0.09 ± 0.0c	1.62 ± 0.0a	1.60 ± 0.0a	1.41 ± 0.0d
Amla	0.16 ± 0.0b	0.18 ± 0.0	0.18 ± 0.0a	1.57 ± 0.0b	1.55 ± 0.0b	1.57 ± 0.0ab
Kokum	0.18 ± 0.0ab	0.18 ± 0.0	0.17 ± 0.0a	1.62 ± 0.0a	1.62 ± 0.0a	1.60 ± 0.0a
25/75 A/G	0.19 ± 0.0a	0.18 ± 0.0	0.14 ± 0.0b	1.61 ± 0.0ab	1.61 ± 0.0a	1.53 ± 0.0b
50/50 A/G	0.18 ± 0.0ab	0.18 ± 0.0	0.17 ± 0.0a	1.59 ± 0.0ab	1.62 ± 0.0a	1.60 ± 0.0a
25/75 K/G	0.18 ± 0.0ab	0.19 ± 0.0	0.12 ± 0.0b	1.62 ± 0.0a	1.61 ± 0.0a	1.48 ± 0.0c
50/50 K/G	0.17 ± 0.0ab	0.19 ± 0.0	0.17 ± 0.0a	1.62 ± 0.0a	1.62 ± 0.0a	1.53 ± 0.0b

[1] Mean ± standard error
[2] Different lowercase letters in each column indicate significant differences among the samples (p<0.05).
[3] The blends used were 25 mL amla with 75 mL grape (25/75 A/G), 50 mL amla with 50 mL grape (50/50 A/G), 25 mL kokum with 75 mL grape (25/75 K/G), and 50 mL kokum with 50 mL grape (50/50 K/G).

The results of the current study indicated that even at low dilutions, amla-grape and kokum-grape blends had high anti-hyperglycemic properties through the inhibition of key carbohydrate digestive enzymes α-amylase and α-glucosidase. This results in a slower breakdown of complex sugars into simpler forms thereby maintaining glucose homeostasis, which is specifically critical as soluble sugar content in grape and grape juice is considerably high and addition of amla and kokum can potentially reduce its glycemic load.

5.4.4. Angiotensin-I-converting enzyme (ACE) inhibitory activity

The ACE inhibitory activity was measured in a dose-dependent manner using undiluted, half-, and one-fifth dilutions of the samples, and the inhibitory activity was expressed in

percentages (%). The ACE inhibitory activity of the undiluted, half-diluted, and one-fifth diluted samples ranged from 98.7 % to 100 %, 92.1 % to 100 %, and 63.4 % to 100 % respectively, and statistical differences in ACE inhibitory activity were observed among the undiluted, half- and one-fifth diluted samples ($p<0.05$) (Figure 5.3). Among the undiluted samples, the amla, kokum, and amla-grape combinations (25/75 A/G and 50/50 A/G) had higher ACE inhibitory activity at 100% ($p<0.05$). Among the half-diluted samples, the amla, kokum, and amla-grape and kokum-grape combinations had higher ACE inhibitory activity ranging from 98.2 % to 100% when compared to the grape sample which had the lowest ACE inhibitory activity at 92.1% ($p<0.05$). Among the one-fifth diluted samples, the amla-grape blend (50/50 A/G) and amla sample had higher ACE inhibitory activity at 99.9% and 100% respectively ($p<0.05$), while the amla-grape and kokum-grape blends (25/75 A/G and 25/75 K/G) had the lowest ACE inhibitory activity at 65.8 % and 63.4 % respectively. In a previous study, LAB-fermented milk supplemented with amla extract had higher ACE inhibitory activity when compared to fermented milk without amla (Kanik et al, 2021). In another study using albino rat models, a poly-herbal formulation containing kokum was found to have high ACE inhibitory activity when compared to the control group (Ghelani, Patel, Gokani & Rachchh, 2014). These results indicated that the incorporation of amla and kokum as bioactive food ingredients can potentially improve the anti-hypertensive relevant benefits in food and beverage synergies. Therefore, amla-grape and kokum-grape blends with high antioxidant, anti-hyperglycemic, and anti-hypertensive functional qualities can be advanced for commercial optimization and utilization to develop health-focused functional beverages.

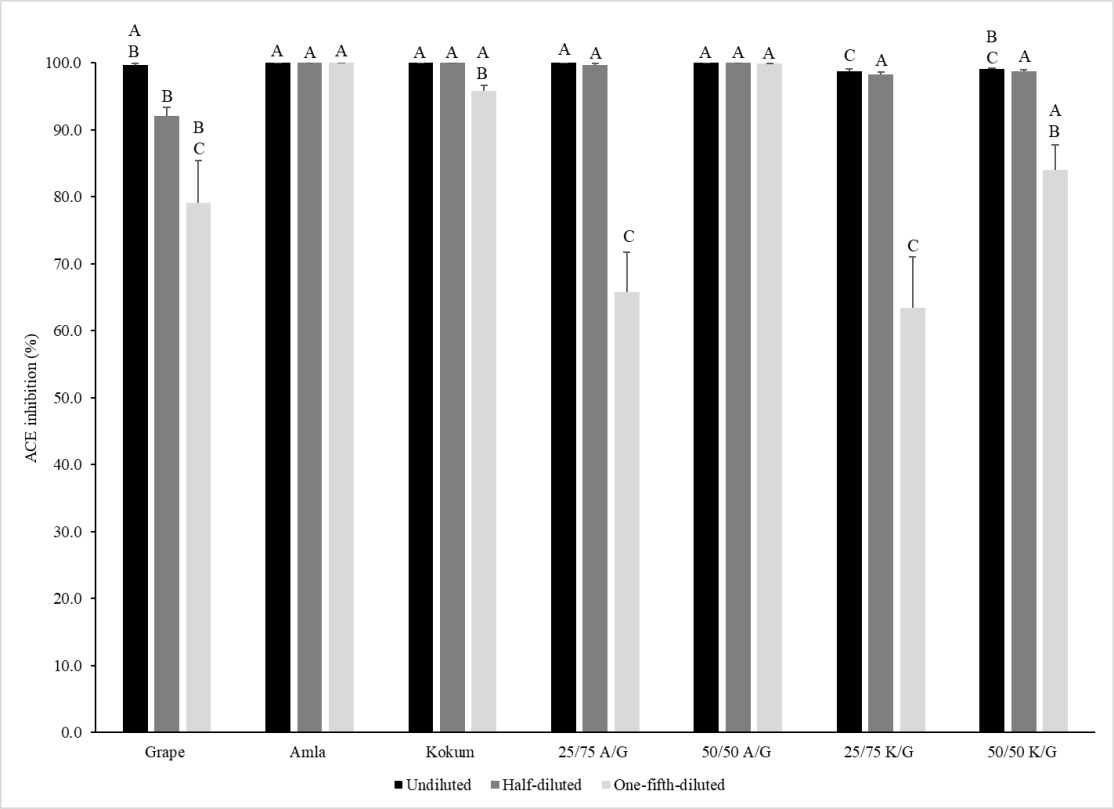


Figure 5.3. Angiotensin-I-converting enzyme (ACE) inhibitory activity (%) of the samples. Different letters indicate significant differences among the samples (p<0.05). The blends used were 25 mL amla with 75 mL grape (25/75 A/G), 50 mL amla with 50 mL grape (50/50 A/G), 25 mL kokum with 75 mL grape (25/75 K/G), and 50 mL kokum with 50 mL grape (50/50 K/G).

5.4.5. Antimicrobial activity

The antimicrobial activity of the individual fruit extracts and fruit synergies was measured against serovars of *Salmonella*, *Listeria* and *E. coli* and the minimal inhibitory concentration (MIC) was expressed in milligram gallic acid equivalents per milliliter (mg GAE/mL). The MIC of the samples against the *Salmonella*, *Listeria*, and *E. coli* serovars ranged from 0.33 to 6.75 mg GAE/mL, 0.17 to 2.20 mg GAE /mL, and 0.33 to 4.39 mg GAE /mL respectively, and statistical differences in MIC were observed among the samples except for the grape extract which did not show any antimicrobial activity towards the *Salmonella*, *Listeria*, and *E. coli* serovars (Table 5.3).

Among the *Salmonella* serovars, the kokum and kokum-grape blends (25/75 K/G and 50/50 K/G) had significantly lower MIC against *S. enterica* subsp. *enterica* serovar Enteritidis and *S. enterica* subsp. *enterica* serovar Typhimurium, with the MIC values ranging from 0.33 to 0.78 mg GAE /mL ($p<0.05$), while the amla-grape blends (25/75 A/G and 50/50 A/G) had the highest MIC values ranging from 5.86 to 6.75 mg GAE /mL. Among the *Listeria* serovars, the kokum and kokum-grape blends (25/75 K/G and 50/50 K/G) had significantly lower MIC against *L. monocytogenes* serovar 1/2a and *L. monocytogenes* serovar 1/2b, with MIC values ranging from 0.17 to 0.25 mg GAE /mL, while the amla-grape blends (25/75 A/G and 50/50 A/G) had the highest MIC, with values ranging from 1.46 to 2.19 mg GAE /mL. Among the *E. coli* serovars, the kokum and kokum-grape blends had significantly lower MIC against *E. coli* serovar O157:H7 and *E. coli* serovar O26:H11, with the values ranging from 0.33 to 0.49 mg GAE /mL ($p<0.05$), while the amla-grape blends had the highest MIC, with values ranging from 2.93 to 4.39 mg GAE /mL.

Table 5.3. Minimal inhibitory concentration (MIC) of samples expressed in milligram gallic acid equivalents per milliliter (mg GAE/mL).

Samples	**Minimal inhibitory concentration[1,2]**					
	Salmonella		*Listeria*		*E. coli*	
	S. enterica subsp. *enterica* serovar Enteritidis	*S. enterica* subsp. *enterica* serovar Typhimurium	*L. monocytogenes* serovar 1/2a	*L. monocytogenes* serovar 1/2b	*E. coli* serovar O157:H7	*E. coli* serovar O26:H11
Grape	NA	NA	NA	NA	NA	NA
Amla	3.31 ± 0.0c	2.48 ± 4.1b	0.83 ± 0.0c	1.65 ± 0.0ab	1.65 ± 0.0c	2.48 ± 4.1ab
Kokum	0.33 ± 0.0f	0.33 ± 0.0c	0.17 ± 0.0d	0.17 ± 0.0b	0.33 ± 0.0f	0.33 ± 0.0b
25/75 A/G	6.75 ± 0.0a	6.75 ± 0.0a	1.69 ± 0.0a	1.69 ± 0.0ab	3.38 ± 0.0a	3.38 ± 0.0ab
50/50 A/G	5.86 ± 0.0b	5.86 ± 0.0a	1.46 ± 0.0b	2.20 ± 0.7a	2.93 ± 0.0b	4.39 ± 1.5a
25/75 K/G	0.78 ± 0.0d	0.78 ± 0.0bc	0.20 ± 0.0d	0.20 ± 0.0b	0.39 ± 0.0e	0.39 ± 0.0b
50/50 K/G	0.49 ± 0.0e	0.49 ± 0.0bc	0.18 ± 0.1d	0.25 ± 0.0b	0.49 ± 0.0d	0.49 ± 0.0b

NA- no activity

[1] Mean value ± standard error

[2] Different letters in each column indicate significant differences among samples ($p<0.05$).

In an earlier study, lemon balm decoctions were found to have antimicrobial activity against gram negative and gram-positive bacteria with MIC values ranging from 0.20 to 1.50 mg mL^{-1} (Carocho et al, 2015). In another study, Fucoidan, a polysaccharide from brown algae was added to apple juice and the resulting functional beverage showed antimicrobial activity against *L. monocytogenes* and *S. enterica* serovar Typhimurium (Poveda-Castillo, Rodrigo, Martínez & Pina-Pérez, 2018). In the current study, no antimicrobial activity was found in 100 % grape extracts, however the addition of amla and kokum extracts resulted in antimicrobial activity of the amla- and kokum- grape functional beverages against the *Salmonella*, *Listeria*, and *E. coli* serovars that were tested.

5.5. Conclusion

Synergizing phenolic rich fruits in widely consumed plant-based foods and beverages can offer exciting strategies to improve their food safety relevant antimicrobial and human health protective functional qualities. Under-utilized and traditionally consumed fruits such as amla and kokum have health protective benefits in terms of *in vitro* antioxidant, anti-hyperglycemic, and anti-hypertensive properties. Furthermore, these fruits also display food safety-relevant benefits in terms of antimicrobial activity against the foodborne pathogens *Salmonella*, *Listeria*, and *E. coli*. In this study, integration of these fruits in grape juice improved the anti-hyperglycemic and anti-hypertensive properties. Additionally, these fruits provided value-added antimicrobial properties to the grape juice against common foodborne pathogens, which has significant relevance in food safety applications. Therefore, amla-grape, and kokum-grape synergies can be targeted in future animal models or clinical- based studies to validate the outcomes of this *in vitro* screening and optimization. Such fruit synergies have wider commercial application, specifically in the emerging health-focused food and beverage industry worldwide.

CHAPTER 6. OZONE ELICITED PHENOLIC BIOACTIVES IN GRAPES AND HEALTH RELEVANT SCREENING TARGETED FOR TYPE 2 DIABETES USING *IN VITRO* ASSAY MODELS

6.1. Abstract

Grapes and grape derived products such as beverages are excellent sources of phenolic antioxidants with human health relevant medicinal properties. These phenolic bioactives with antioxidant function can be screened and targeted for the dietary management of oxidative stress-linked chronic diseases, such as early stages of type 2 diabetes. The aim of this study was to enhance phenolic antioxidant-linked medicinal properties in grapes (*Vitis vinifera* L.) through pre-harvest ozone treatment. Further the goal was to improve its bioactive functionality that can be potentially targeted for post-prandial glucose management through dietary intake. In this study, eight grape cultivars were treated with ozone at pre-harvest stage. Following harvest, cold water extracts of grapes were evaluated for total soluble phenol content, phenolic profile, antioxidant capacity, and α-amylase, and α-glucosidase enzyme inhibitory activities using *in vitro* assay models. Overall, all red grape cultivars and one white grape cultivar (Vignoles) showed high total soluble phenolic content and antioxidant activity when compared with other white grape cultivars. Ozone treatment significantly enhanced total soluble phenolic content in Vignoles and Frontenac grape cultivars. High α-amylase and high α-glucosidase enzyme inhibitory activities were also found in all grapes with Vignoles having the highest inhibitory activity even at one-fifth dilution. In this study, Vignoles, St Croix, Frontenac, De Chaunac, and Marechal Foch grape cultivars showed high phenolic bioactive-linked functionalities in *in vitro* assay models and have the potential to be targeted as medicinally active foods and ingredients for management of early stages type 2 diabetes. Further, these results suggest that stress

induction with ozone at preharvest stage has potential to improve such phenolic bioactive-linked medicinal properties in specific grape cultivars like Vignoles and Frontenac.

6.2. Introduction

Grapes are grown worldwide and are consumed either as a table fruit or as a processed non-alcoholic or alcoholic beverage (wine). The global production of table grapes is expected to be around 21.0 million metric tons with the production at 984,000 tons in the United States (USDA, 2016). Among the 59 cultivars of commercial grapes that are grown and consumed, some of the popular cultivars in the United States are St Croix, Edelweiss, Frontenac, La Crescent, Marquette, Marechal Foch, St Pepin and Concord (USDA, 2011). The popularity of grapes among consumers is growing rapidly due to its flavor, taste, and diverse human health relevant medicinal properties. Grapes are a rich source of human health relevant phenolic compounds which have diverse medicinal values and varies widely with type of grape (white and red), genotype, geographical origin, oenological practices, and exposure to biotic or abiotic stresses (Frémont, 2000). The main difference between red and white grape varieties is that red grapes have higher anthocyanin content than white grapes. However, in both cases, grape skins are a rich source of anthocyanins, hydroxycinnamic acids, flavanols and flavonol glycosides (Kammerer, Claus, Carle & Schieber, 2004; Ramchandani, Chettiyar & Pakhale, 2010).

The human health relevant phenolic compounds of grapes are secondary metabolites that have diverse roles in plant growth, lignification, pigmentation, pollination and biotic and abiotic stress tolerance (Fraga et al, 2010; Frémont, 2000). Further, these same phenolic compounds are effective antioxidants and can be utilized in dietary strategies to counter oxidative-stress linked diseases such as early stages of type 2 diabetes and cardiovascular diseases (Leonard et al, 2003). Phenolic metabolites of grapes can quench super oxide radical O2 - and hydrogen peroxide

H2O2 and can also inhibit prostaglandin production which in turn reduces inflammation (Martinez & Moreno, 2000). Phenolics also have a dual role in improving mitochondrial function and providing protection against diet induced obesity and insulin resistance (Lagouge et al, 2006). The major mechanisms by which phenolics can quench free radicals include non-specific (electron transfer) and specific interactions based on structural conformation of phenolic acids which allow these compounds to interact with target proteins, including enzymes, membrane proteins, transcription factors and membrane receptors that are involved in countering chronic oxidative stress (Fraga et al, 2010). Grapes also have a low glycemic index and when consumed have shown to reduce hyperglycemia, and improve pancreatic β cell function (Zunino, 2009). Further, previous research has found that grapes and beverages derived from grapes have inhibitory activity against enzymes like α-amylase and αglucosidase and thus has the potential to reduce postprandial hyperglycemia (Hogan et al, 2010; Kwon, Apostolidis & Shetty, 2008). Therefore, grapes and grape derived non-alcoholic beverages can be screened and targeted as medicinally active plant and food source for the dietary management of early stages of type 2 diabetes and associated complications.

Several strategies involving plants endogenous defense responses against biotic and abiotic stresses (UV-B, Ozone, and chemical) has been identified and utilized to improve phenolic biosynthesis and antioxidant activity in grapes during pre- and postharvest stages (Gonzalez-Barrio et al, 2006; Portu, López-Alfaro, Gómez-Alonso, López & Garde-Cerdán, 2015; Ruiz-Garcia et al, 2012; Wang et al, 2013). The major goals of these previous studies were to enhance phenolic biosynthesis to improve biotic and abiotic stress tolerance and fruit quality of grapes. But same strategy involving stress-induction can be used to improve human health relevant phenolic antioxidant profile and subsequent medicinal properties in grapes and in foods

derived from grapes. Therefore, the major aim of this study was to improve health relevant phenolic bioactives and antioxidant activity in eight grape cultivars (both red and white) through ozone treatment and to compare it with untreated grapes. Further, the specific goal was to evaluate the potential impact on phenolic bioactive enrichment in grapes by screening for human health benefits using in vitro assay models for targeting dietary management of early stages of type 2 diabetes.

6.3. Materials and Methods

6.3.1. Materials

Eight grape cultivars were used in this study that included both red and white grapes. The red grape cultivars included Marechal Foch, Frontenac, De Chaunac and St Croix while the white grape varieties were La Crescent, Edelweiss, Brianna and Vignoles. Porcine pancreatic alpha–amylase (EC 3.2.1.1), rat intestinal alpha–glucosidase (EC 3.2.1.20), 2, 2-diphenyl-1-picrylhydrazyl (DPPH), and phenolic acid standards were purchased from Sigma Chemical Co (St. Louis, MO).

6.3.2. Treatments

The field experiments with red and white grape cultivars were carried out at Mac's Creek Winery & Vineyards, Lexington, Nebraska in 2014. Each of the grape cultivars were arranged into ozone and untreated (control) groups. The sample groups were selected by row blocks which consisted of three rows for each group having the control group in between the ozone group in order to minimize drift or overlap between the groups. For the ozone treatment, ozone was sprayed onto the grapes at the rate of 25 gallons per acre, once every 10 days. A total of six rounds of ozone spraying were done with the help of an air blast mist sprayer. The treated

(ozone) and untreated grapes were stored and transported to North Dakota State University at -20°C prior to phenolic bioactive and functionality analysis.

6.3.3. Sample extraction

For the cold-water extraction procedure, 40 g of each of the grape cultivars were weighed and each sample was then homogenized with 100 mL of distilled water using a Waring blender for 5 minutes at low speed. The homogenized samples were then centrifuged at 8,500 rpm for 20 minutes after which the supernatant was collected and then re-centrifuged at the same rpm for 15 minutes. The cold-water extracts were stored at 4°C during the period of the biochemical analysis.

6.3.4. Total soluble phenolic content

A modified protocol was used to measure the total soluble phenolic content as described earlier (Shetty et al, 1995). In this protocol, 0.5 mL of sample extracts were added to their respective test tubes. For the control 0.5 mL of distilled water was added instead of sample. This was followed by the addition of 0.5 mL of distilled water, 1 mL of 95% ethanol to all the tubes. Then 0.5 mL of 50% (v/v) Folin- Ciocalteu reagent was added to each tube. This was followed by the addition of 1 mL 5% sodium carbonate. The contents in each tube were mixed using a vortex and were incubated under dark conditions for 60 minutes at room temperature. After incubation the absorbance was checked using a spectrophotometer (Genesys, Thermo Fisher Inc.) set at 725nm. The absorbance values were converted to total soluble phenolic content and expressed in milligram per gram of fresh weight with the help of standard curve that was established using different concentrations of gallic acid in 95% ethanol.

6.3.5. Antioxidant capacity through DPPH (2, 2, diphenyl1-picrylhydrazyl) radical scavenging assay

For this assay as described earlier (Kwon et al, 2006), 1.25 mL of 60 mM DPPH (in 95% ethanol) was added to 0.25 mL of sample. The tubes were gently tapped to ensure mixing and were incubated for 5 minutes, after which they were centrifuged for 1 minute at 13,000 rpm and the absorbance of the supernatant was measured at 517nm using a UV spectrophotometer (Genesys). Each sample had a corresponding control which contained 0.25 mL of 95% ethanol. Based on the absorbance readings, the percentage of inhibition was calculated using the formula:

$$\% \text{ inhibition} = \frac{\text{Absorbance}^{\text{control}} - \text{Absorbance}^{\text{extract}}}{\text{Absorbance}^{\text{control}}} \times 100$$

6.3.6. Alpha-glucosidase inhibitory activity

The assay followed here was based on the protocol as described earlier (Kwon et al, 2006). A total of 10 µL, 25 µL and 50 µL of each sample extract was pipetted into 96 well micro titer plates and used as undiluted, half diluted and one-fifth diluted extracts respectively. The half dilution and one-fifth dilutions were made up to a total of 50 µL in volume by adding 25 µL and 40 µL of 0.1M potassium phosphate buffer (pH 6.9), respectively. Each sample extract had a corresponding control of 50 µL of phosphate buffer. Finally, the volume in all the wells was made up to 100 µL by the addition of 50 µL of phosphate buffer in each well including the controls. Then 100 µL of 0.1M potassium phosphate buffer (pH 6.9) containing α glucosidase enzyme (1 U/mL) was added to each well and incubated at 25°C for 10 minutes. After this 50 µL of 5 mM p-nitrophenyl-αD- glucopyranoside solution in 0.1 M phosphate buffer (pH 6.9) was added to each well at timed intervals. The reaction mixtures were then incubated at 25° C for 5 min. Absorbance readings were taken before (0 minute) and after (5 minute) incubation period

using a micro plate reader (Thermomax, Molecular device Co., Virginia, USA) set at 405nm. The percentage of α glucosidase inhibitory activity was calculated based on the absorbance readings of the sample extract and their respective controls using the formula:

$$\% \text{ inhibition} = \frac{(Abs^{control}\ 5min - Abs^{control}\ 0\ min) - (Abs^{extract} 5min - Abs^{extract} 0\ min)}{(Abs^{control}\ 5min - Abs^{control}\ 0\ min)} \times 100$$

6.3.7. Alpha-amylase enzyme inhibitory activity

The assay followed here was based on the protocol as described earlier (Kwon et al, 2006). Similar to α-glucosidase, undiluted, half-diluted and one-fifth diluted extract samples were used for this assay. The dilutions were done using distilled water. The buffer used was 0.1M sodium phosphate (pH 6.9) with 0.006M sodium chloride added to it. Around 500 µL of each sample extract was added to test tubes while the control tubes had 500 µL of buffer only. Additionally, each sample extract had a corresponding sample blank tube which contained 500 µL of the sample extract. Then of 500 µL of porcine pancreatic amylase (0.5 mg/ mL buffer) was added to all the tubes except for the sample blank tubes and the tubes were incubated at 25°C for 10 minutes. After incubation, 500 µL of 1% starch (1 g/100 mL buffer) was added to all the tubes and incubated for 10 minutes. The reaction was then stopped by the addition of 1 mL of 3, 5 dinitro salicylic acid and the tubes were placed in a boiling water bath for 10 minutes after which the tubes were taken out and cooled to room temperature. The reaction mixture in the tubes was then diluted by adding 10 mL of distilled water and the absorbance was measured at 540 nm. The percentage of inhibition of α amylase enzyme was calculated using the formula:

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

6.3.8. HPLC analysis of phenolic profiles

Two milliliter of fruit extracts were filtered through a 0.2 µm filter and then centrifuged for 5 min. A 5 µL volume of sample was injected using Agilent ALS 1200 auto sampler into Agilent 1260 series HPLC (Agilent Technologies, Palo Alto, CA equipped with DAD 1100 diode array detector). The solvents used for gradient elution were (A) 10 mM phosphoric acid (pH 2.5) and (B) 100% methanol. The methanol concentration was increased to 60% for the first 8 min and to 100% over the next seven minutes, 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 was used was Agilent Suppelco SB-C18 250 x 4.6 mm i.d., with packing material of 5 µm particle size at a flow rate of 0.700 mL/min at ambient temperature. During each run the chromatogram was recorded at 254 nm and 306 nm and integrated using Agilent Chemstation enhanced integrator. Pure standards of gallic acid, protocatechuic acid, catechin, chlorogenic acid, caffeic acid, quercetin derivatives, resveratrol and p-coumaric acid (purchased from Sigma Chemical Co., St. Louis, MO) in 100% methanol were used to calibrate the standard curve and retention times.

6.3.9. Statistical analysis

The complete biochemical analysis was repeated four times. Analysis at every time point from each experiment was carried out in triplicates. Means, standard errors, and standard deviations were calculated from replicates within the experiments and analyses were done using Microsoft Excel XP. The data were analyzed with analysis of variance (ANOVA) of Statistical Analytical Software (SAS version 9.4; SAS Institute, Cary, NC). Differences among cultivars and treatments and cultivar × treatments interactions were determined by the Tukey's least mean square test at the 0.001 probability level.

6.4. Results

6.4.1. Total soluble phenolic content

The total soluble phenolic content of grape extracts was determined using Folin-Ciocalteau method. Overall, significantly higher (p<0.001) total soluble phenolic content was observed in red grapes when compared to white grape cultivars, with exception in Vignoles (Figure 6.1). Significant differences (p<0.001) in total soluble phenolic content between cultivars, treatments, and cultivar × treatment interactions were found in this study (Table 6.1). Ozone treatment was most effective for the red grape cultivar Frontenac, which had significantly higher total soluble phenolic content of 1.37 mg GAE/ g F.W. (p<0.001) when compared to the untreated grapes.

Table 6.1. Analysis of variance (ANOVA) for total soluble phenolic content (TSP), antioxidant activity (AA), α-amylase, and α-glucosidase inhibitory activity between cultivars (eight), treatments (two), and cultivar × treatment interactions.

		TSP	**AA**	**α-amylase inhibitory activity**			**α-glucosidase inhibitory activity**		
	df			Undiluted	1:2 dilution	1:5 dilution	Undiluted	1:2 dilution	1:5 dilution
Cultivar	7	***	***	***	***	***	***	***	***
Treatment	1	***	***	*	***	*	**	***	***
Cultivar x treatment	7	***	***	NS[a]	*	**	***	***	***

*p<0.05; ** p<0.01; *** p<0.001; [a] Not significant

Similarly, among the white grape cultivars, ozone treatment enhanced the total soluble content of the Vignoles cultivar nearly two-fold (1.83 mg GAE/g F.W) which was significant when compared to untreated grapes (p<0.001).

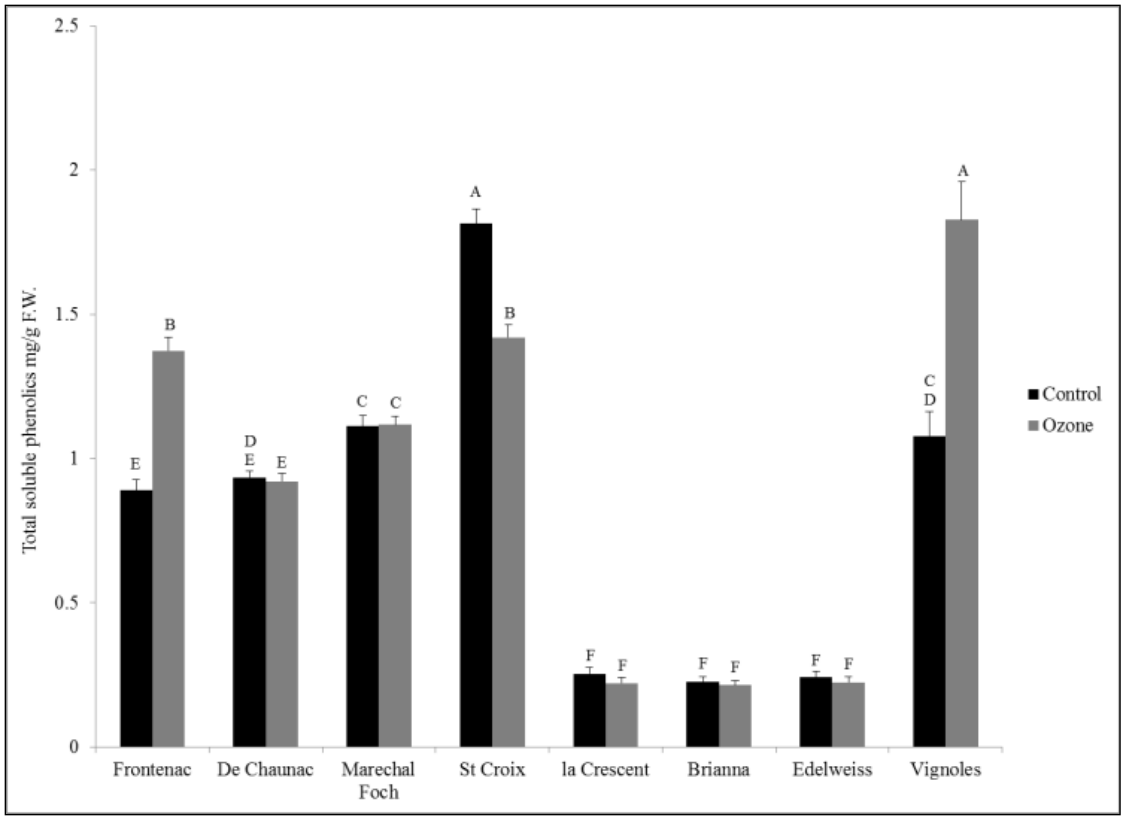


Figure 6.1. Total soluble phenolic content (mg GAE/ g F.W.) of eight red and white grape cultivars with two different pre-harvest treatments (control and ozone). Different capital letters represent significant differences between cultivar × treatment interactions at $p<0.001$ probability levels.

Among the red grape cultivars, untreated St Croix had the highest total soluble phenolic content of 1.81 mg GAE/ g F.W., which was significantly higher ($p<0.05$) than the ozone treated grape. White grape cultivars such as La Crescent, Brianna, and Edelweiss had lower baseline total soluble phenolic content ranging from 0.19 to 0.27 mg GAE/g F.W. Ozone treatment also did not result in any improvement in total soluble phenolic content in these white grape cultivars.

6.4.2. Total antioxidant activity

The total antioxidant activity of the grape cultivars was measured by their ability to quench the DPPH radical. Similar to the total soluble phenolic content, significant differences ($p<0.001$) in total antioxidant activity between cultivars, treatments, and cultivar × treatment

interactions were also observed (Table 6.1). In general, the antioxidant activity was significantly higher in the red grape cultivars (80-90% DPPH inhibition) when compared to the white grape cultivars (12-25% DPPH inhibition) (Figure 6.2). However, among the white grape cultivars, Vignoles had very high antioxidant activity (91% DPPH inhibition) which is in the same range as red grape cultivars. Among red grape cultivars, De Chaunac with ozone treatment had significantly (p<0.001) higher antioxidant activity when compared to the untreated grapes. On the contrary, the untreated Marechal Foch and Frontenac cultivar had significantly higher antioxidant activity (88%) when compared to the ozone treated grapes (p<0.001).

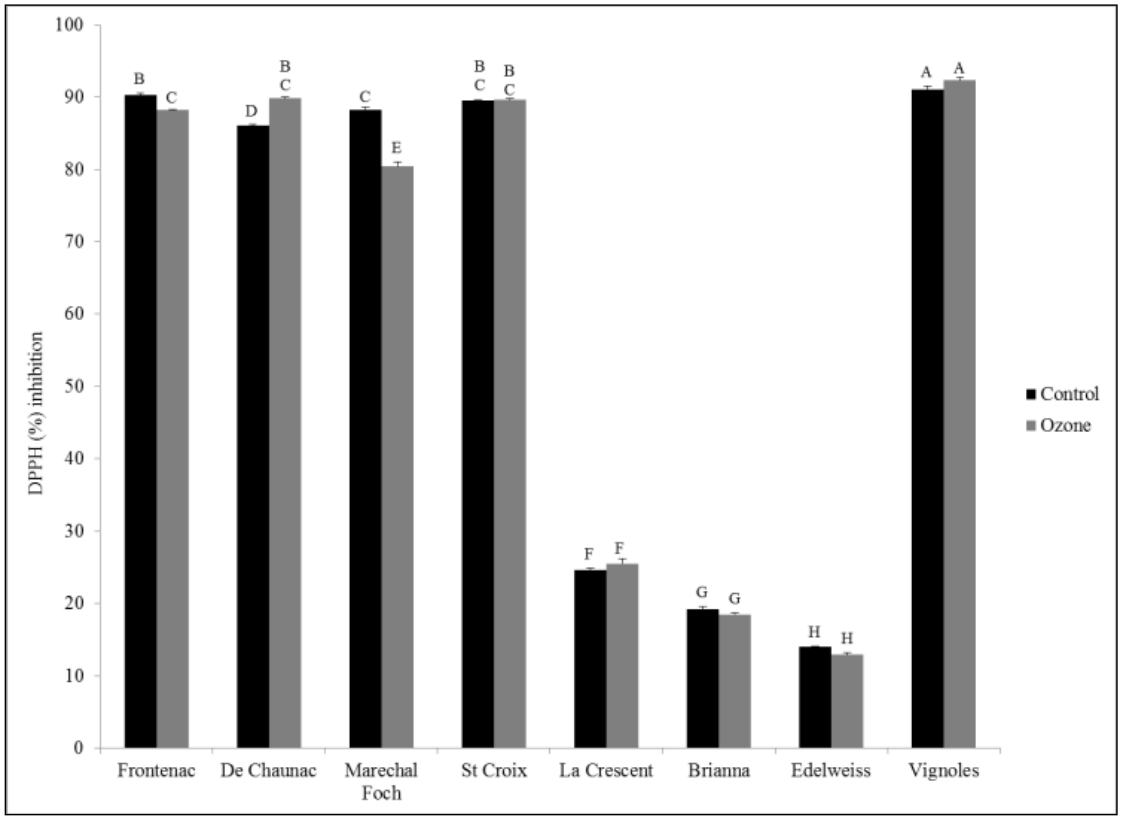


Figure 6.2. Total antioxidant activity (DPPH radical scavenging % inhibition) of eight red and white grape cultivars with two different pre-harvest treatments (control and ozone). Different capital letters represent significant differences between cultivar × treatment interactions at p<0.001 probability level.

6.4.3. Alpha-amylase inhibitory activity

To determine the role of grape bioactives for improving glucose metabolism, α-amylase inhibitory activity of all grape cultivars with and without ozone treatment was determined through model *in vitro* assay. Significant differences ($p<0.001$) in α-amylase inhibitory activity between cultivars were observed in all dilutions (Table 6.1). In undiluted samples, no significant differences between cultivar × treatment interactions were observed, while in half ($p<0.05$) and one-fifth ($p<0.01$) dilutions, significant differences between cultivar × treatment interactions were observed (Table 6.1). In this study all grape cultivars (both red and white grapes) showed very high αamylase inhibitory activity (70-100% inhibition) with significant dose responses (Figure 6.3 A, B, C).

Among all cultivars, Vignoles exhibited very high α-amylase inhibitory activity even at the one-fifth dilution (88%), which was significantly higher when compared with all other red and white grape cultivars ($p<0.001$). Ozone treated Vignoles grape showed higher trend of α-amylase inhibitory activity in undiluted and half diluted sample but was not statistically significant when compared with untreated grapes. Ozone treated Frontenac grape showed significantly higher α-amylase inhibitory activity when compared with untreated grapes ($p<0.001$) at half dilution. At one-fifth dilution untreated Edelweiss had significantly higher α-amylase inhibitory activity when compared to ozone treated grapes ($p<0.001$).

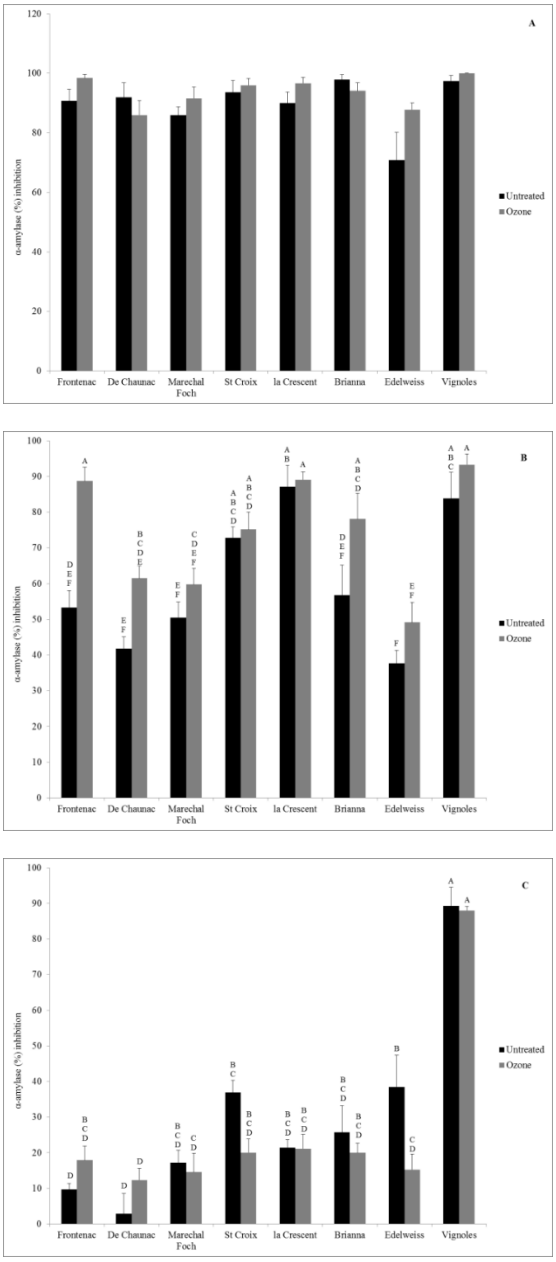


Figure 6.3. Alpha-amylase enzyme inhibitory activity (% inhibition) with three different doses (undiluted A, half dilution B, and one-fifth dilution C) of eight red and white grape cultivars with two treatments (control and ozone). Different capital letters represent significant differences between cultivar × treatment interactions at $p<0.05$ and $p<0.01$ probability levels. No significant differences in α-amylase inhibitory activity between cultivar × treatment interactions were observed in undiluted samples.

6.4.4. Alpha-glucosidase inhibitory activity

The inhibitory activity of α-glucosidase which is a key enzyme involves in human glucose metabolism was also determined using *in vitro* assay model. Significant differences in α-glucosidase inhibitory activity between cultivar × treatment interactions ($p<0.001$) were observed in all dilutions (Table 6.1). Similar to α-amylase inhibitory activity, all red and white grape cultivars had very high α-glucosidase inhibitory activity in this study (Figure 6.4 A, B, C).

Like soluble phenolic content, antioxidant activity, and α-amylase inhibitory activity, white grape cultivar Vignoles had very high α-glucosidase inhibitory activity even at one-fifth dilution (99%), which was significantly higher than other red and white grape cultivars ($p<0.001$). Among red grape cultivars, higher α-glucosidase inhibitory was found in Marechal Foch, and St. Croix ($p<0.001$). Positive correlation between total soluble phenolic content and α-glucosidase inhibitory activity was observed in all grape cultivars. Vignoles, St Croix, Frontenac, De Chaunac, and Marechal Foch showed high α-glucosidase inhibitory activity, while La Crescent, Brianna, and Edelweiss had moderate αglucosidase inhibitory activity at one-fifth dilution. Untreated Edelweiss grape resulted in significantly higher α-glucosidase inhibitory activity when compared to ozone treated Edelweiss grape at undiluted and at one-fifth dilution ($p<0.001$).

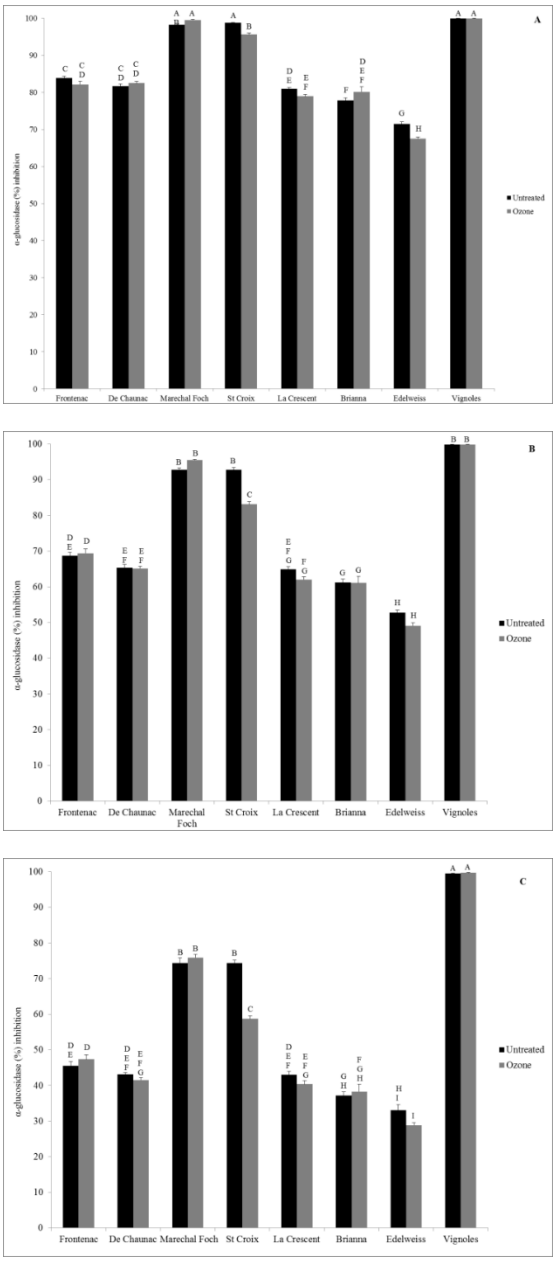


Figure 6.4. Alpha-glucosidase enzyme inhibitory activity (% inhibition) with three different doses (undiluted A, half dilution B, and one-fifth dilution C) of eight red and white grape cultivars with three different pre-harvest treatments (control and ozone). Different capital letters represent significant differences between cultivar × treatment interactions at $p<0.001$ probability level.

6.4.5. Phenolic profile through HPLC

High performance liquid chromatography (HPLC) analysis was carried out to determine phenolic acid content in red and white grape cultivars. In this study, catechin and gallic acid were the two major phenolic acids found in red and white grape cultivars except for Brianna and Edelweiss, whereas only catechin was detected (Table 6.2).

Table 6.2. Concentrations (µg/mL) of two major phenolic compounds (catechin and gallic acids) determined using high performance liquid chromatography (HPLC) method of eight grape cultivars with two treatments (control and ozone).

Cultivar	**Treatment**	**Catechin (µg/mL)**	**Gallic Acid (µg/mL)**
Frontenac	Control	13.11	4.99
	Ozone	12.02	5.02
De Chaunac	Control	11.63	4.88
	Ozone	12.92	5.52
Marechal Foch	Control	14.07	4.83
	Ozone	13.07	5.32
St Croix	Control	12.77	6.19
	Ozone	11.85	5.75
La Crescent	Control	5.97	0.55
	Ozone	8.42	0.55
Brianna	Control	6.67	ND[a]
	Ozone	6.21	ND
Edelweiss	Control	5.14	ND
	Ozone	5.05	ND
Vignoles	Control	16.60	6.92
	Ozone	16.69	6.86

[a] Not Detected

In all red and white grape cultivars concentrations of catechin were 2-3 folds higher than the gallic acid concentrations. Similar to total soluble phenolic content, the catechin concentrations in white grape cultivars such as La Crescent, Brianna, and Edelweiss were significantly lower compared to all red grape cultivars and Vignole white grapes. No significant differences in catechin and gallic acid concentrations were found with ozone treatment. A higher concentration of catechin was found in ozone treated La Crescent grape when compared with untreated grape.

6.5. Discussion

Rich and diverse phenolic bioactive profiles in grape offer significant health benefits and can be targeted as medicinally active plant food to manage oxidative stress-induced diseases such as non-communicable chronic diseases, especially for type 2 diabetes and associated macro-vascular complications such as cardiovascular disease (Leonard et al, 2003; Yang & Xiao, 2013). The medicinal properties of grape and in grape derived beverages are mostly due to the phenolic bioactive profile and high antioxidant activity (Georgiev, Ananga & Tsolova, 2014). But the phenolic profile as well as antioxidant activity varies widely among different red and white grape cultivars and results in varying quality of fruit as well as alcoholic and non-alcoholic beverages (Frémont, 2000). Furthermore, the growing conditions, horticultural practices, environment, and post-harvest storage also dictate the health relevant bioactive profile of the grape and can lead to heterogeneous fruit and wine quality. In this study, significant differences in phenolic antioxidant-linked functionalities for potential dietary management of early stages of type 2 diabetes between red and white grape cultivars were observed. But among all grape cultivars, Vignoles (white grape cultivar) showed very high phenolic bioactive-linked functionalities for potentially improving glucose metabolism based on indications from in vitro assays when

compared to all other white and red grape cultivars. Positive correlation between total soluble phenolic content, antioxidant activity, α-glucosidase, and α-amylase enzyme inhibitory activity was also observed in Vignoles.

Overall, higher total soluble phenolic content and higher berry antioxidant capacity was observed in red grape cultivars when compared with white grape cultivars. Lower concentration of anthocyanin and other phenolic acids were observed in white grapes and in white wines in previous studies (Kammerer et al, 2004; Kwon et al, 2008; Vinson & Hontz, 1995). But white grape cultivar, Vignoles showed similar level of total soluble phenolic content and higher antioxidant activity like other red grape cultivars evaluated in this study. The most interesting finding of this study was the significant increase in total soluble phenolic content in ozone treated Vignoles grape. Increased concentrations of several anthocyanins and flavonols were observed in grapes with foliar application of phenylalanine and urea in grape vines (Portu et al, 2015). Similarly, higher accumulation of viniferins was found in white grape 'Superior' with gas ozone treatments (Gonzalez-Barrio et al, 2006). Although ozone treatment did not result in increasing soluble phenolic content in all grape cultivars, this strategy might be effective for selected cultivars such as white grape Vignoles and red grape Frontenac. Therefore, from the findings of this study we can hypothesize that the response to stress-induction through treatments such as ozone for improving phenolic antioxidant is very much dependent on grape genotypes along with other phenotypic factors. Overall, all red grape cultivars and Vignoles white grape had very high antioxidant (DPPH radical scavenging activity) activity and thus showed potential to be utilized as dietary antioxidants to counter oxidative stress-linked chronic diseases such as type 2 diabetes. Higher antioxidant activity (DPPH) of crude polyphenolic extracts of seedless and seeded Indian grapes was also found in a previous study (Ramchandani et al, 2010).

Similar to total soluble phenolic content and total antioxidant activity, higher inhibitory activity of enzymes such as α-glucosidase, and α-amylase was also observed in all red and white grape cultivars (without dilution). Although Vignoles white grape had highest α-glucosidase, and α-amylase enzyme inhibitory activities, these did not improve with ozone treatment. One possible reason for not finding significant differences with stress induction is due to very high baseline value of both α-glucosidase, and α-amylase enzyme inhibitory activities in this white grape cultivar. Even after one-fifth dilution Vignoles had high α-glucosidase, and α-amylase inhibitory activities. Similarly, other red grape cultivars such as St. Croix, Marechal Foch and Frontenac showed high α-glucosidase inhibitory activity. Improvement of αamylase inhibitory activity was found in ozone treated Frontenac grape and it positively correlated with total soluble phenolic content. Therefore, the high α-amylase enzyme inhibitory activity of Frontenac and Vignoles might be due to their high soluble phenolic content. Therefore, Vignoles white grape cultivar along with other red grape cultivars such as St. Croix, Marechal Foch and Frontenac can be potentially targeted for dietary management of early stages of type 2 diabetes as reflected from these in vitro assay models. Higher α-glucosidase inhibitory activity was observed previously in red wines (Kwon et al, 2008). Similarly, antidiabetic properties in polyphenolic extracts of red wine were also observed in rat model (Al-Awwadi et al, 2004). High phenolic antioxidant content and high enzyme inhibitory activity in grapes relevant for improving glucose metabolism would allow this fruit to be targeted as a medicinally active plant and as a dietary choice to improve post-prandial glucose metabolism. Not only is this relevant for maintaining glucose homeostasis but due to high and rich antioxidant profiles, grape and grape derived food products can be targeted to counter chronic oxidative stress and inflammation associated with type 2 diabetes.

In this study, we only found catechin and gallic acid in all red and white grape cultivars. Previous research reported catechin, gallic acid, quercetin, coumaric acid, caffeic acids and resveratrol in different grape cultivars (Kennedy, 2008; Pastrana-Bonilla, Akoh, Sellappan & Krewer, 2003). Use of different organic solvents, extraction methods, concentrations, and HPLC protocols might have resulted in the detection of other phenolic acids in these previous studies. Catechin and gallic acid were two major phenolic acids observed in this study and are known to have significant health benefits when consumed as part of the diet (Frankel, Waterhouse & Teissedre, 1995). White grape cultivars such as La Crescent, Brianna, and Edelweiss had lower concentrations of catechin, and this observation positively correlated with phenolic antioxidant linked functionalities in these cultivars. Therefore, the bioactive functionalities relevant for dietary management of type 2 diabetes might be due to the concentration and compositions of phenolic acids in red and white grape cultivars. Overall, this study showed that red grape cultivars and white grape cultivar such as Vignoles with higher phenolic antioxidant-linked functionalities are an ideal choice to be targeted as medicinally active food source for dietary management of early stages type 2 diabetes and to potentially mitigate associated oxidative stress-linked complications. The impact of stress induction to improve phenolic antioxidant-linked functionalities in grape cultivars depends on their genetic profile and variability in the growing conditions.

6.6. Conclusion

Grapes are one of the most popular fruits in the world and are rich in human health relevant medicinal properties. This rich composition of phenolic-linked medicinal properties in grape potentially offers significant health benefits and can be targeted to manage oxidative stress-linked chronic diseases such as type 2 diabetes. Different strategies involving endogenous

defense response pathways of plants have been developed to improve phenolic antioxidant profile in fruits and other food crops. Ozone treatments provide significant protection against biotic and abiotic stresses in plants including grapes. Same strategy can be used to improve medicinal properties in fruits during pre- and postharvest stages. In this study we observed very high phenolic antioxidant-liked functionalities relevant for dietary management of type 2 diabetes in red and white grape cultivars using in vitro assay models. Improvement of these phenolic antioxidant-linked functionalities with ozone treatment was also observed in selected red and white grape cultivars such as Frontenac and Vignoles. Among all red and white grape cultivars, Vignoles had very high phenolic antioxidant-linked functionalities and could be used for future animal, clinical and epidemiological studies targeting to improve human glucose metabolism. This present study provides initial insights and strong foundation to utilize stress-induced strategy such as ozone treatment to improve human health relevant medicinal properties in selected red and white grape cultivars. Future studies with different cultivars, other stress treatments, and under different locations are needed to validate the findings of this study and to advance this innovative concept. This innovative strategy will help grape growers and wineries to improve medicinal properties and shelf-life of grape fruits and foods and beverages derived from grapes.

CHAPTER 7. COMPARING PHENOLIC-LINKED ANTIOXIDANT, ANTIHYPERGLYCEMIC, AND ANTIHYPERTENSIVE PROPERTIES OF MILLED AND UNMILLED FLAXSEED

7.1. Abstract

Common flaxseed is used in a wide range of food processing applications and has diverse human health-related benefits due to its rich phytochemical profile. Therefore, targeting flaxseed for dietary support strategies to counter chronic oxidative stress, hyperglycemia, and hypertension has health protective dietary relevance. Based on this rationale, the aim of this study was to investigate the effect of post-harvest milling on the phenolic linked antioxidant and anti-hyperglycemic properties of different flaxseed cultivars, milled and processed sources using *in vitro* screening strategy. A processed lignan derived from flaxseed was also used for comparison. Milled and unmilled flaxseed from commercial sources (Bob's Red mill Premium and Bob's Red Mill Organic) and two locally grown cultivars (Linott and Pembina) from organic cultivation system, and a dried lignan powder were extracted in cold water or with 12% ethanol. Total soluble phenolic content, phenolic profile, antioxidant activity, antihyperglycemic-relevant α-amylase, and α-glucosidase inhibitory, and antihypertension-relevant angiotensin-I-converting (ACE) enzyme inhibitory activities were determined using *in vitro* assay models. Though aqueous extracts of milled flaxseed had higher total soluble phenolic content and moderate antioxidant activity, the anti-hyperglycemic properties of flaxseed were higher in the ethanol extracts. Moderate anti-hypertensive relevant ACE inhibitory activity was also found in lignan derived from flaxseed. Therefore, aqueous extracts of milled flaxseeds with high phenolic-linked antioxidant activity can be combined synergistically with other food ingredients, while ethanol extracts of flaxseeds and processed lignan can be utilized in nutraceutical applications to counter

chronic inflammation, hyperglycemia, and hypertension, which are common health risks associated with type 2 diabetes and other non-communicable chronic diseases.

Keywords: Antioxidant, Flaxseed, Hypertension, Type 2 diabetes, Phenolics

7.2. Introduction

Flaxseed (*Linum usitatissimum* L) is utilized as an oilseed and fiber crop and is currently gaining interest as a functional food and food ingredient source due to its rich health and nutritionally relevant bioactive profile (Toure & Xueming, 2010; Rabetafika et al, 2011). Canada is the largest producer of flaxseed at 38% of total global production followed by China, United States, and India (Toure & Xueming, 2010; Kasote, 2013). Flaxseed has good nutritional profile due to its high alpha-linolenic acid and dietary fiber content, as well as high quality protein profile (Rabetafika et al, 2011). Flaxseed contains 40 to 45% oil, 20-25% fiber, 20-25% proteins and 1% lignans (Rabetafika et al, 2011). Additionally, flaxseeds are good source of phenolic phytochemicals including phenolic acids, lignans, flavonoids, and tannins (Kasote, 2013). Few previous studies have investigated effect of pre-harvest and post-harvest variables on phenolic phytochemical content of flaxseed (Dabrowski & Sosulski 1984; Oomah et al, 1995).

The total phenolic content of different flaxseed cultivars grown under different locations were varied between 8-10 g/kg of total phenolic acid content of which 5 g/kg were esterified phenolic acids and 3-5 g/kg were etherified phenolic acids (Oomah et al, 1995). Phenolic acids found in defatted flour of flaxseed include p-hydroxybenzoic acid, trans-p-coumaric acid, trans-ferulic acid and trans-caffeic acids, with trans-ferulic acid being the highest concentration at 37.6 mg/100g (Dabrowski & Sosulski 1984). Flaxseeds are specifically a good source of lignans like secoisolariciresinol diglycoside (SDG) which are natural antioxidants that display hydroxyl and peroxyl radical scavenging activity and can also help in the inhibition of lipid peroxidation

(Soleymani, Habtemariam, Rahimi & Nabavi, 2020; Toure & Xueming 2010; Kasote, 2013). SDG is converted to secoisolariciresinol (SECO) and to the mammalian lignans enterodiol (ED) and enterolactone (EL) by bacteria found in the colon of humans and animals (Toure & Xueming 2010; Kasote, 2013; Prasad, 2000). Higher antioxidant activity was found in SECO, ED, and EL when compared to SDG and vitamin E (Prasad, 2000).

Beyond its antioxidant capacity, phenolic phytochemicals can potentially inhibit the activity of carbohydrate digestive enzymes like α-amylase and α-glucosidase, which in turn can potentially help maintaining postprandial blood glucose homeostasis (Baron, 1998; Feng et al, 1996; Liu et al, 2011; Najafian et al, 2011; van de Laar, 2008). Furthermore, phenolic phytochemical can also inhibit the activity of angiotensin I converting enzyme (ACE) thereby helping to manage and control chronic hypertension (Ademiluyi & Oboh 2013; McCue et al, 2005). Therefore, due to such high phenolic–linked antioxidant potentials and wider functional benefits, flaxseeds can be targeted as value-added functional foods or functional ingredient source especially in dietary support strategies to counter chronic oxidative stress and other common health risks associated with non-communicable chronic diseases (NCDs).

Based on these above health protective functional potentials, the aim of this research was to compare the soluble phenolic content, phenolic profile, antioxidant, antihyperglycemic, and antihypertensive properties of milled and unmilled flaxseeds from different sources (commercial and locally grown organic flaxseeds) using *in vitro* assay model-based screening strategy. Two different food grade extractions (aqueous and 12% ethanol) were used to screen and optimize flaxseed-based formulation, which could be integrated in nutraceutical, pharmaceutical or dietary applications, aimed at mitigating common health risks associated with type 2 diabetes and other NCDs.

7.3. Materials and Methods

7.3.1. Extraction of flaxseed and flax lignan

The flaxseed samples used in this study were the commercially sourced from Bob's Red Mill (BRM) premium (BP) and BRM organic (BO) flaxseed, while the Linott (LI) and Pembina (PE) flaxseed cultivars were obtained from a local grower (Kaul Organic Farm, Harvey, ND-58341, USA), and the flax lignan powder (FL) was also obtained from a local source (Rapha Global Corporation, ND). Using a coffee grinder, the flaxseed samples were milled for 5 minutes to obtain a fine and uniform powder. The unmilled (UN) and milled (MI) flaxseed and flax lignan samples were then extracted in duplicate using aqueous (AQ) or 12% ethanol (ET) based on the extraction procedure as described previously published study (Christopher et al, 2018). For the extraction procedure, 1 g of the flaxseed samples were blended in 50 mL of cold water or 12% ethanol using a Waring Blender set at low speed for 5 minutes. The samples were then poured into 50 mL centrifuge tubes and centrifuged at 8,500 rpm for 20 min after which the supernatant was collected and re-centrifuged at 8,500 rpm for 15 min. The final supernatant containing the flaxseed and flax lignan extracts were collected and stored at 4°C during the *in-vitro* analysis.

7.3.2. Total soluble phenolic content

The total soluble phenolic (TSP) content of the flaxseed and flax lignan extracts was measured using the Folin-Ciocalteu method based on the protocol as described in previously published study (Shetty et al, 1995). For this protocol, 0.5 mL of the extracts were analyzed by adding 1 mL of 95% ethanol, 0.5 mL of 50% (v/v) Folin- Ciocalteu reagent and 1 mL of 5% sodium carbonate in that respective order to each of the extracts. For the control, 0.5 mL of distilled water was used instead of the extract. The extracts and control were then mixed using a

vortex machine and incubated for one hour under dark conditions followed by the measurement of their absorbance at 725nm using a UV-visible spectrophotometer (Genesys 10S UV-VIS spectrophotometer, Thermo Scientific, NY). Using a standard curve based on different concentrations of gallic acid in 95% ethanol, total soluble phenolic content was determined and expressed in milligram gallic acid equivalents per gram dry weight (mg GAE/g DW).

7.3.3. Phenolic profile

The phenolic profile of the flaxseed and flax lignan extracts was measured using high performance liquid chromatography (HPLC) technique in which 5 µL of the extracts was injected using an Agilent ALS 1200 auto-extractor into an Agilent 1260 series (Agilent Technologies, Palo Alto, CA) HPLC equipped with a D1100 CE diode array detector. The solvents used for gradient elution were 10 mM phosphoric acid (pH 2.5) and 100% methanol. The methanol concentration was increased to 60% for the first 8 min, then to 100% over the next 7 min, then decreased to 0% for the next 3 min which was then maintained for 7 min with a total run time of 25 min per extract. 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 0.7 mL/min at room temperature. The absorbance values were recorded at 214 nm, 230 nm, 260 nm, and 306 nm and the chromatogram was integrated using Agilent Chem station enhanced integrator. Pure standards of benzoic acid, gallic acid, protocatechuic acid, ferulic acid and catechin in 100% methanol were used to calibrate the respective standard curves and retention times. The phenolic compounds detected in the extracts were expressed in micro gram per gram dry weight (µg/G DW).

7.3.4. Antioxidant activity

The antioxidant activity of the flaxseed and flax lignan extracts was measured by their radical scavenging activity against 2, 2-Azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) (A1888-5G, Sigma-Aldrich) and 2, 2-Dipheny-1—Picryl hydrazyl (DPPH) (D9132-5G, Sigma-Aldrich) free radicals. The protocol for the DPPH scavenging assay was followed as described in a previous study (Kwon et al, 2006). According to the protocol, 1.25 mL of 60 mM DPPH (in 95% ethanol) was added to 0.25 mL of the extracts and vortexed to ensure mixing. Each extract had a corresponding control containing 0.25 mL of 95% ethanol instead of the extract. The extracts and their controls were incubated for 5 min followed by centrifugation at 13,000 rpm for one min, after which the supernatant was collected and the absorbance at 517nm was measured using a UV-visible spectrophotometer (Genesys 10S UV-VIS spectrophotometer, Thermo Scientific, NY). The protocol for the ABTS scavenging assay was followed as described in a previous study (Re et al, 1999). In this protocol, 1 mL of ABTS radical cation (in 95% ethanol) was added to 0.05 mL of the extracts and vortexed to ensure mixing. For the control 0.05 mL of 95% ethanol was used instead of the extract. The extracts and their corresponding controls were incubated for 3 min followed by centrifugation at 13,000 rpm for one min after which the supernatant was collected and the absorbance at 734 nm was measured using a UV-visible spectrophotometer (Genesys 10S UV-VIS spectrophotometer, Thermo Scientific, NY). For both the DPPH and ABTS radical scavenging assays the absorbance values of the samples along with their corresponding controls were used to calculate the percentage of radical scavenging of each sample using the following formula:

$$\% \text{ Antioxidant activity} = \frac{\text{Control}^{\text{absorbance}} - \text{Sample}^{\text{absorbance}}}{\text{Control}^{\text{absorbance}}} \times 100$$

7.3.5. Alpha-amylase inhibitory activity

The protocol used to measure the α-amylase enzyme inhibitory activity of the flaxseed and flax lignan extracts was based on the procedure as described in a previous study (Kwon et al, 2006). The α-amylase inhibitory activity of the extracts was measured in a dose dependent manner using undiluted, half diluted and one-fifth diluted extracts. The buffer used in this assay was 0.1 M sodium phosphate (pH 6.9) containing 0.006 M sodium chloride. In this protocol, 500 µL of the undiluted, 1:2 and 1:5 diluted extracts were added to their respective tubes while the control tube had 500 µL of distilled water. Also, each extract had a corresponding blank tube containing 500 µL of the extract and 500 µL of distilled water. Then 500 µL of porcine pancreatic α-amylase (0.5 mg/mL buffer) (EC 3.2.1.1, purchased from Sigma Chemical Co, St Louis, MO) was added to all the extracts and control tubes except for the blank tubes. The tubes were shaken to ensure mixing and then incubated for 10 min at 25°C. After incubation 500 µL of the substrate (1% starch in buffer) was added to the extract, control, and blank tubes and incubated for 10 min at 25°C. This was followed by the addition of 1 mL of 3, 5 dinitro salicylic acid, and the tubes were incubated in a boiling water bath for 10 min to stop the reaction. The tubes were then cooled down to room temperature and the reaction mixture in all the tubes was diluted with distilled water until the absorbance values in the control tubes reached between 1.0 and 1.2. The absorbance values of all the tubes were measured at 540 nm using a UV-visible spectrophotometer (Genesys 10S UV-VIS spectrophotometer, Thermo Scientific, NY). The absorbance values of the extract, sample blank and control tubes were then used to calculate the percentage of enzyme inhibitory activity of the extracts using the following formula:

$$\% \text{ inhibition} = \frac{\text{Control}^{\text{absorbance}} - (\text{Extract}^{\text{absorbance}} - \text{Blank}^{\text{absorbance}})}{\text{Control}^{\text{absorbance}}} \times 100$$

7.3.6. Alpha-glucosidase inhibitory activity

The protocol used to measure the α-glucosidase enzyme inhibitory activity of the flaxseed and flax lignan extracts was based on the procedure as described earlier (Kwon et al, 2006). The α-glucosidase enzyme inhibitory activity was measured in a dose-dependent manner using undiluted, half diluted, and one-fifth diluted extracts. The extracts were diluted with 0.1M potassium phosphate buffer (pH 6.9) and 50 µL of the undiluted, half, and one-fifth diluted extracts were aliquoted into 96-well microtiter plates. Each extract had a corresponding control containing 50 µL buffer and the volume in all the wells was made up to a final volume of 100 µL by the addition of 50 µL of the buffer. After this 100 µL of buffer containing yeast α-glucosidase enzyme (1 U/mL) (EC 3.2.1.20, purchased from Sigma Chemical Co, St. Louis, MO) was added to each well and the plates were incubated for 10 min at 25°C. This was followed by the addition of 50 µL of the substrate 5 mM p-nitrophenyl-α-D- glucopyranoside solution (prepared in buffer) to each well followed by a 5-min incubation at 25°C. The absorbance of all the wells was measured at 405 nm using a microplate reader (Thermomax, Molecular device Co., Virginia, USA) and the readings were taken before and after the 5-min incubation period and were assigned as the 0-min and 5-min readings respectively. The absorbance values of the samples and their controls were used to calculate the percentage of enzyme inhibitory activity of the samples using the following formula:

$$\% \text{ inhibition} = \frac{(\text{Control}^{\text{abs}}\,5 \text{ min} - \text{Control}^{\text{abs}}\,0 \text{ min}) - (\text{Extract}^{\text{abs}}\,5 \text{ min} - \text{Extract}^{\text{abs}}\,0 \text{ min})}{(\text{Control}^{\text{abs}}\,5 \text{ min} - \text{Control}^{\text{abs}}\,0 \text{ min})} \times 100$$

7.3.7. Angiotensin-I-converting enzyme (ACE) inhibitory activity

The protocol used to measure the ACE inhibitory activity of the flaxseed and flax lignan extracts was followed as described in a previous study (Kwon et al, 2006). The substrate used was hippuryl-histidyl-leucine (HHL) and the enzyme ACE was obtained from rabbit lung (EC

3.4.15.1). To 50 µL of the extracts, 200 µL of 1M NaCl-borate buffer (pH 8.3) containing 2 mU of ACE was added and then incubated at 37°C for 10 min. After this, 100 µL of 5mM HHL substrate was added to each tube and incubated for 1 h at 37°C. The reaction was stopped by the addition of 150 µL of 0.5N HCl. The hippuric acid formed due to ACE activity was detected using the HPLC method for which, 5 µL of the reaction mixtures were injected using Agilent ALS 1200 autosampler into an Agilent 1260 series (Agilent Technologies, Palo Alto, CA) HPLC equipped with a D1100 CE diode array detector. The solvents used for the gradient were a combination of 10 mM phosphoric acid (pH 2.5) and 100% methanol. For the first 8 min the methanol concentration was increased to 60% then to 100% for 5 min and finally to 0% for the next 5 min and the total run time was 18 min for each sample. 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 0.7 mL/min at room temperature. The absorbance was measured at 228 nm and the chromatogram was integrated using Agilent Chemstation (Agilent Technologies) enhanced integrator for detection of hippuric acid. A hippuric acid standard was used to calibrate standard curve and retention time and the percentage of ACE inhibition was calculated using the formula:

$$\% \text{ inhibition} = \frac{(\text{Control}^{abs} - \text{Extract}^{abs})}{(\text{Control}^{abs} - \text{Blank}^{abs})} \times 100$$

7.3.8. Statistical analysis

All *in vitro* analysis of the TSP content, antioxidant, anti-hyperglycemic, and anti-hypertensive properties as well as phenolic profiling, were performed in duplicate and from two different extractions (repeat run). The means and standard error were calculated using Microsoft Excel XP. The analysis of covariance was done using Statistical Analysis Software (SAS version 9.4, SAS Institute, Cary, NC). Significant differences across samples (unmilled and milled),

extractions (aqueous and 12% ethanol), and sample×extraction interactions were determined using Tukey's least square means separation at the 0.05 probability level.

7.4. Results and Discussion

7.4.1. Total Soluble Phenolic (TSP) content

The TSP content of the flaxseed and lignin extracts ranged from 4.72 to 12.34 mg GAE/g DW and statistical significance were observed between all sample × extraction interactions (p<0.05) (Figure 7.1). The TSP content of the aqueous extracts of flax lignan (FL-AQ) and milled Pembina (PE-MI-AQ) was higher at 12.34 and 11.75 mg GAE/g DW respectively when compared to the rest of the samples (p<0.05), while the 12% ethanol extracts of unmilled Linott (LI-UN-ET) had the lowest TSP content at 4.72 mg GAE/g DW (Figure 7.1). The total phenolic content of flaxseed can vary due to factors such as cultivar, year, and location of the crop. In a previous study, the cultivar ×location ×year interactions were found to contribute to a large variation in total phenolic content among eight flaxseed cultivars (Oomah et al, 1995). The TSP content of flaxseed can also vary based on the type of solvent used in the extraction. In an earlier study, the TSP content of a defatted flaxseed extracted with either 80% or 100 % methanol or ethanol was found to range from 13.6 to 32.6 mg GAE/g (Anwar & Przybylski, 2012), while in another study, the TSP content of water and 100% methanol extracts of a non-defatted local flaxseed cultivar were found to be at 1.17 mg GAE/g and 1.90 mg/GAE, respectively (Alu'datt, Rababah, Ereifej & Alli, 2013).

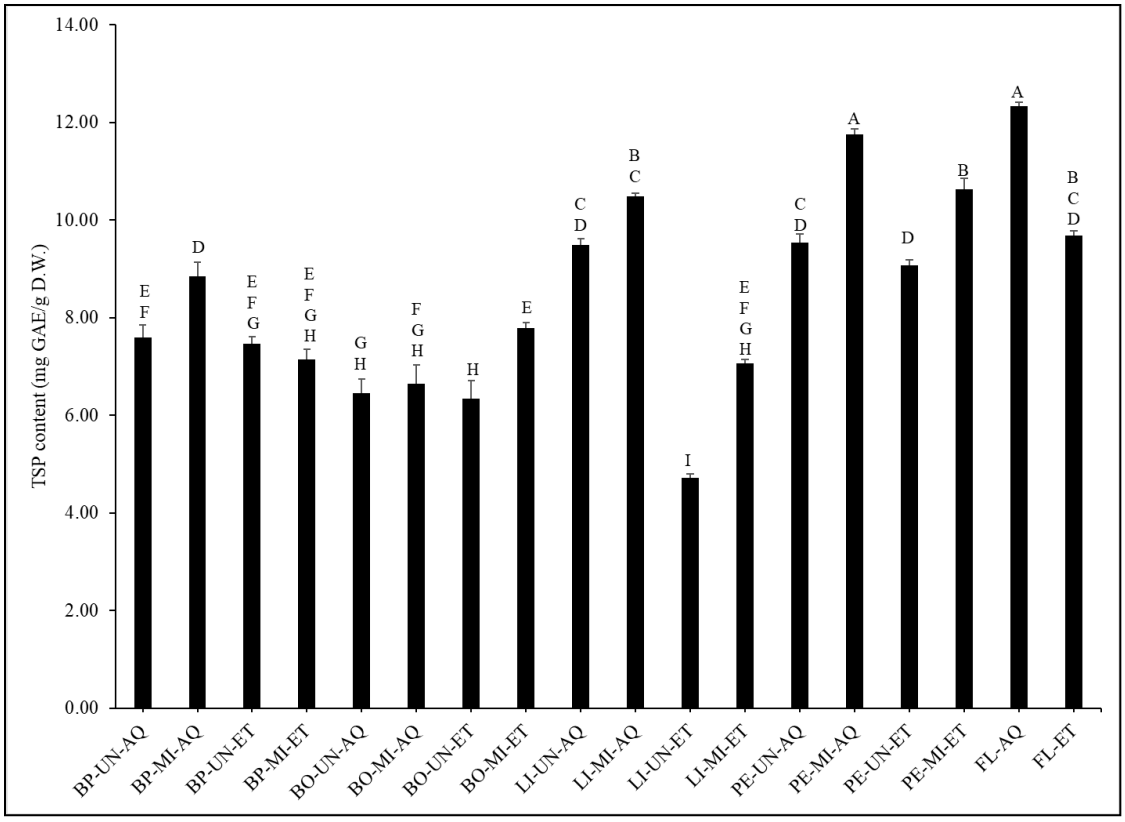


Figure 7.1. Total soluble phenolic content of unmilled (UN) and milled (MI), aqueous (AQ) and 12% ethanol (ET) extracts of Bob's Red Mill Premium (BP), Bob's Red Mill Organic (BO), Linott (LI), and Pembina (PE) flaxseed, and flax lignan powder (FL). TSP content is expressed in milligram gallic acid equivalent per gram dry weight (mg GAE/g DW). Different letters indicate statistical differences between sample× extraction interactions (p<0.05).

Apart from the type of solvent used in the extraction of flaxseed, several post-harvesting strategies such as milling or grinding of flaxseed can potentially affect the TSP content and bioactivity. Milling or grinding can increase the availability of bioactive components present in flaxseed hence making milled flaxseed an ideal food ingredient source that can be used to enhance the functionality of foods (Edel, Aliani & Pierce, 2015). The results of the current study indicated that the milling of flaxseeds followed by its aqueous extraction resulted into higher TSP content when compared to the corresponding unmilled flaxseed cultivar that was extracted in 12% ethanol (p< 0.05). Furthermore, among all the cultivars analyzed, Pembina, a locally grown cultivar, and flax lignan powder, which is also produced locally, have the potential to be

utilized to develop functional food ingredients or can be integrated in nutraceuticals for wider health and dietary benefits.

7.4.2. Phenolic profile

The phenolic compounds detected in flaxseeds were dihydroxybenzoic acid, gallic acid, benzoic acid, and catechin, with their concentrations ranging from 0 to 11.2 µg/g, 9.2 to 24.7 µg/g, 0 to 13.1 µg/g, and 0.7 to 2.5 µg/g respectively (Table 7.1). Statistical differences were observed across all sample ×extraction interactions for all the detected compounds ($p<0.05$) (Table 7.1). The dihydroxybenzoic acid content of the aqueous extract of flax lignan (FL-AQ) was significantly higher at 11.2 µg/g when compared to the flaxseed samples ($p<0.05$), while the aqueous extract of unmilled BRM premium (BP-UN-AQ) had the lowest dihydroxybenzoic acid content at 1.02 µg/g (Table 7.1). Dihydroxybenzoic acid was detected in the 12% ethanol extracts of unmilled and milled Linott (LI-UN-ET and LI-MI-ET), but not in the aqueous extracts, thereby indicating that solvent extraction with 12% ethanol could have potentially released this phenolic acid. In other flaxseed cultivars, dihydroxybenzoic acid was detected in both aqueous and 12% ethanol extracts. The gallic acid content of the aqueous extracts of unmilled and milled Pembina (PE-UN-AQ and PE-MI-AQ) was statistically higher at 24.7 and 23.3 µg/g respectively when compared to the rest of the samples ($p<0.05$), while 12% ethanol extract of flax lignan (FL-ET) had the lowest gallic acid content at 9.2 µg/g (Table 7.1). The benzoic acid content of the 12% ethanol and aqueous extracts of flax lignan (FL-ET and FL-AQ) was higher at 13.1 and 12.6 µg/g respectively when compared to the rest of the samples ($p<0.05$), while the aqueous extract of unmilled BRM premium (BP-UN-AQ) had the lowest benzoic acid content at 0.3 µg/g (Table 7.1).

Table 7.1. Individual phenolic compounds of flaxseed and flax lignan extracts in ug/g DW.

Samples	**Phenolic compounds detected[a,b]**			
	Dihydroxybenzoic acid	Gallic acid	Benzoic acid	Catechin
BP-UN-AQ	1.04 ± 0.1h	17.93 ± 0.5cdef	0.26 ± 0.0f	2.19 ± 0.0abc
BP-MI-AQ	1.20 ± 0.0gh	21.43 ± 0.2abc	2.66 ± 0.1c	1.15 ± 0.0h
BP-UN-ET	1.46 ± 0.0g	16.49 ± 0.3defg	ND	1.51 ± 0.1fg
BP-MI-ET	1.56 ± 0.1g	15.72 ± 0.2efgh	0.89 ± 0.0e	1.17 ± 0.0h
BO-UN-AQ	3.52 ± 0.0f	13.69 ± 0.2ghij	ND	1.86 ± 0.1de
BO-MI-AQ	1.73 ± 0.2g	12.09 ± 0.1hijk	1.74 ± 0.1d	1.65 ± 0.0ef
BO-UN-ET	8.55 ± 0.6bc	10.35 ± 0.2jk	ND	1.78 ± 0.0def
BO-MI-ET	6.33 ± 0.0e	11.17 ± 0.6ijk	0.89 ± 0.0e	2.23 ± 0.1ab
LI-UN-AQ	ND	17.20 ± 1.2defg	ND	0.72 ± 0.0i
LI-MI-AQ	ND	14.84 ± 0.2fghi	ND	0.80 ± 0.0h
LI-UN-ET	4.35 ± 0.0f	15.81 ± 0.8efgh	ND	0.72 ± 0.0i
LI-MI-ET	3.91 ± 0.1f	14.72 ± 0.4fghi	ND	0.72 ± 0.0i
PE-UN-AQ	7.71 ± 0.1cd	24.67 ± 0.8a	ND	2.44 ± 0.0a
PE-MI-AQ	5.72 ± 0.1e	23.25 ± 0.2ab	9.10 ± 0.4b	1.84 ± 0.0de
PE-UN-ET	9.04 ± 0.0b	19.91 ± 0.1bcd	ND	1.99 ± 0.1bcd
PE-MI-ET	8.04 ± 0.3bcd	19.43 ± 0.1cde	ND	2.39 ± 0.1a
FL-AQ	11.17 ± 0.6a	15.48 ± 2.3fgh	12.56 ± 0.3a	1.88 ± 0.1cde
FL-ET	6.89 ± 0.3de	9.17 ± 0.4k	13.11 ± 0.1a	1.29 ± 0.1gh

[a] Mean value ± standard error

[b] Different lower-case letters in each column indicate statistical differences between sample× extraction interactions (p<0.05).

ND- not detected, UN-unmilled, MI- milled, AQ- aqueous, ET- 12% ethanol, BP- Bob's Red Mill Premium, BO- Bob's Red Mill Organic, LI- Linott, and PE- Pembina, FL- flax lignan powder.

The catechin content of the aqueous extracts of unmilled Pembina (PE-UN-AQ) and 12% ethanol extracts of milled Pembina (PE-MI-ET) was higher at 2.5 and 2.4 µg/g respectively when compared to other flaxseed extracts, while the aqueous and 12% ethanol extracts of unmilled and milled Linott (LI-UN-AQ, LI-MI-AQ, LI-UN-ET, and LI-MI-ET) had the lowest catechin content ranging from 0.7 to 0.8 µg/g (Table 7.1). In earlier studies, several free and bound forms of phenolic compounds have been detected in flaxseed, including gallic, hydroxybenzoic, vanillic, protocatechuic, syringic, coumaric, ferulic, caffeic, and sinapic acids (Alu'datt et al, 2013; Dabrowski & Sosulski 1984; Hao & Beta, 2012; Herchi et al, 2011). The

phenolic profile can vary among the flaxseed varieties and furthermore, the phenolic compounds are mostly concentrated in the flax hull rather than in the endosperm (Hao & Beta, 2012). Like in the case of TSP content, the phenolic profile of flaxseed can be affected by the type of solvent used in the extraction as well as post-harvest procedures such as milling or grinding. The results of the current study indicated that the phenolic profile and content can vary depending on the cultivar, type of solvent used in extraction (aqueous and 12% ethanol) as well as post-harvest milling of the sample. Furthermore, among all the flaxseed analyzed, Pembina, a locally North Dakota grown cultivar, had good phenolic profile and higher phenolic acid content. Therefore, Pembina can be further targeted in future clinical studies to determine its health protective functional benefits and for wider value-added applications.

7.4.3. Antioxidant activity

The antioxidant activity of the flaxseed and flax lignan extracts was measured *in-vitro* via the ABTS and DPPH radical scavenging activity and the activity was expressed in percentages. The values for the ABTS scavenging activity ranged from 28.2% to 99.9% and statistical differences in scavenging activity were observed across all sample× extraction interactions ($p<0.05$) (Figure 7.2). The ABTS scavenging activity of the 12% ethanol and aqueous extracts of flax lignan (FL-ET and FL-AQ) was significantly higher at 99.9% and 98.9% respectively when compared to the flaxseed samples ($p<0.05$), while the aqueous extract of milled Linott (LI-MI-AQ) had the lowest ABTS scavenging activity at 28.2 % (Figure 7.2). The values for the DPPH scavenging activity ranged from 14.7 % to 47.9 % and statistical differences in scavenging activity were observed across all sample× extraction interactions ($p<0.05$) (Figure 7.3).

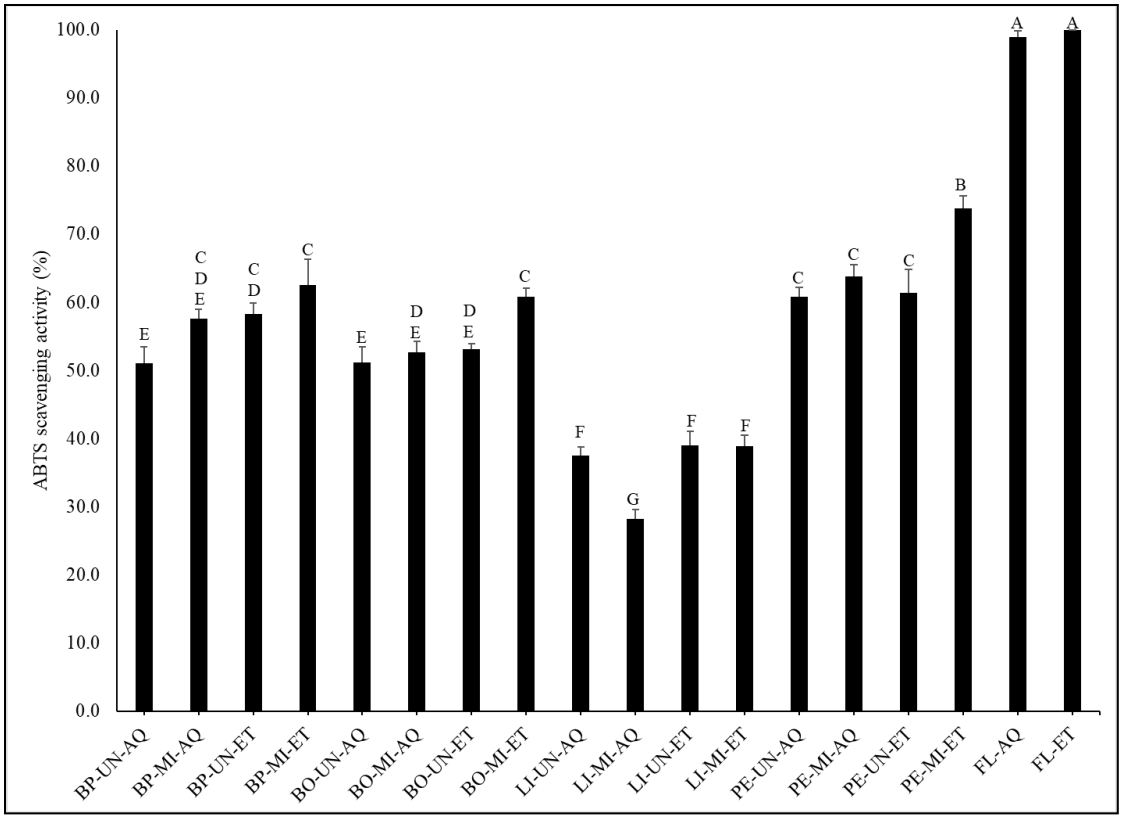


Figure 7.2. 2, 2-Azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) scavenging activity of unmilled (UN) and milled (MI), aqueous (AQ) and 12% ethanol (ET) extracts of Bob's Red Mill Premium (BP), Bob's Red Mill Organic (BO), Linott (LI), and Pembina (PE) flaxseed, and flax lignan powder (FL). Scavenging activity is expressed in percentages (%). Different letters indicate statistical differences between sample× extraction interactions (p<0.05).

Like ABTS scavenging activity, the DPPH scavenging activity of the aqueous and 12% ethanol extracts of flax lignan (FL-AQ and FL-ET) was significantly higher at 47.9% and 47.5% respectively when compared to the flaxseed extracts (p<0.05). Aqueous extract of milled Linott (LI-MI-AQ) had the lowest DPPH scavenging activity at 14.7% (Figure 7.3). Additionally, a weak positive correlation was observed between the TSP content and the ABTS or DPPH scavenging activity (r = 0.44).

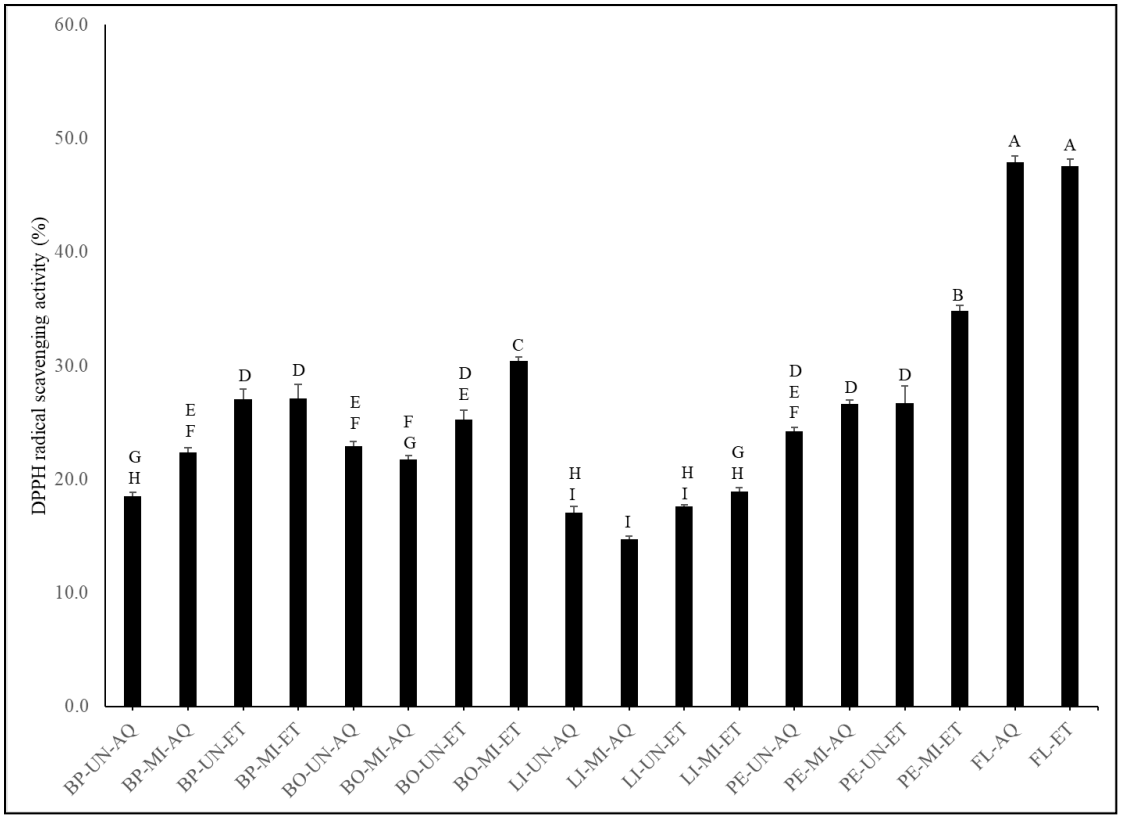


Figure 7.3. 2, 2-Dipheny-1-Picryl hydrazyl (DPPH) scavenging activity of unmilled (UN) and milled (MI), aqueous (AQ) and 12% ethanol (ET) extracts of Bob's Red Mill Premium (BP), Bob's Red Mill Organic (BO), Linott (LI), and Pembina (PE) flaxseed, and flax lignan powder (FL). Scavenging activity is expressed in percentages (%). Different letters indicate statistical differences between sample× extraction interactions (p<0.05).

In an earlier study, the DPPH scavenging activity of flaxseed subjected to different post-harvest processing steps (soaking, roasting, malting, two-phase solvent extraction, and dehulling) was found to range from 19.6% to 49.5%, and a strong positive correlation was found between the total phenolic content and antioxidant activity (r= 0.96) (Yadav, Khatak, Singhania & Bishnoi, 2020). The results of the current study indicated that aqueous or 12% ethanol extracts of flax lignan provided maximum ABTS and DPPH scavenging activity. Hence flax lignan can be incorporated into the diet to potentially mitigate the chronic oxidative stress- linked health complications. This study also provides the rationale for further animal model studies to target specific associated chronic diseases.

7.4.4. Alpha-amylase inhibitory activity

Overall, low α-amylase inhibitory activity was observed with the values ranging from 1.3% to 9.9% and statistical differences in activity were observed only across different flaxseed samples and flax lignan powder ($p<0.05$) (Table 7.2). Across samples, flax lignan had significantly higher α-amylase inhibitory activity ($p<0.05$) while no significant differences were observed across the aqueous and 12% ethanol extracts. In an earlier study, flax seedcoat extracts prepared using microwave assisted extraction showed α-amylase inhibitory activity at 45.7 % and the activity was attributed to the lignan content (Hano et al, 2013). The same study concluded that the mechanism of inhibition of α-amylase activity was due to mixed inhibition wherein the flaxseed lignans could interact directly with the enzyme forming an inactive enzyme–inhibitor complex, thereby inducing an enzyme conformation change which prevents the formation of the enzyme–substrate intermediate, or the lignan could also interact with the enzyme–substrate intermediate to give a sterile ternary enzyme–substrate–inhibitor (Hano et al, 2013). Another study suggested that inhibitory action of phytochemicals can be due to its antioxidant activity which causes a reduction of disulfide bridges (oxidized cysteines) present on the surface of the enzyme, leading to a change in protein structure and potential inhibition of enzyme activity (McCue et al, 2004). The results of the current study indicated that flax lignan has the potential to be utilized in dietary strategies to manage chronic hyperglycemia without causing harmful side effects. However, flaxseed or flax lignan must first undergo suitable extraction procedures or processing steps in order provide optimum α-amylase inhibitory activity relevant for dietary or therapeutic benefits.

Table 7.2. Alpha-amylase, α-glucosidase, and angiotensin-I-converting enzyme (ACE) inhibitory activities of flaxseed and flax lignan extracts (%).

Sample	**Enzyme inhibitory activity[a,b,c]**		
	α-amylase	α-glucosidase	ACE
BP-UN-AQ	1.6 ± 1.0B	1.4 ± 0.4de	13.9 ± 3.4efgh
BP-MI-AQ	4.1 ± 1.2B	2.7 ± 0.5de	28.6 ± 1.9cde
BP-UN-ET	2.3 ± 0.9B	5.3 ± 1.4bcde	11.7 ± 2.2fgh
BP-MI-ET	1.9 ± 1.0B	4.3 ± 0.8cde	17.8 ± 1.9defg
BO-UN-AQ	2.7 ± 1.0B	1.0 ± 0.5e	14.1 ± 1.3efgh
BO-MI-AQ	3.9 ± 1.4B	1.4 ± 0.5de	26.2 ± 5.1cdef
BO-UN-ET	2.6 ± 0.8B	5.6 ± 0.7bcd	11.7 ± 3.6fgh
BO-MI-ET	5.2 ± 1.3B	4.8 ± 0.9bcde	31.0 ± 3.1cd
LI-UN-AQ	1.9 ± 1.1B	3.7 ± 0.8cde	49.5 ± 1.0b
LI-MI-AQ	1.3 ± 0.7B	3.6 ± 1.4cde	38.4 ± 3.3bc
LI-UN-ET	3.6 ± 1.3B	3.2 ± 1.0cde	4.4 ± 1.4gh
LI-MI-ET	1.8 ± 1.2B	3.0 ± 0.8cde	13.4 ± 2.2fgh
PE-UN-AQ	4.1 ± 1.1B	1.5 ± 0.4de	18.5 ± 2.5defg
PE-MI-AQ	4.5 ± 1.5B	3.6 ± 0.6cde	35.4 ± 1.1bc
PE-UN-ET	1.8 ± 0.6B	7.4 ± 0.9abc	NA
PE-MI-ET	4.4 ± 1.2B	8.9 ± 0.7ab	34.0 ± 4.2c
FL-AQ	10.0 ± 0.7A	4.1 ± 1.2cde	64.8 ± 2.9a
FL-ET	9.5 ± 1.0A	11.5 ± 1.6a	0.6 ± 0.2h

[a] Mean value ± standard error

[b] Different upper-case letters indicate statistical differences in α-amylase inhibitory activity across samples (p<0.05).

[c] Different lower-case letters in each column indicate statistical differences in α-glucosidase and angiotensin-I-converting enzyme inhibitory activity between sample× extraction interactions (p<0.05).

NA-no activity, UN-unmilled, MI- milled, AQ- aqueous, ET- 12% ethanol, BP- Bob's Red Mill Premium, BO- Bob's Red Mill Organic, LI- Linott, and PE- Pembina, FL- flax lignan powder.

7.4.5. Alpha-glucosidase inhibitory activity

Like the result of α-amylase inhibitory activity, low α-glucosidase inhibitory activity was observed with the values ranging from 1% to 11.6%. A statistically significant differences in α-glucosidase inhibitory activity were observed across all sample× extraction interactions (p<0.05) (Table 7.2). The α-glucosidase inhibitory activity of 12% ethanol extract of flax lignan (FL-ET) was statistically higher at 11.6% when compared to all flaxseed extracts (p<0.05), while aqueous extract of unmilled Bob's Red Mill Organic (BO-UN-AQ) had the lowest inhibitory activity at

1%. In an earlier study, flax seedcoat extracts prepared using microwave assisted extraction, showed α-glucosidase inhibitory activity at 52.4% and the activity was attributed to the lignan content (Hano et al, 2013). Like α-amylase inhibition, the mechanism of inhibition of α-glucosidase is through competitive inhibition by binding of phytochemicals to the active site of the enzyme, which can change the enzyme configuration leading to loss of enzyme activity (Chen, Wu, Zhang, Yin & Zhang, 2020; Gong et al, 2020; Hossain, Das, Ghosh & Sil, 2020). The results of the current study indicated that flax lignan can be utilized in dietary strategies to potentially counter chronic hyperglycemia. However, like in the case of α-amylase inhibition, the optimization of different extraction methods and post-processing steps is necessary to improve the α-glucosidase inhibitory activity of flaxseed and flax lignan. This study provides the rationale and foundation for further animal model studies to advance health targeted solutions of these ingredients.

7.4.6. Angiotensin-I-converting enzyme (ACE) inhibitory activity

The ACE inhibitory activity of the flaxseed and flax lignan extracts was measured *in-vitro* and expressed in percentages. Overall, low to moderate ACE inhibitory activity was observed with the values ranging from 0.6% to 64.8% (Table 7.2). The ACE inhibitory activity of aqueous extract of flax lignan (FL-AQ) was statistically higher at 64.8% when compared to all flaxseed extracts ($p<0.05$), while interestingly, the 12% ethanol extract of flax lignan (FL-ET) had the lowest activity at 0.6% (Table 7.2). In a previous study, Sprague Dawley male rats treated with SDG, an important lignan found in flaxseed, showed a decrease in angiotensin I-induced rise in the systolic, diastolic, and mean arterial pressures (Prasad, 2013). Apart from phytochemicals, protein present in flaxseed can also display ACE inhibitory activity, especially after post-harvest extraction or enzymatic treatment which breaks down the flaxseed proteins to

release the bioactive peptides (Wu, Wang, Qi, & Guo, 2019). The results of the current study indicated that flax lignan can be targeted in dietary strategies to manage hypertension, however the optimization of different extraction methods and post-processing steps are necessary to improve the ACE inhibitory activity of flaxseed or flax lignin, specifically for their value added dietary and therapeutic applications. These improvements must be integrated with animal model studies to further rationalized health-targeted applications.

7.5. Conclusion

Flaxseed and flax lignan with rich phenolic phytochemicals profile can be utilized as functional food and food ingredient source targeting wider dietary and therapeutic benefits. However, cultivar variations, post-harvest processing strategies, and different solvent-based extraction protocols can ultimately affect the phenolic linked bioactivity of flaxseed due to increase or decrease in availability of these phytochemical compounds. The result of this study indicated that these above-mentioned variables have significant impact on phenolic-linked antioxidant, anti-hyperglycemic and anti-hypertensive properties of flaxseed. Milled aqueous extracts of a local North Dakota grown flaxseed (Pembina) had higher bioavailability of soluble phenolic compounds, while flax lignan showed anti-hyperglycemic and anti-hypertensive properties. Future studies with different organic solvent-based extraction procedures are required to find optimum anti-hyperglycemic and anti-hypertensive benefits of flaxseed and flax lignan, with the goal of formulating NCD-countering food ingredients or nutraceuticals.

CHAPTER 8. ETHNIC FOOD PERSPECTIVE OF NORTH DAKOTA COMMON EMMER WHEAT AND RELEVANCE FOR HEALTH BENEFITS TARGETING TYPE 2 DIABETES

8.1. Abstract

Ancient grains with ethnic food origins are gaining renewed interest in contemporary food design due to its balanced nutritional profiles and health benefits. The "North Dakota Common Emmer Wheat" (*Triticum dicoccum*), a tetraploid species, had ethnic origins with German immigrants from Russia migrating to North Dakota in late 19th century. Targeting such grains with ethnic origins that are rich in fibers, amino acids, minerals, and other bioactive compounds has significant merit for advancing health benefits against emerging diet-linked chronic diseases. Based on this rationale, phenolic-linked antioxidant and antihyperglycemic properties of North Dakota Common Emmer Wheat was compared with those of other commercial wheat cultivars in order to integrate it into a health-targeted food design based on past ethnic food insights. Aqueous extracts of the North Dakota Common Emmer Wheat (with and without hull) and two other commercial wheat varieties, Barlow and Coteau, were analyzed before and after milling. The total soluble phenolic content, phenolic acid profile, protein content, antioxidant activity, type 2 diabetes relevant α-amylase, and α-glucosidase enzyme inhibitory activities were determined using *in vitro* assay models. North Dakota Common Emmer Wheat with hull had highest total soluble phenolic content and associated antioxidant and antihyperglycemic properties (before and after milling) when compared to the other commercial wheat cultivars. These results indicate that North Dakota Common Emmer Wheat with hull can be integrated into a health-targeted contemporary food design as a part of dietary

support against chronic hyperglycemia and oxidative stress associated with early stages type 2 diabetes.

Key words: Antioxidant, Enzyme Inhibitors, Ethnic Wheat, North Dakota Common Emmer, Phenolics, Type 2 Diabetes

8.2. Introduction

The origin of cultivated emmer wheat (*Triticum dicoccum*) lies in parts of the Fertile Crescent including Syria, Egypt, and Turkey. Ancient emmer wheat was domesticated around 8000 BC and was closely associated with Neolithic agriculture (Luo et al, 2007; Zaharieva et al, 2010). The wild progenitor of emmer wheat, *Triticum dicoccoides* (Schrank) is said to have originated from Israel and the near East (Luo et al, 2007; Nevo, 2014). From this center of origin, cultivation of emmer spread to the other parts of the world such as Mediterranean regions of Europe and Northern Africa, Eastern Europe and Russia, and Indian subcontinent during 60000 BC to 3000 BC (Luo et al, 2007). Cultivation of emmer wheat was an integral part of early agriculture in Europe (Luo et al, 2007). Introduction and domestication of the free-threshing wheat slowly replaced the emmer wheat from its original geographical distribution. However, emmer wheat was extremely popular in Egypt until Greco-Roman period because of its higher stress tolerance and the dietary preference associated with superior bread quality (Zaharieva et al, 2010). In Italy, emmer wheat was cultivated until recently and was commonly referred as “Pharaoh's wheat”. Currently, emmer wheat is cultivated in over 17 countries and is grown as a major crop only in Ethiopia and as a minor crop in India and Italy (Zaharieva et al, 2010; Stallknecht et al, 1996). In the United States, landraces of emmer were introduced by the East-European German origin immigrant farmers during the late 19th century (Figure 8.1 A). The introduction of emmer in the Northern Great Plains during the late 19th century was mainly

due to its high resilience against extreme climate and its value as both human food and animal feed. In the early 1900s, thousands of acres of land in the Midwestern and Western regions of the United States were utilized for emmer wheat cultivation (Figures 8.1 B, C), but now this wheat variety is grown only under small acreage in the states of Montana and North Dakota,

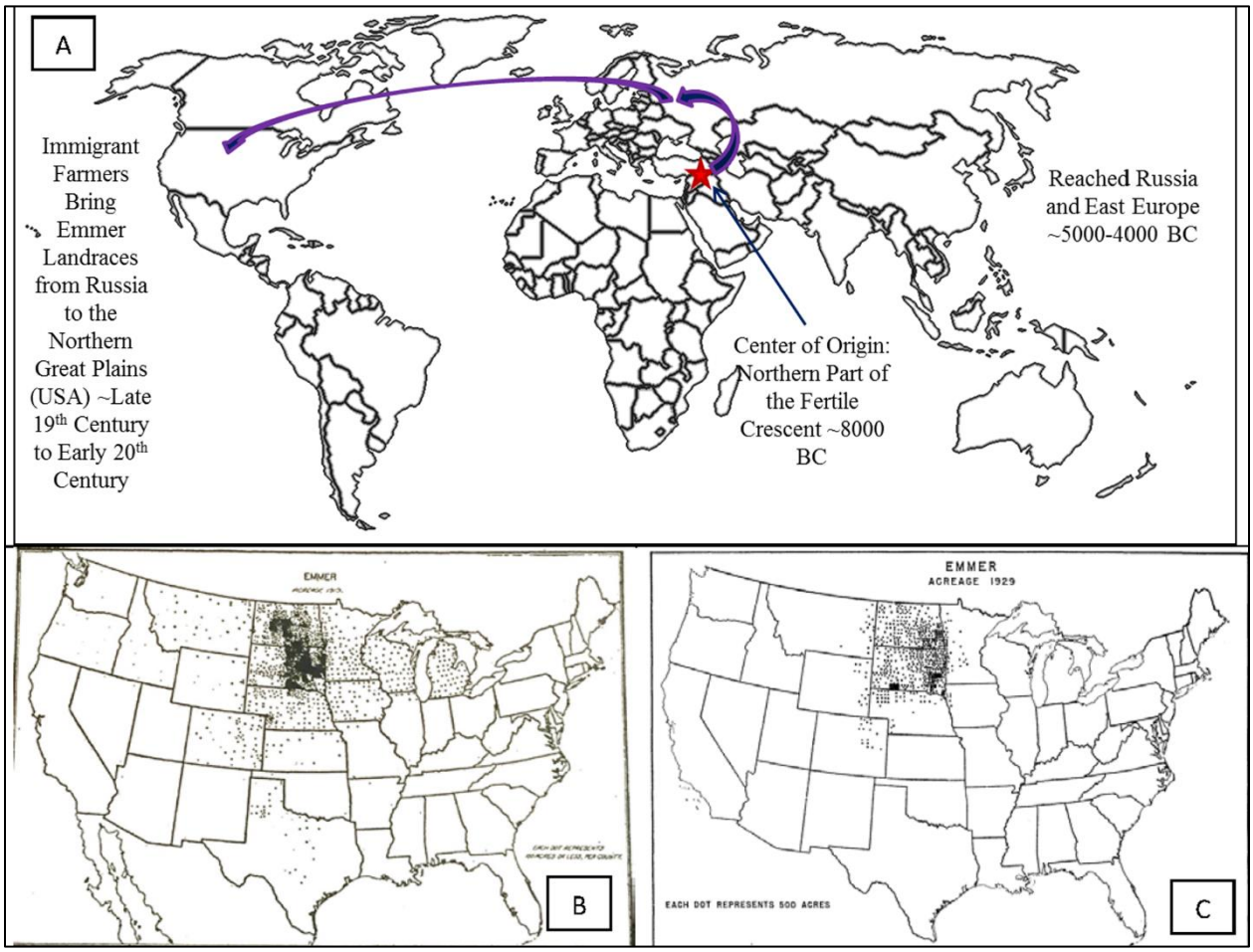


Figure 8.1. The origin of emmer wheat and its first domestication in parts of the fertile crescent around 8000 BC. From its center of origin, cultivation of emmer spread in different parts of Europe, North Africa, and Asia between 6000 and 3000 BC. (A) In the United States especially in the Northern Great Plain region, emmer wheat was introduced by Russian–German immigrant farmers in the late 19th century (adapted and modified from Zaharieva et al, 2010). In the early 20th century emmer was extensively cultivated in the Northern Great Plain region as ethnic food source and as animal feed. (B) Distribution of emmer wheat cultivation in the early 20th century (1919) in the United States. (C) Distribution of emmer wheat cultivation in the early 20th century (1929) in the United States. Each dot represents 500 acres. Currently emmer wheat including North Dakota Common Emmer is only cultivated by few farmers in North Dakota and in Montana.

with Cenex emmer and common emmer wheat being the two commercially available emmer varieties (Stallknecht et al, 1996; Zaharieva et al, 2010). One reason for the decline in emmer wheat production in the Northern Great Plains could be due to the replacement by high yielding free threshing wheat varieties and difficulty associated with commercial dehulling of emmer (Zaharieva et al, 2010). The beneficial traits of emmer wheat from an agronomic and nutritional standpoint include its tolerance against biotic and abiotic stresses, the high concentration of micronutrients, superior grain quality, rich amino acid profile, and high storage protein content of the seed grains (Çakmak et al, 2004; Chatzav et al, 2010; Chen et al, 2013; Peleg et al, 2005; Peleg et al, 2008). Crop domestication has inadvertently led to the reduction of genetic diversity, leading to the loss of desirable traits which are important for the plant response to biotic and abiotic stresses (Lobel et al, 2011; Nevo, 2014; Peleg et al, 2005; Richards, 2000). The key metabolites responsible for the plant response to biotic and abiotic stress are secondary metabolites (Edreva et al, 2008).

In general, cultivars of food crops resilient against stress have a higher concentration of these secondary metabolites providing protection against abiotic and biotic stress-induced oxidative stresses (Sarkar & Shetty, 2014). These secondary bioactive compounds when consumed as a part of diet can potentially play a beneficial role in protection against oxidative stress-linked chronic diseases (Sarkar & Shetty, 2014). Owing to diverse health benefits and bioactive functionalities, these phenolic bioactives from plant-based food sources are gaining increasing attention (Huang, Ou & Prior, 2005; Manach, Scalbert, Morand, Remesy, & Jimenez, 2004; Prior, Wu & Schaich, 2005; Fraga, Galleano, Verstraeten & Oteiza, 2010). In wheat, phenolic acids, carotenoids, flavonoids, tocopherols, and tocotrienols are largely responsible for protection against oxidative stress (Abdel-Aal & Rabalski, 2008; Beta, Nam, Dexter &

Sapirstein, 2005; Fung et al, 2002; Lampi, Nurmi, Ollilainen & Piironen, 2008; Montonen, Knekt, Järvinen, Aromaa & Reunanen, 2003; Serpen et al, 2008). Emmer wheat with its late 19th century roots in North Dakota as ethnic food of German immigrants from Russia has potential health benefits. This ethnic origin wheat significantly has higher total phenolic acid content, as well as associated antioxidant activity when compared to Einkorn and other wheat cultivars traditionally used for making breads (Serpen et al, 2008). Therefore, North Dakota Common Emmer Wheat with superior abiotic stress resilient traits and with higher health relevant nutritional profile is ideal target to design foods for better health outcomes of the population experiencing higher prevalence of diet-linked noncommunicable chronic diseases (NCDs) such as type 2 diabetes.

Bioactive compounds of wheat have shown potential to inhibit carbohydrate digestive enzymes such as α-amylases and α-glucosidases (Feng et al, 1996; Heidari, Zareae & Heidarizadeh, 2005; Liu, Deseo, Morris, Winter & Leach, 2011) which in turn can help to maintain postprandial glucose homeostasis (Baron, 1998; Van de Laar, 2008). Therefore, targeting whole grain ethnic emmer rich in phenolic bioactive for designing health-targeted food relevant for dietary uses against type 2 diabetes has significant merit. Bread made from ethnic emmer is generally dense and has lower gluten content. Furthermore, emmer with hull can also be incorporated alone or in combination with other commercial and dehulled wheat flour in ethnic flatbreads such as naan, roti, chapatti, and tortilla which are traditional staple food of Middle East, Indian subcontinent, and Mexico. Such value-added use of ethnic emmer in contemporary ethnic flatbreads with health benefits can be targeted for improved diets to counter type 2 diabetes and other NCDs that are prevalent among population in these countries. Based on this perspective and rationale, the goal of this study was to screen antidiabetic properties of

North Dakota Common Emmer Wheat with its ethnic origins before and after milling process. This study provides biochemical rationale to develop wheat-based healthy staple and ethnic foods by integrating high-phenolic and high-antioxidant ethnic emmer from North Dakota to support the health and well-being of communities.

8.3. Materials and Methods

8.3.1. Chemicals used

Other than mentioned, all chemicals were purchased from Sigma Chemical Co (St. Louis, MO).

8.3.2. Wheat samples

North Dakota Common Emmer Wheat and Coteau were obtained from local grower (Kaul Organic Farm, Harvey, ND-58341, USA). Whole grain seeds from 2015 crop year were used for this study. Whole grain of North Dakota Common Emmer Wheat was dehulled manually. Barlow wheat sample was obtained from North Dakota Research Experiment Station (Fargo, ND).

8.3.3. Sample extraction

The wheat grain samples were milled using a WonderMill^{TM} Grain Mill (WonderMill, Pocatello, ID). The wheat samples before and after milling were extracted using cold water extraction procedure. In this procedure, 10 g of each of the wheat samples were weighed and then homogenized with 50 mL of distilled water using a Waring blender for 5 minutes. The samples were then centrifuged at 8,500 rpm for 20 minutes for two times. The sample extracts were then stored at 4°C during the period of the biochemical analysis.

8.3.4. Total soluble phenolic content

The total soluble phenolic (TSP) content was measured using a modified Folin–Ciocalteu method (Shetty, Curtis, Levin, Witkowsky & Ang, 1995). In this protocol, 0.5 mL of wheat extracts were added to their respective sample test tubes. This was followed by the addition of 1 mL of 95% ethanol, 0.5 mL of 50% (v/v) Folin–Ciocalteu reagent, and 1 mL 5% sodium carbonate to each tube. The contents in each tube were mixed using a vortex mixer and then incubated under dark conditions for 60 min. After incubation, the absorbance of the sample was determined using a UV-visible spectrophotometer (Genesys 10S UV-VIS spectrophotometer, Thermo Scientific, NY) at 725 nm. Total phenolic content was expressed in milligram equivalents of gallic acid per gram of dry weight.

8.3.5. Total protein content

The total protein content of the wheat samples was measured using a modified version of the Bradford assay (Bradford, 1976). The dye reagent (Bio-Rad protein assay kit II, Bio-Rad Laboratory, Hercules, CA) was diluted in 1:4 with distilled water after which 5 mL of the diluted dye was added to 50 μL of the wheat samples, mixed thoroughly, and incubated in the dark for 5 minutes. The absorbance was then measured at 595nm using a UV-visible spectrophotometer. Protein content was expressed as milligram per gram dry weight (mg/g DW).

8.3.6. Total antioxidant activity

The total antioxidant activity was measured using three different assays: 2, 2-Dipheny-1—Picryl hydrazyl (DPPH) free radical scavenging assay, 2, 2-Azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) radical cation assay, and oxygen radical absorbance capacity (ORAC) assay. For the DPPH assay as earlier (Kwon et al, 2006), 1.25 mL of 60mM DPPH (in 95% ethanol) was added to 0.25 mL of sample in microcentrifuge tubes. The tubes were vortexed to

ensure mixing and then incubated for 5 minutes. The absorbance of the supernatant was measured at 517nm using a UV-visible spectrophotometer. For the ABTS assay as described earlier (Re et al 1999), the same procedure was followed except in this case 1 mL of ABTS (in 95% ethanol) was added to 0.05 mL of wheat grain sample or control (95% ethanol), and the absorbance was measured at 734nm using a UV-visible spectrophotometer. The percentage of inhibition for both DPPH and ABTS was calculated using the formula:

$$\% \text{ inhibition} = \frac{\text{Absorbance}^{\text{control}} - \text{Absorbance}^{\text{extract}}}{\text{Absorbance}^{\text{control}}} \times 100$$

The percentages of inhibitory activity obtained from the DPPH and ABTS radical scavenging assays were then expressed as milli molar equivalents of trolox per gram of dry weight (mM TE/g DW).

The ORAC assay was based on a protocol described earlier (Huang, Ou, Hampsch-Woodill, Flanagan & Prior, 2002). The Zen Bio (Zen Bio Inc. Research Triangle Park, NC, USA) ORAC assay kit was used to perform the assay. Briefly, 25 μL of wheat extract (or trolox standards) and 150 μL of fluorescein working solution diluted in 75mM phosphate buffer, pH 7.4 were pipetted into the well of microplate. The mixture was preincubated for 10 min at 37ºC, and then 25 μL of 153 mM 2,2'-azobis-2-methyl-propanimidamide, dihydrochloride (AAPH) was added rapidly using a multichannel pipette. The fluorescence (λex = 485 nm; λem = 520 nm) was recorded every minute for 30 minute using BioTek Synergy H1 Hybrid Multimode fluorescence reader (BioTek Instruments, Inc., Winooski, VT, USA). Area under the curve (AUC) and net AUC of sample was calculated by subtracting blank AUC value and was plotted against Trolox standard net AUC. The ORAC values of the wheat samples were also expressed in mM TE/g dry weight of sample.

8.3.7. Alpha-amylase enzyme inhibitory activity

The assay followed here was based on the protocol as described earlier (Kwon et al, 2006). Undiluted, half and one-fifth diluted sample extracts were used for this assay. The buffer used was 0.1M sodium phosphate (pH 6.9). A volume of 500 µL of each sample extract was added to test tubes while the control tubes had 500 µL of buffer only. Then 500 µL of porcine pancreatic amylase (0.5 mg/mL buffer) was added to all the tubes except for the sample blank and blank tubes and incubated at 25°C for 10 minutes. After incubation, 500 µL of 1% starch (1 g/100 mL buffer) was added to all the tubes and incubated for 10 minutes. The reaction was then stopped by the addition of 1 mL of 3, 5 dinitro salicylic acid, and the tubes were placed in a boiling water bath for 10 minutes. The reaction mixture in the tubes was then diluted by adding 10 mL of distilled water to adjust the absorbance of the control to 1.0, and the absorbance was measured at 540nm using a UV-VIS Genesys spectrophotometer. The percentage of inhibition of α-amylase enzyme activity was calculated based on the following formula:

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

8.3.8. Alpha-glucosidase enzyme inhibitory activity

The assay followed here was based on the protocol as described earlier (Kwon et al, 2006). A volume of 50 µL, 25 µL, and 10 µL of each sample extract was pipetted into 96-well microtiter plates. Then 100 µL of 0.1M potassium phosphate buffer (pH 6.9) containing α-glucosidase enzyme (1 U/mL) was added to each well and incubated at 25°C for 10 minutes. After this, 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 then incubated at 25°C for 5 min. Absorbance readings were taken before (0 minute) and after (5 minute)

incubation period using a microplate reader (Thermomax, Molecular device Co., Virginia, USA) set at 405nm. The percentage of inhibition of α-glucosidase enzyme activity was calculated based on the following formula:

$$\% \text{ inhibition} = \frac{(Abs^{control}\ 5min - Abs^{control}\ 0\ min) - (Abs^{extract} 5min - Abs^{extract} 0\ min)}{(Abs^{control}\ 5min - Abs^{control}\ 0\ min)} \times 100$$

8.3.9. High-performance liquid chromatography analysis of phenolic acid profiles

Two milliliter of sample extracts were filtered through a 0.2 μm filter and then centrifuged for 5 min. A 5 μL volume of sample was injected using Agilent Automatic Liquid Sampler (ALS) 1100 auto sampler into Agilent 1260 series high-performance liquid chromatography (HPLC) (Agilent Technologies, Palo Alto, CA equipped with DAD 1100 diode array detector). The solvents used for gradient elution were (A) 10 mM phosphoric acid (pH 2.5) and (B) 100% methanol. The methanol concentration was increased to 60% for the first 8 min and to 100% over the next 7 minutes, 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 x 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 306 nm and 333 nm and integrated using Agilent Chemstation enhanced integrator. Pure standards in 100% methanol were used to calibrate the standard curve and retention times.

8.3.10. Statistical analysis

The entire experiment was repeated two times. Analysis at every time point from each experiment was carried out in triplicates. Means, standard errors, and standard deviations were calculated using Microsoft Excel XP. The data were analyzed for analysis of variance using Statistical Analytical Software (SAS version 9.4; SAS Institute, Cary, NC). Significant

differences among wheat samples before and after milling (treatment) and wheat sample × treatment interactions were determined using Tukey's least square means separation at the 0.05 probability level.

8.4. Results and Discussion

8.4.1. Total soluble phenolic content and total antioxidant activity

North Dakota Common Emmer originating from ethnic German immigrants of the late 19th century migrating from Russia to North Dakota was evaluated for phenolic bioactive-linked health benefits. To advance health-targeted ethnic food design, it is important to screen the health-relevant phenolic bioactive profile and associated functionalities of targeted foods. Significant differences in TSP content were observed among wheat samples between treatments (before and after milling) and between wheat sample × treatment interactions ($p < 0.05$). North Dakota Common Emmer with hull had significantly higher TSP content ($p < 0.05$), followed by dehulled emmer, Coteau, and Barlow (Table 8.1). The wheat samples after milling gave a significantly higher TSP content than before milling ($p < 0.05$). Previous studies have reported that TSP content can vary among wheat varieties due to locations, genotypic factors, and stages of grain processing (Abdel-Aal & Rabalski, 2008; Beta et al, 2005; Lu et al, 205; Serpen et al, 2008). The TSP content of emmer wheat reported in these studies ranged from 0.51 mg/g to 2.32 mg/g dry weight with ferulic acid being the dominant phenolic acid (Abdel-Aal & Rabalski, 2008; Li, Shewry & Ward, 2008). The high TSP content of hulled North Dakota Common Emmer Wheat in the current study has significant relevance for designing health-targeted ethnic foods from whole grain emmer for dietary support strategies against type 2 diabetes and associated complications. In addition, the high TSP content of North Dakota Common Emmer Wheat after milling might be beneficial as it can be combined with other refined or nonrefined

grain flours to develop additional health benefits for ethnically relevant staple foods such as tortilla, roti, naan, and other flat breads.

Table 8.1. Total soluble phenolic content and antioxidant activity of wheat samples before and after milling.

Wheat sample	TSP*	ABTS†	DPPH‡	ORAC§
Before milling				
Emmer with hull	1.08ab	0.65a	0.22a	4.9a
Emmer without hull	0.89e	0.49b	0.13c	5.2a
Barlow	0.82f	0.48b	0.12c	4.9a
Coteau	0.84ef	0.49b	0.14c	4.9a
After milling				
Emmer with hull	1.11a	0.63a	0.21a	5.0a
Emmer without hull	1.03cb	0.46b	0.12c	4.9a
Barlow	0.97cd	0.47b	0.13c	4.9a
Coteau	0.96d	0.49b	0.16b	4.8a

Different lower-case letters in each column indicate significant wheat sample x treatment interactions (p<0.05).
*Total soluble phenolic content expressed in mg GAE/g DW.
†2, 2-Azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) radical scavenging assay expressed in mM TE/g DW
‡2, 2-Dipheny-1-picryl hydrazyl radical scavenging assay expressed in mM TE/g DW
§Oxygen radical absorbance capacity assay expressed in mM TE/g DW

In this study, the antioxidant activity of the wheat grain samples was determined using DPPH, ABTS, and ORAC radical scavenging assays. Significant differences in ABTS radical scavenging activity were observed among wheat samples between treatments and wheat sample × treatment interactions ($p < 0.05$). North Dakota Common emmer with hull had the highest ABTS radical scavenging activity (Table 8.1). The samples before milling gave a significantly higher ABTS scavenging activity than after milling ($p < 0.05$). A strong positive correlation

between TSP content and ABTS radical scavenging activity were observed before milling (r = 0.98) and after milling (r = 0.82) (Table 8.2). For the DPPH radical scavenging assay, significant differences in radical scavenging activity were observed among wheat samples ($p < 0.05$) but not between treatments. North Dakota Common Emmer with hull had the highest DPPH radical scavenging activity followed by Coteau, emmer without hull, and Barlow (Table 8.1). Before milling, there was a strong positive correlation observed between TSP content and DPPH radical scavenging activity (r = 0.97) (Table 8.2). The moderate to strong correlation between TSP and antioxidant activity suggests that phenolic content is linked to the total antioxidant activity of the samples. For the ORAC assay, there were no significant differences observed in antioxidant activity among the samples (Table 8.1). An earlier study reported a negative correlation (r = −0.35) between phenolic content and antioxidant activity of the water extracts of two wheat cultivars, and a strong positive correlation between phenolic content and antioxidant activity of 80% methanolic extracts for the whole grain and hulled wheat fractions (Zieliński & Kozlowska, 2000). Like in the case of TSP content, the antioxidant activity of wheat can also vary depending on the location and growing conditions, genotypic factors, and stages of grain processing (Abdel-Aal & Rabalski, 2008; Beta et al, 2005; Serpen et al, 2008; Lu et al, 2015). An earlier study reported that the ABTS and DPPH scavenging activities of emmer wheat cultivar "Vernal" are in the range of 21.3 μmole/g and 3.2 μmole/g, respectively (Abdel-Aal & Rabalski, 2008). Another study reported the ABTS scavenging activity of emmer cultivars/accessions ranging from 19 μmole/g to 23.84 μmole/g (Serpen et al, 2008). In our study, high antioxidant activity was observed in North Dakota Common Emmer Wheat, which indicated a potential for protective function of emmer bioactives against chronic oxidative stresses, which is commonly associated with type 2 diabetes. Therefore, emmer wheat can be targeted in ethnic foods to improve diet

against chronic inflammation and associated macrovascular and microvascular complications linked to type 2 diabetes.

Table 8.2. Pearson's correlation analysis of wheat samples before and after milling.

Wheat samples	**ABTS**	**DPPH**	**ORAC**	**TPC***	**α-amylase inhibition**			**α-glucosidase inhibition**		
					1/5 dilution	1/2 dilution	Un-diluted	1/5 dilution	1/2 dilution	Un-diluted
Before milling										
TSP	0.98	0.97	-0.09	-0.38	0.39	0.73	0.12	0.96	0.89	0.93
ABTS		0.99	-0.29	-0.50	0.21	0.58	0.02	0.96	0.96	0.97
DPPH			-0.34	-0.52	0.15	0.53	0.02	0.94	0.96	0.98
ORAC				0.79	0.87	0.59	0.56	-0.22	-0.54	-0.38
TPC					0.51	0.18	0.82	-0.59	-0.66	-0.45
α amylase										
1/5 dilution								0.29		
1/2 dilution									0.33	
undiluted										0.13
After milling										
TSP	0.82	0.68	0.89	0.11	-0.72	0.53	-0.08	0.59	0.97	0.98
ABTS		0.96	0.71	0.23	-0.76	0.91	-0.63	0.04	0.67	0.82
DPPH			0.47	0.47	-0.55	0.89	-0.75	-0.11	0.48	0.64
ORAC				-0.36	-0.92	0.52	-0.01	0.49	0.92	0.96
TPC					0.46	0.09	-0.30	0.00	-0.06	-0.08
α amylase										
1/5 dilution								-0.12		
1/2 dilution									0.35	
undiluted										-0.08

ABTS, 2, 2-Azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid); DPPH, 2, 2-Dipheny-1—Picryl hydrazyl; ORAC, oxygen radical absorbance capacity; TPC, total protein content; TSP, total soluble phenolic.

8.4.2. Total protein content

There were no significant differences in protein content among the samples, with significant differences between treatments and sample × treatment interactions ($p < 0.05$). Wheat samples had significantly higher protein content before milling than after milling ($p < 0.05$), and North Dakota Common Emmer without hull (before milling) had higher protein content ($p < 0.05$) (Figure 8.2). An earlier study reported that hulled wheat cultivars of emmer wheat tend to

have higher protein content than their corresponding dehulled counterparts (Piergiovanni, Laghetti & Perrino, 1996). Another study reported wild type emmer varieties to have higher protein content than the domesticated emmer varieties (Chatzav et al, 2010).

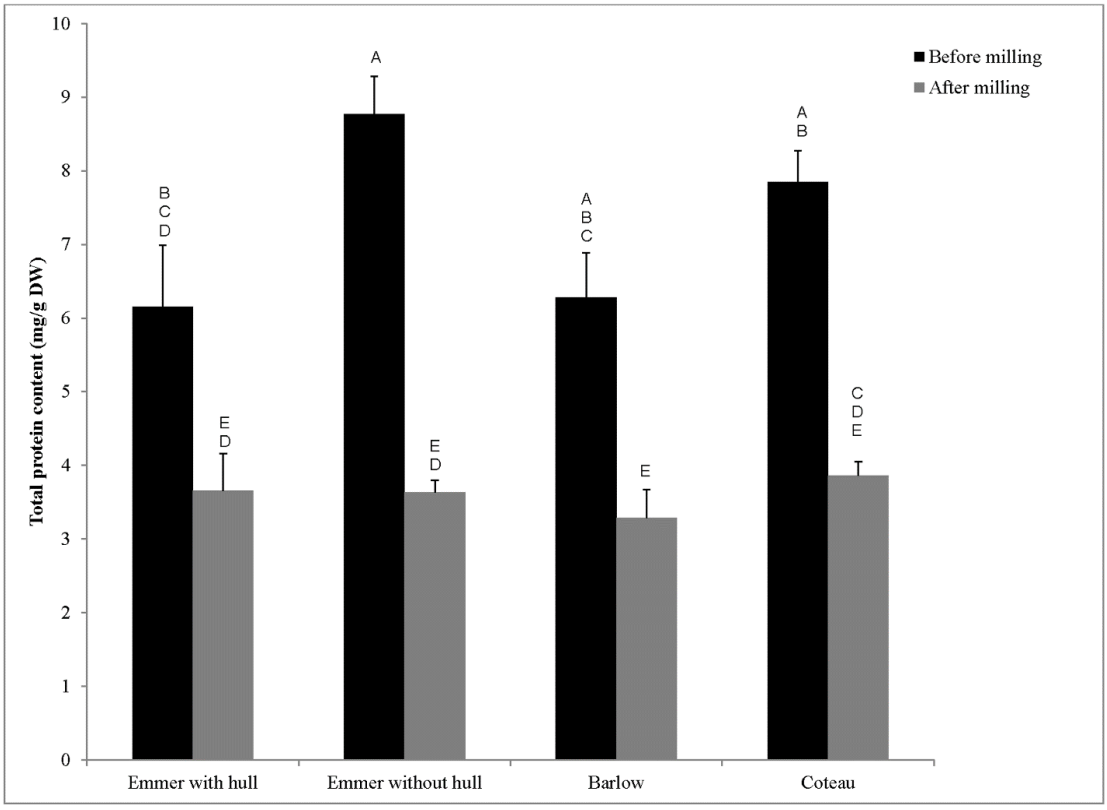


Figure 8.2. Total protein content (mg/g dry weight) of North Dakota Common Emmer with and without hull, and it was compared with commercial wheat cultivars such as Barlow and Coteau before and after milling. Different uppercase letters represent significant differences in protein content between wheat sample × treatment interactions (before and after milling) at 95% confidence level. Protein content of wheat is an important quality parameter and determines its market value. North Dakota Common Emmer without hull had higher protein content before milling and with milling protein content of all wheat sample reduced significantly.

8.4.3. Alpha-amylase enzyme inhibitory activity

The α-amylase inhibitory activity was determined for the undiluted, half and one-fifth dilutions of the wheat grain. For the undiluted and half-diluted wheat samples, no significant differences in α-amylase inhibitory activity were observed (Figure 8.3). For the half-diluted

samples, a moderately positive correlation was observed between TSP content and α-amylase inhibitory activity before milling (r = 0.73) and after milling (r = 0.53) (Table 8.2).

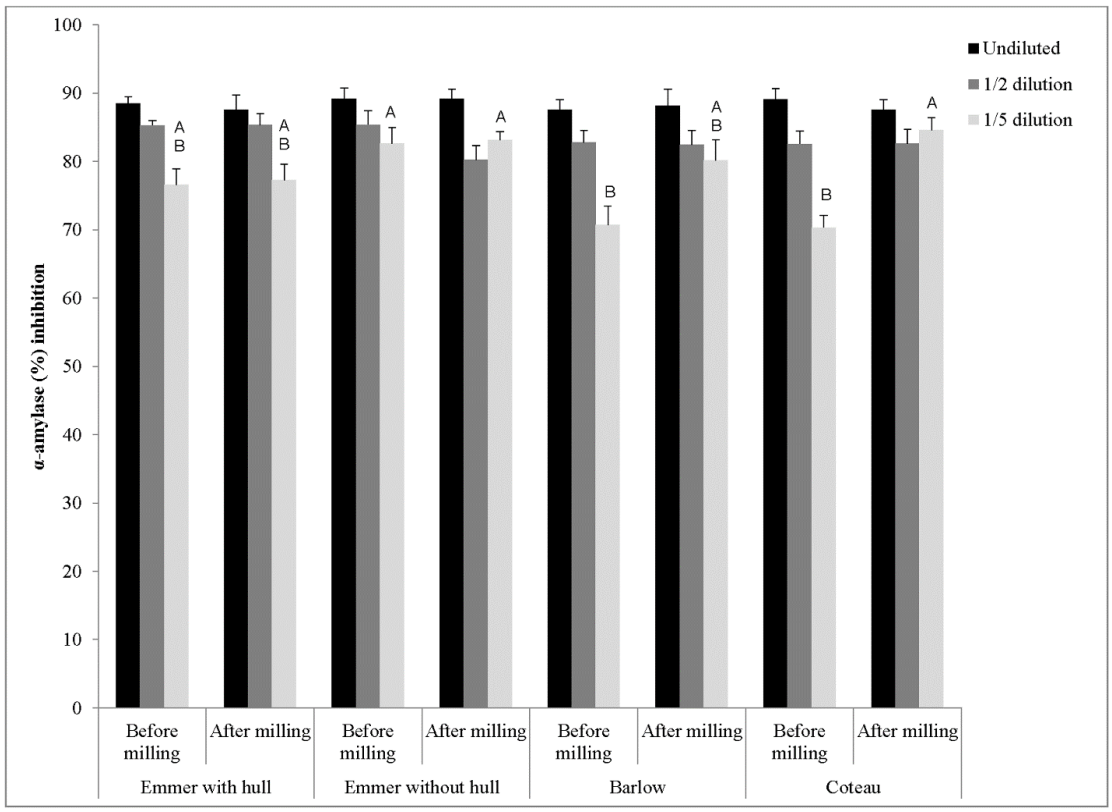


Figure 8.3. Dose-dependent (undiluted, half, and one-fifth) response in α-amylase inhibitory activity (% inhibition) of North Dakota Common Emmer with and without hull and was compared with commercial wheat cultivars such as Barlow and Coteau before and after milling. No statistically significant differences were observed between wheat sample and treatments in undiluted and half diluted extracts. However, wheat sample with one-fifth dilution had statistically significant differences between wheat sample × treatment interactions (before and after milling) at 95% confidence level and represented by different uppercase letters. Alpha-amylase is a key digestive enzyme responsible for *in vivo* carbohydrate metabolism, and inhibition of this enzyme is critical for managing postprandial glucose homeostasis.

For the one-fifth diluted samples significant differences in α-amylase inhibitory activity were observed among samples, between treatments, and sample × treatment interactions (p < 0.05). Dehulled emmer had highest α-amylase inhibitory activity followed by Coteau, hulled emmer, and Barlow (Figure 8.3), and the samples after milling gave a significantly higher α-

amylase inhibitory activity than before milling ($p < 0.05$). The high α-amylase inhibitory activity of all samples has significant relevance for its potential dietary and therapeutic support to potentially maintain postprandial glucose homeostasis, which is essential for prevention and overall management of early stages type 2 diabetes.

8.4.4. Alpha-glucosidase enzyme inhibitory activity

The α-glucosidase inhibitory activity was measured among undiluted, half diluted and one-fifth diluted samples before and after milling. For the undiluted samples, significant differences in α-glucosidase inhibitory activity were observed among samples, between treatments (before and after milling) ($p < 0.05$), and between sample × treatment interactions. Hulled North Dakota Common Emmer Wheat had the highest α-glucosidase enzyme inhibitory activity followed by Coteau, dehulled emmer, and Barlow (Figure 8.4), and the samples before milling had significantly higher α-glucosidase enzyme inhibitory activity than after milling ($p < 0.05$). There was a strong positive correlation between TSP content and α-glucosidase inhibitory activity before milling ($r = 0.93$) and after milling ($r = 0.98$) (Table 8.2.). For the half and one-fifth diluted samples, there were significant differences in α-glucosidase inhibitory activity among sample and between treatments as well as significant sample × treatment interactions ($p < 0.05$). Hulled North Dakota Common Emmer wheat had the highest α-glucosidase inhibitory activity followed by dehulled emmer, Barlow, and Coteau (Figure 8.4). An earlier study attributed the α-glucosidase inhibitory activity of wheat bran and wheat germ to the presence of phosphatidic acids that could bind to the substrate binding site on the enzyme thereby inhibiting enzymatic activity (Liu et al, 2011). However, in the current study, the moderate to strong positive correlation between TSP and α-glucosidase inhibitory activity suggests that the phenolic

content could play a vital role in inhibition of this key enzyme responsible for breakdown of carbohydrates and uptake of glucose in the small intestine.

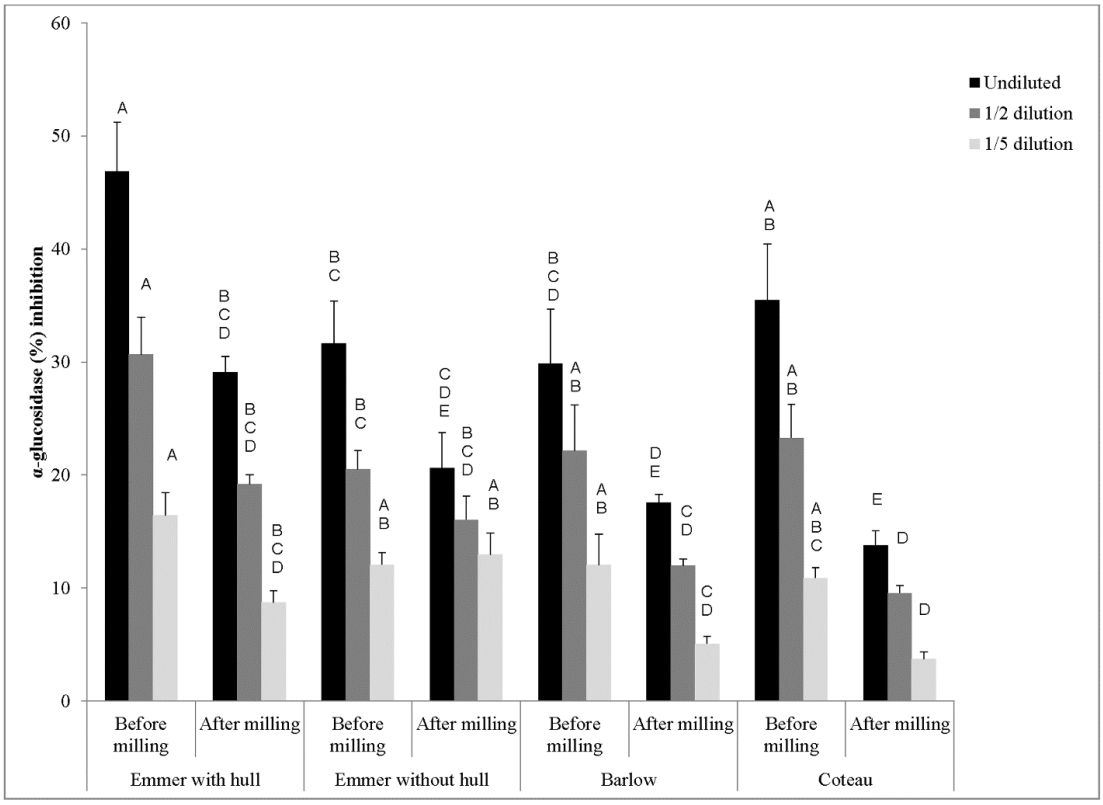


Figure 8.4. Dose-dependent (undiluted, half, and one-fifth) response in α-glucosidase inhibitory activity (% inhibition) of North Dakota Common Emmer with and without hull and was compared with commercial wheat cultivars such as Barlow and Coteau before and after milling. Statistically significant differences between wheat sample × treatment interactions (before and after milling) were observed in all dilutions at 95% confidence level and represented by different uppercase letters. Alpha-glucosidase is a key digestive enzyme responsible for *in vivo* carbohydrate metabolism, and by inhibiting the activity of this enzyme, the release and absorption of glucose in the bloodstream can be delayed which is critical for managing postprandial glucose level in the blood.

High α-amylase and moderate α-glucosidase inhibitory activity of North Dakota Common Emmer Wheat with hull in this study has significant relevance for its potential use in health relevant ethnic food design targeting against chronic hyperglycemia commonly associated with type 2 diabetes. Therefore, flour of emmer wheat with hull can be strategically utilized with other grain flours to make common wheat-based ethnic staple foods targeting communities who

have higher burden of hyperprocessed diet and lifestyle-linked NCDs, such as type 2 diabetes, cardiovascular disease, and obesity.

8.4.5. Phenolic acid profile

Major phenolic acids of samples were determined using HPLC method. Gallic acid was the major phenolic acid found in all samples with significant differences in gallic acid content among samples, between treatments (before and after milling) as well as significant sample × treatment interactions ($p < 0.05$). Hulled North Dakota Common Emmer had higher gallic acid content followed by dehulled emmer, Coteau, and Barlow (Table 8.3) ($p < 0.05$).

Table 8.3. Phenolic acid profile of wheat samples before and after milling

Wheat grain samples	***m*-coumaric acid***	**Gallic acid†**
Before milling		
Emmer with hull	10.14±0.58a	7.23±0.31b
Emmer without hull	ND‡	3.66±0.01c
Barlow	ND	2.86±0.03cd
Coteau	ND	3.35±0.06cd
After milling		
Emmer with hull	7.32±0.09b	7.99±0.24a
Emmer without hull	ND	2.83±0.02cd
Barlow	ND	2.32±0.05d
Coteau	ND	2.53±0.14d

Different lower-case letters indicate significant wheat sample × treatment interactions ($p < 0.05$).
*Mean ± standard error expressed in μg/g.
†Not detected.

Interestingly, the phenolic acid, m-coumaric acid, was detected only in hulled North Dakota Common Emmer before and after milling and not in dehulled emmer and the other wheat samples Barlow and Coteau. This result indicates that m-coumaric acid is predominantly found

in the hull and not in the grain. In this study, we did not observe other phenolic acids commonly found in wheat grains such as ferulic acid, benzoic acid, and catechin, and the reason for this could be due to the food grade extraction method (cold water extraction) and the HPLC protocol utilized in this study which only allowed for the detection of gallic acid and m-coumaric acid.

8.5. Conclusion

Wheat is one of the leading staple cereal crops in the world and wheat-based foods are part of traditional diets in many ethnic communities meeting critical nutritional needs. However, increasingly many parts of the world are facing new health challenges of food security and public health due to NCD epidemics such as type 2 diabetes resulting from higher intake of refined macronutrients such as soluble carbohydrates and related lifestyles causes. Emmer wheat originating with the 19th century migration to North Dakota is tolerant to many biotic and abiotic stresses and based on this stress-linked metabolic rationale emmer wheat with higher soluble phenolic and antioxidant content can be targeted for improving diets against diet-linked chronic diseases, such as type 2 diabetes. North Dakota Common Emmer Wheat had significantly high phenolic antioxidant-linked antihyperglycemic properties in hull both before and after milling. This suggests that North Dakota Common Emmer Wheat with hull can be combined with other wheat and non-wheat grain flours to improve human health-relevant nutritional qualities of wheat based on ethnically relevant staple foods such as tortilla, roti, naan, and other flatbreads. Future fermentation and processing studies of North Dakota Common Emmer with lactic acid bacteria could improve phenolic antioxidant-linked antihyperglycemic properties. This study provides a framework to advance knowledge of ethnic grains like North Dakota Common Emmer which has relevance for building food security and resilience with global climate change.

CHAPTER 9. IMPROVING PHENOLIC-LINKED ANTIOXIDANT, ANTIHYPERGLYCEMIC AND ANTIBACTERIAL PROPERTIES OF EMMER AND CONVENTIONAL WHEAT USING BENEFICIAL LACTIC ACID BACTERIA

9.1. Abstract

Beneficial lactic acid bacteria (LAB)-based fermentation is an effective bioprocessing approach to improve human-health-targeted functional benefits of plant-based food substrates, such as cereal grains. Previously, we observed high phenolic bioactive-linked antioxidant and anti-hyperglycemic properties in whole grain Emmer (hulled). In this study, beneficial LAB (*Lactiplantibacillus plantarum*) was recruited to ferment (0–72 h) aqueous extracts (0.4 g/mL concentration) of previously optimized hulled Emmer wheat and conventional red spring wheat cv. Barlow. The fermented and unfermented (control) wheat extracts were analyzed for phenolic content, phenolic profile, antioxidant activity, and antihyperglycemic properties (α-amylase and α-glucosidase enzyme inhibitory activity) using in vitro assay models. Additionally, antimicrobial activity against pathogenic bacteria *Helicobacter pylori*, and potential prebiotic activity supporting the growth of beneficial *Bifidobacterium longum* were also investigated. Improvement in antioxidant activity and antihyperglycemic functional benefits were observed, while soluble phenolic content remained high after 72 h fermentation. Antimicrobial activity against *H. pylori* was also observed in 48 and 72 h fermented wheat extracts. This study provides an insight into the efficacy of LAB-based fermentation as a safe bioprocessing tool to design health-targeted functional foods and ingredients from underutilized whole grains like Emmer for targeting type 2 diabetes dietary benefits.

Key words: Ancient Wheat, Antimicrobial, Antioxidant, Antihyperglycemic, Lactic Acid Bacteria, Fermentation, Phenolics

9.2. Introduction

The fermentation of cereal grains with beneficial lactic acid bacteria (LAB) is a useful bioprocessing approach to enhance nutritional, organoleptic, and bioactive properties, which has wider relevance in health-targeted food applications. There are many dietary advantages associated with the LAB-based fermentation of whole and processed grains, such as (i) the enhanced mobilization and solubilization of health-relevant bioactive nutrients, (ii) the improvement in the stability of the functional properties of bioactive compounds, (iii) the addition of potential prebiotic and probiotic functions, (iv) the food-safety benefits associated with the improved antimicrobial property, and (v) the removal of undesirable toxins or antinutrients (Skrajda-Brdak, Konopka, Tańska & Czaplicki, 2019; Zhou et al, 2020). Such improvements with LAB fermentation have dual functional benefits for enhancing post-harvest preservation or shelf-life and for the improvement of human-health-targeted nutritional qualities of cereal-grain-based food substrates (Rakhmanova, Khan & Shah, 2018).

Globally, there is a renewed interest in the fermentation-based bioprocessing of cereal grains, which is mostly driven by an increasing consumer awareness of the potential health benefits of fermented foods and beverages. Additionally, cereal grains that are rich in human-health-protective bioactive compounds, dietary fibers, and minerals are also good substrate sources for supporting the growth and viability of beneficial bacteria, such as LAB. Therefore, targeting a LAB-based fermentation strategy to enhance the human-health-relevant bioactive and nutritional qualities of cereal-grain-based functional foods and ingredients and concurrently improving their keeping qualities have significant merit. These cereal-grain-based fermented foods with enhanced nutritional profile and bioactive functionality have wider relevance for their value-added integration in dietary support strategies to help address an increasing prevalence of

diet- and lifestyle-influenced chronic diseases, such as type 2 diabetes, cardiovascular diseases, obesity, and dyslipidemia.

Among different cereal grains, whole wheat is an excellent substrate source that supports the growth of single or mixed bacterial starter cultures, including bacterial combined cultures as well as probiotic cultures containing beneficial human gut microbes such as LAB (Charalampopoulos, Pandiella & Webb, 2002; Gobetti, 1998; Paramithiotis, Chouliaras, Tsakalidou & Kalantzopoulos, 2005; Paramithiotis, Gioulatos, Tsakalidou & Kalantzopoulos, 2006; Plessas et al, 2011; Robert et al, 2006). The fermentation of wheat with LAB potentially enhances its bioactive functionality due to beneficial changes in the nutritional, rheological, and organoleptic properties of the acidified dough matrix, along with an improvement in the shelf-life of wheat-based fermented foods (Arendt, Ryan & Dal Bello, 2007; Clarke, Schober, Dockery, O'Sullivan & Arendt, 2004; Corsetti et al, 2000; Dal Bello et al, 2007; Gül, Özçelik, Sağdıç & Certel, 2005; Leenhardt, Levrat-Verny, Chanliaud & Rémésy, 2005; Palacios, Haros, Sanz & Rosell, 2008; Thiele, Gänzle & Vogel, 2002). Emmer, an ancient and underutilized wheat, has a good nutritional and bioactive profile, thereby making it an attractive choice as a source for functional food and dietary ingredients with human-health-relevant benefits (Çakmak et al, 2004; Chatzav et al, 2010; Chen et al, 2013; Christopher, Sarkar, Zwinger & Shetty, 2018b; Nevo, 2014; Peleg et al, 2008; Stallknecht et al, 1996; Serpen et al, 2008; Zaharieva et al, 2010). Previously, we reported high phenolic bioactive-linked antioxidant and antihyperglycemic functionalities in aqueous extracts of hulled Emmer wheat with its potential relevance as a dietary target against type 2 diabetes (Christopher et al, 2018b). Additionally, another study found that the fermentation of Emmer flour, gelatinized Emmer flour, and Emmer malt with *Lactobacillus, Weissella* and *Pediococcus* spp., improved the physical, chemical, nutritional, and

sensory properties with the aim of developing commercial Emmer-based fermented beverages with probiotic functions (Coda, Rizzello, Trani & Gobbetti, 2011). These studies suggest that Emmer wheat is a promising substrate source for designing LAB-based fermented foods and beverages with diverse human-health-targeted functional benefits.

Therefore, the aim of this current study was to recruit *Lactiplantibacillus plantarum* (previously known as *Lactobacillus plantarum*), a widely distributed LAB in the plant-based food matrix, to ferment previously optimized (Christopher et al, 2018b) aqueous extracts of hulled North Dakota common Emmer and modern-day hard red spring wheat cv. Barlow to improve the antioxidant, antihyperglycemic, and antimicrobial properties. The potential efficacy of LAB fermentation to modulate the total soluble phenolic content (TSP), phenolic profile, antioxidant activity, glycemic control via α-amylase and α-glucosidase enzyme inhibitory activities, antimicrobial activity against *Helicobacter pylori*, and prebiotic property supporting the growth of beneficial *Bifidobacterium longum* were determined using rapid in vitro assay models. The wider goal was to provide insights on how such a bioprocessing approach involving LAB-based fermentation strategy can positively influence the health-targeted bioactive-linked functional properties such as the composition of health-relevant phenolic compounds and the associated antioxidant, antihyperglycemic, antimicrobial, and prebiotic properties, which can be subsequently integrated into dietary support strategies targeting wider health benefits.

9.3. Materials and Methods

9.3.1. Preparation of wheat extracts

In this study, the previously screened wheat varieties (Christopher et al, 2018b), hulled North Dakota Common Emmer and hard red spring wheat cv. Barlow, were obtained from Kaul Organic Farms (Harvey, ND-58341, USA) and from the North Dakota Research Experiment

Station (North Dakota State University Wheat Breeding Program, Fargo, ND-58108) respectively. The year of harvest for these wheat varieties was 2017. Using a WonderMill (WonderMill, Pocatello, ID, USA), the wheat grains (Emmer with hull and Barlow without hull) were milled at the pastry setting to obtain a fine flour. Extraction was done in duplicate using the cold-water extraction procedure as described earlier (Christopher et al, 2018b). For this study, 20 g of the wheat flour was blended in 50 mL distilled cold water in a Warring Blender at low speed for 5 min. The concentration of the Emmer and Barlow wheat extracts was 0.4 g/mL. The extracts were centrifuged at 8500 rpm for 20 min, and the supernatant was collected and re-centrifuged at 8500 rpm for 15 min, and the extracts were stored at 4 °C. Prior to fermentation, the wheat extracts were pasteurized in a water bath at 70 °C for 20 min, after which the pasteurized extracts were immersed in an ice bath to ensure rapid cool-down to room temperature.

9.3.2. Bacterial strains used

The bacterial strains used in this study were *Lactiplantibacillus plantarum* (ATCC 8014), *Bifidobacterium longum* (ATCC 15708), and *Helicobacter pylori* (ATCC 43579). The LAB and *H. pylori* cultures were stored as frozen stocks in their respective MRS (Difco), and *H. pylori* special peptone (HPSP) broths, with both media containing 20% glycerol as the cryoprotectant. The revival and growth of the bacterial stocks was done according to a protocol described earlier (Ranilla et al, 2017). For the HPSP broth, 10 g L^{-1} of special peptone (Oxoid, Basingstoke, UK), 5 g L^{-1} of sodium chloride (Fisher Scientific, Waltham, MA, USA), 5 g L^{-1} of yeast extract (Difco), and 5 g L^{-1} of beef extract (Difco) were dissolved in distilled water and autoclaved. The MRS broth was prepared according to the manufacturer's specifications and autoclaved. The MRS and HPSP agar plates were prepared by the addition of 15 g L^{-1} of

granulated agar (Difco) to the respective broths, followed by autoclaving. For revival of the frozen bacterial stocks, 100 µL of thawed LAB and *H. pylori* stocks were inoculated in 10 mL sterile MRS or HPSP broth and incubated at 37 °C for 24 h, after which 100 µL of the 24 h culture was re-inoculated into 10 mL of sterile MRS or HPSP broth and incubated at 37 °C for another 24 h. The cultures were then used in the respective fermentation, antimicrobial, and prebiotic assays.

9.3.3. Wheat fermentation

Fermentation was done based on a protocol as described earlier (Ankolekar, Pinto, Greene & Shetty, 2012) in which 10 mL of the revived *L. plantarum* culture was added to 90 mL of pasteurized wheat extracts in sterile 125 mL flasks. For the control or unfermented extracts, 10 mL of sterile MRS broth was added. The extracts were then incubated in duplicate at 37 °C for a period of 72 h, and samples were collected at the 0, 24, 48, and 72 h time points of fermentation for in vitro analysis. The growth of *L. plantarum* was measured at each time point by serially diluting the fermented extracts, followed by plating onto MRS agar plates. The plates were incubated anaerobically at 37 °C for 48 h in BBL GasPak jars (Becton, Dickinson & Co., Sparks Glencoe, MD, USA) containing the BD GasPak EZ anaerobe container system sachets (Becton, Dickinson & Co), and the number of colonies was expressed in log CFU/mL. The samples collected at each time point were centrifuged at 8500 rpm for 15 min, after which the supernatant was collected, and the pH of one the duplicates of the fermented extract was adjusted using 1M NaOH to match its control (unfermented extract, pH 6.3–6.6), while an equal amount of distilled water was added to the duplicate of the fermented extract to maintain natural acidic pH (without adjustment). The unfermented and fermented extracts (with and without pH adjustment) were analyzed for their TSP content, antioxidant activity, and enzyme inhibitory

activity against α-amylase and α-glucosidase at the 0, 24, 48, and 72 h time points of the fermentation period. Also, samples of the unfermented and fermented extracts (with and without pH adjustment) at each time point was filtered through sterile 0.22 µm syringe filters (Millipore Corp, Burlington, MA, USA) and stored at −20 °C for later analysis of phenolic compounds, antimicrobial activity, and prebiotic function.

9.3.4. Total soluble phenolic content

The soluble phenolic content of the unfermented and fermented wheat extracts (0.4 g/mL) was determined using the Folin–Ciocalteu method based on a protocol as described previously (Shetty et al, 1995). For this assay, 0.5 mL aliquots of the unfermented and fermented wheat extracts were taken into respective glass tubes, after which 1 mL of 95% ethanol, 0.5 mL of 50% (*v/v*) Folin–Ciocalteu reagent, and 1 mL of 5% sodium carbonate were added sequentially. The tubes were mixed by a vortex machine and incubated for 60 min under dark conditions. The absorbance values were measured at 725 nm with a UV-visible spectrophotometer (Genesys 10S UV-VIS spectrophotometer, Thermo Scientific, New York, NY, USA). Using a standard curve of different concentrations of gallic acid in 95% ethanol, the absorbance values of the extracts were converted and expressed as TSP content in milligrams of gallic acid equivalents per gram of dry weight (mg GAE/g DW).

9.3.5. Phenolic compound characterization (high-performance liquid chromatography assay)

The profile of the phenolic compounds was determined using a high-performance liquid chromatography (HPLC) assay method, in which 5 µL of the unfermented and fermented wheat extracts was injected using an Agilent ALS 1200 auto-extractor into an Agilent 1260 series (Agilent Technologies, Palo Alto, Santa Clara, CA, USA) HPLC equipped with a D1100 CE

diode array detector. The solvents used for gradient elution were 10 mM phosphoric acid (pH 2.5) and 100% methanol. The methanol concentration was increased to 60% for the first 8 min, then to 100% over the next 7 min, then decreased to 0% for the next 3 min and was maintained for 7 min with a total run time of 25 min per injected sample run. 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 0.7 mL/min at room temperature. The absorbance values were recorded at 214 nm, 230 nm, 260 nm, and 306 nm, and the chromatogram was integrated using Agilent Chem station enhanced integrator. Pure standards of benzoic acid, gallic acid, protocatechuic acid, ferulic acid, and catechin in 100% methanol were used to calibrate the respective standard curves and retention times. The phenolic compounds detected in the extracts were expressed in microgram per gram of dry weight (μg/g DW). Chromatograms of detected phenolic compounds of fermented and unfermented wheat extracts are included in the result and discussion section.

9.3.6. Antioxidant activity assay

The antioxidant activity of the unfermented and fermented wheat extracts was measured by their scavenging activity against the free radicals 2, 2-dipheny-1-picryl hydrazyl (DPPH) (D9132-5G, Sigma-Aldrich, St. Louis, MO, USA), and 2, 2-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) (A1888-5G, Sigma-Aldrich) respectively. The DPPH scavenging assay was based on a protocol as described earlier (Kwon et al, 2006), in which 0.25 mL of the wheat extracts was added to 1.25 mL of 60 mM DPPH prepared in 95% ethanol, while the controls had 0.25 mL of 95% ethanol instead of the sample extract. After 5 min of incubation, the extracts and their corresponding controls were centrifuged at 13,000 rpm for 1 min. and the absorbance values of the supernatants was measured at 517nm using a UV-visible spectrophotometer (Genesys 10S UV-VIS spectrophotometer, Thermo Scientific, NY). The ABTS scavenging assay

was based on a protocol as described earlier (Re et al, 1999), in which 0.05 mL of the wheat extracts was added to 1 mL of ABTS prepared in 95% ethanol, while the controls had 0.05 mL of 95% ethanol in 1 mL ABTS. After 2 min of incubation, the extracts and their controls were centrifuged at 13,000 rpm for 1 min, and the absorbance values of the supernatant was measured at 734 nm with a UV-visible spectrophotometer (Genesys 10S UV-VIS spectrophotometer, Thermo Scientific, NY). For the ABTS and DPPH assays, Trolox was prepared in 95% ethanol and serially diluted to give different concentrations (10, 5, 2.5, 1.25, 0.625, and 0.3125 mg/mL), which was used as the positive control. The absorbance values from the DPPH and ABTS radical scavenging assays were used to calculate the percentage of antioxidant activity for each extract using the following formula:

$$\% \text{ inhibition} = \frac{\text{Absorbance}^{\text{control}} - \text{Absorbance}^{\text{extract}}}{\text{Absorbance}^{\text{control}}} \times 100$$

9.3.7. Alpha -amylase enzyme inhibitory activity

The α-amylase enzyme inhibitory activity of the unfermented and fermented wheat extracts was measured based on a protocol as described earlier (Kwon et al, 2006), and the activity was measured in a dose-dependent manner using undiluted, half, and one-fifth diluted sample extracts. The sample extracts were diluted using distilled water, and 500 µL of undiluted and diluted extracts were added to respective 1 mL glass tubes, while the control tubes had 500 µL of 0.1M sodium phosphate buffer (containing 0.006M sodium chloride at pH 6.9). Each extract had a corresponding sample blank tube containing only 500 µL of the sample extract and buffer instead of the enzyme. Then 500 µL of porcine pancreatic α-amylase (0.5 mg/mL buffer) (EC 3.2.1.1, purchased from Sigma Chemical Co, St Louis, MO, USA) was added only to the sample and control tubes and incubated for 10 min at 25 °C. After incubation, 500 µL of the

substrate (1% starch in buffer) was added to all of the tubes and incubated again for 10 min at 25 °C. Then 1 mL of 3, 5 dinitro salicylic acid was added, and the tubes were incubated in a boiling water bath for 10 min to stop the reaction. After removing the tubes from the water bath and cooling them down to room temperature, 10 mL of distilled water was added to all the tubes to ensure that the absorbance values in the control tubes ranged between 1.0 and 1.2, and the absorbance of all the tubes was measured at 540 nm using a UV-visible spectrophotometer (Genesys 10S UV-VIS spectrophotometer, Thermo Scientific, NY). A positive control with acarbose was prepared in distilled water and serially diluted to give different concentrations (10, 5, 2.5, 1.25, 0.625, and 0.3125 mg/mL), which was included in the study for comparison. The absorbance values were then used to calculate the percentage of enzyme inhibitory activity of the fermented and unfermented sample extracts using the following formula:

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

9.3.8. Alpha-glucosidase enzyme inhibitory activity

The α-glucosidase enzyme inhibitory activity of the unfermented and fermented wheat extracts was measured based on a protocol as described earlier (Kwon et al, 2006). The enzyme inhibitory activity was measured in a dose-dependent manner at undiluted, half, and one-fifth dilutions of the sample extracts. The extracts were diluted with 0.1M potassium phosphate buffer (pH 6.9) in 96-well micro titer plates in which 50 µL, 25 µL, and 10 µL of each extract was pipetted, and the final volume was increased to 50 µL by the addition of potassium phosphate buffer. Each extract had a corresponding control of 50 µL (buffer instead of sample), and the volume in all the wells was made to up to a final volume of 100 µL by the addition of 50 µL of buffer. Then 100 µL of buffer containing yeast α-glucosidase enzyme (1 U/mL) (EC 3.2.1.20,

purchased from Sigma Chemical Co, St. Louis, MO, USA) was added to each well and incubated for 10 min at 25 °C, after which 50 µL of the substrate and 5 mM p-nitrophenyl-α-d-glucopyranoside solution (prepared in buffer) was added to each well, followed by 5 min of incubation at 25 °C. A positive control with acarbose was prepared in distilled water and serially diluted to give different concentrations (10, 5, 2.5, 1.25, 0.625, and 0.3125 mg/mL), which were included in the study for comparison. The absorbance of all of the wells was measured at 405 nm using a microplate reader (Thermomax, Molecular Devices, San Jose, CA, USA) at the 0- and 5-min time point of the 5 min incubation period, and the absorbance values were used to calculate the percentage of enzyme inhibitory activity using the following formula:

$$\% \text{ inhibition} = \frac{(Abs^{control}\ 5min - Abs^{control}\ 0\ min) - (Abs^{extract} 5min - Abs^{extract} 0\ min)}{(Abs^{control}\ 5min - Abs^{control}\ 0\ min)} \times 100$$

9.3.9. Antimicrobial activity

The antimicrobial activity of the unfermented and fermented wheat extracts against *Helicobacter pylori* was measured using the agar diffusion method (Ranilla et al, 2017). Frozen *H. pylori* culture was revived and streaked onto HPSP agar plates with the help of sterile cotton swabs. Then sterile 12.7 mm paper discs (BBL Taxo, Becton, Dickinson & Co, Sparks, MD, USA) were placed on the HPSP agar plates, and 100 µL of the filter-sterilized wheat extracts from the 0, 24, 48, and 72-h time points were added to their respective paper discs with each plate having a control disc of sterile water. The plates were incubated at 37 °C for 48 h in BBL GasPak jars (Becton, Dickinson & Co) containing BD GasPak Campy container system sachets (Becton, Dickinson & Co, Sparks, MD, USA) to help maintain a microaerophilic environment. After incubation, the plates were examined for any zones of inhibition (no growth) around the discs, and the diameter of the zones of inhibition was measured in millimeters.

9.3.10. Prebiotic activity

The potential prebiotic activity of the unfermented and fermented wheat extracts was measured using the LAB proliferation prebiotic assay to determine the growth of *Bifidobacterium longum* (Ranilla et al, 2017). Frozen *B. longum* culture was revived and diluted 100-fold by the addition of 100 µL of culture to 9.9 mL sterile water. This diluted culture was used as the inoculum for the LAB proliferation assay, in which 1 mL of the filter-sterilized extracts were added to their respective tubes containing 9 mL sterile MRS broth, while the control tube had 1 mL of sterile water instead of the extract. This was followed by the inoculation of 100 µL of the diluted *B. longum* culture to each tube, and the tubes were incubated for 48 h, during which time the growth of *B. longum* was measured at the 0, 6, 12, 24, and 48 h time points. At each assay time point, 100 µL of the unfermented and fermented extracts containing *B. longum* were serially diluted in sterile water, and the dilutions plated onto MRS agar plates followed by incubation at 37 °C for 48 h in anaerobic BBL GasPak jars (Becton, Dickinson & Co., Sparks, MD, USA) containing BD GasPak EZ anaerobe container system sachets with indicator (Becton, Dickinson & Co) to help maintain an anaerobic environment. The number of colonies in the control and extract plates were counted and expressed as log CFU/mL.

9.3.11. Statistical analysis

Wheat fermentation, characterization of phenolic compounds and all in vitro assays were repeated twice with repeat extractions and duplicate samples. The mean and standard error were calculated from 12 (n) data points using Microsoft Excel XP software. The analysis of covariance was determined using Statistical Analysis Software (SAS version 9.4, SAS Institute, Cary, NC, USA). Statistical mean separation between sample extracts, fermentation time points, and sample extract × fermentation time point interactions were determined using Tukey's test at

a 0.05 probability level. Statistically significant differences between sample extract × fermentation time point interactions are presented in the figures and tables. For ABTS-based antioxidant activity results, statistically significant differences between fermentation time points are presented, as sample extract × fermentation time point interactions had no significant effect on this parameter.

9.4. Results and Discussion

9.4.1. Total soluble phenolic content and phenolic profile

The nutritional and other bioactive-associated health benefits of plant-based fermented foods and beverages largely depends on their ability to retain and stabilize protective functional bioactive compounds like phenolics beyond the basic macronutrient profiles. The increasing demand for plant-based fermented foods and beverages is partly based on the wider perception of consumers about the potential bioactive enrichment and associated improvement of wider health-relevant nutritional and functional benefits. In general, the physio-chemical properties of a fermented food matrix change substantially during fermentation, which influences the composition and functional bioactivity of phenolic compounds present in plant-based food substrates. Therefore, to develop health-targeted fermented foods and beverages, it is important to understand the impact of fermentation on the composition and content of phenolic bioactives and their health-protective functional benefits, such as antioxidant, antihyperglycemic, antimicrobial, and prebiotic properties. In this study, the TSP content of fermented and unfermented wheat extracts (0.4 g/mL) ranged between 0.61 to 0.76 mg GAE/g DW (Figure 9.1).

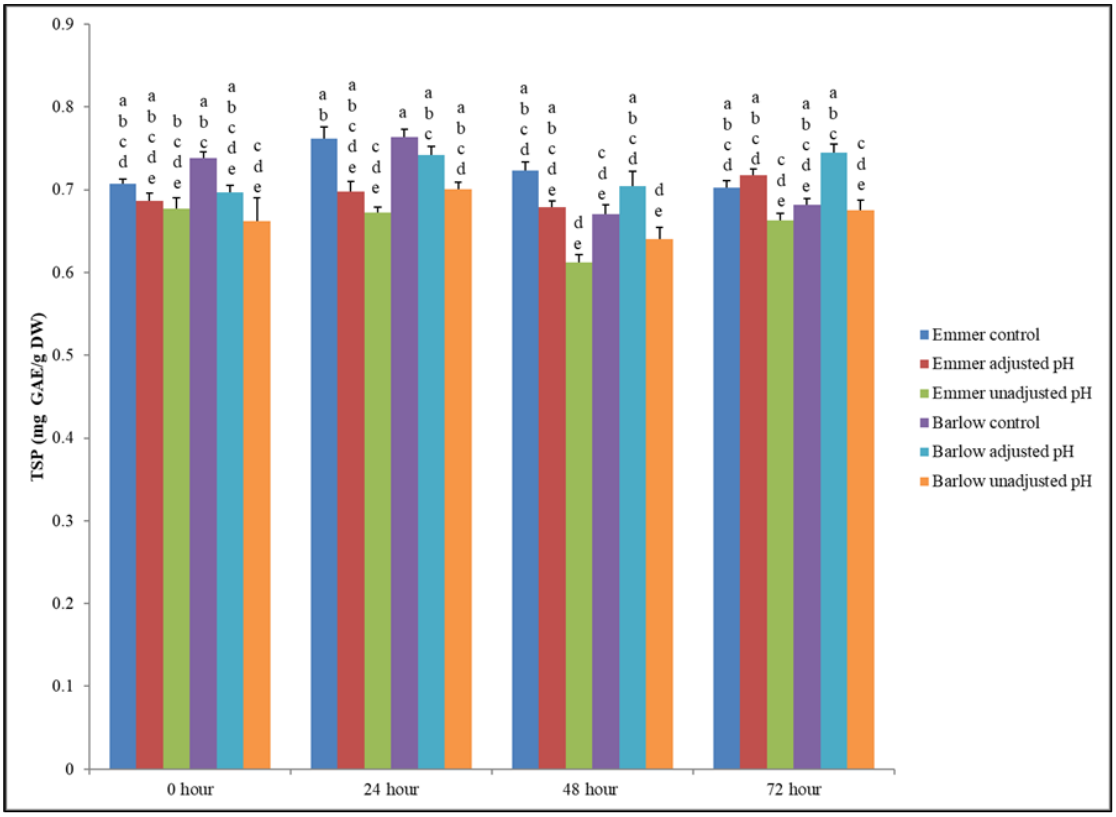


Figure 9.1**.** Total soluble phenolic (TSP) content (mg GAE/g DW) of unfermented (control) and fermented (adjusted pH and unadjusted pH) Emmer and Barlow wheat extracts (0.4 g/mL) at 0, 24, 48, and 72 h fermentation time points. Data is expressed as the mean ± standard error. Different letters indicate statistically significant differences in TSP content between sample extract × fermentation time point interactions ($p < 0.05$).

Statistically significant differences in TSP content were observed between sample extracts, fermentation time points, and sample extract × fermentation time point interactions ($p < 0.05$) (statistically significant effect of 2-way interaction on TSP content is presented in Figure 9.1). During the initial fermentation stages (0–24 h), a higher TSP content was observed in unfermented wheat extracts (control) (Figure 9.1). However, after 72 h of fermentation, fermented wheat extracts (Emmer and Barlow) with adjusted pH had higher TSP content but were statistically at par with the respective controls. In this study, the mean TSP content was comparatively lower among the fermented extracts with acidic pH (without adjustment), which could be due to the effect of acidic condition on the activity of Folin–Ciocalteu, which is a

reducing reagent that requires an alkaline pH to fully bind to the phenolic compounds present in the extracts. Furthermore, the stability of phenolic compounds depends on the structure of the compound and pH of the environment, and a pH close to neutral favors a better stability of the water-soluble phenolics (Friedman & Jürgens, 2000). The TSP content of the unfermented Emmer and Barlow extracts in this study was slightly lower when compared to the results of our previous study (Christopher et al, 2018b). The differences in the TSP content between these two studies was due to the differences in the year of harvest, as environmental factors of each specific crop year can have a significant impact on the stress-protective phenolic content and profile (Brandolini et al, 2013; Heimler et al, 2010; Reddivari et al, 2007). In this current study, the year of harvest for Emmer and Barlow was 2017, while in the previous study the crop years were 2015 and 2016 (Christopher et al, 2018b). Overall, higher retention of soluble phenolic content was observed in all fermented wheat extracts (with adjusted pH) even after 72 h fermentation.

The results also indicate that *L. plantarum*-based fermentation had no detrimental impact on soluble phenolic content of Emmer and Barlow aqueous extracts, and therefore fermentation, as a bioprocessing approach, can be recruited to develop phenolic bioactive-rich functional foods and ingredients from these targeted wheat substrates. In addition to the TSP content, it is important to know that the composition of soluble, bioavailable phenolic compounds in fermented food matrices and their potential structure-function changes during fermentation for better defining phenolic-linked functional benefits. A high-performance liquid chromatography (HPLC)-based analytical technique was used to detect and quantify major phenolic compounds present in the aqueous extracts of fermented and unfermented wheat. The phenolic compounds detected in this study were benzoic acid, gallic acid, protocatechuic acid (hydroxybenzoic acid

group), ferulic acid (hydroxycinnamic acid group), and catechin (flavan-3-ol) (Table 9.1) A chromatogram of individual phenolic compounds is presented in Figure 9.2.

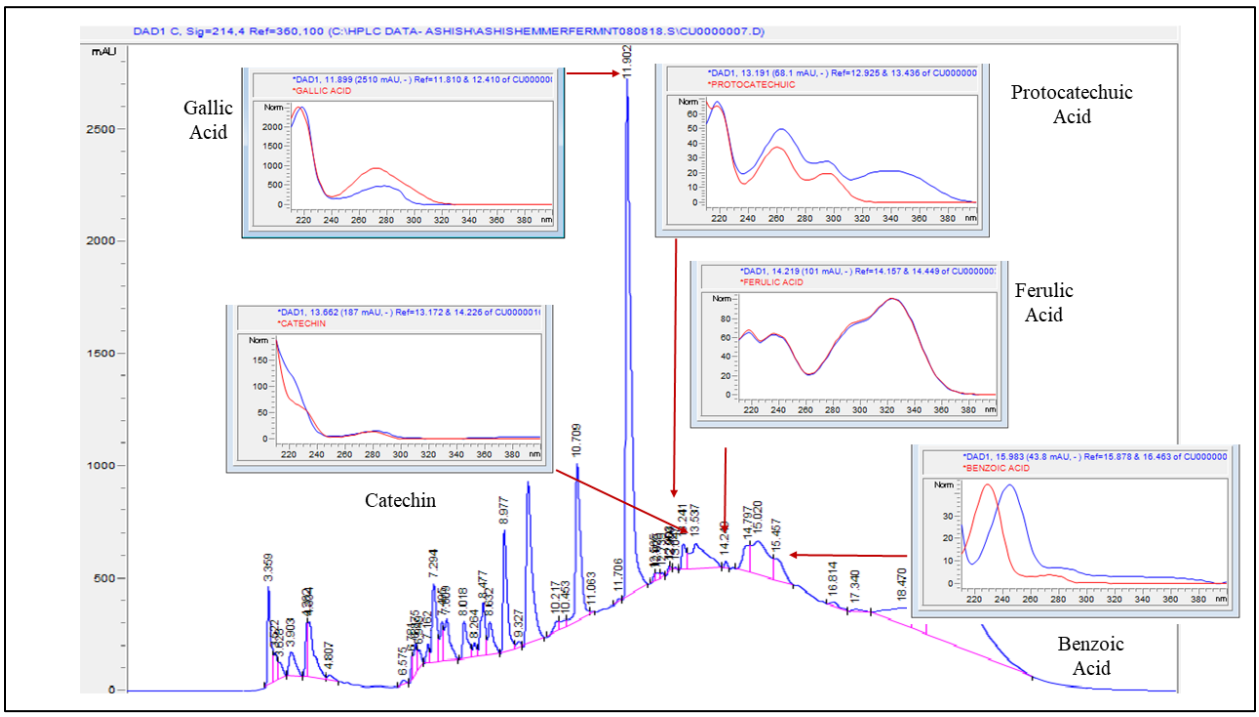


Figure 9.2. Chromatogram of detected phenolic compounds present in unfermented (control) and fermented (adjusted pH and unadjusted pH) Emmer and Barlow wheat extracts (0.4 g/mL) at 0, 24, 48, and 72 h fermentation time points. Analysis was done using high-performance liquid chromatography (HPLC).

Table 9.1. Profile and content (µg/g DW) of individual phenolic compound determined using high-performance liquid chromatography (HPLC).

Time point (hours)	Sample extract[a]		Benzoic acid	Catechin	Gallic acid	Protocatechuic acid	Ferulic acid
0	Emmer	Unfermented	0.88 ± 0.0fghij	9.95 ± 0.0cd	11.50 ± 0.0abcdef	0.31 ± 0.0hij	1.17 ± 0.0ef
		Fermented/ adjusted pH	0.93 ± 0.0defg	9.68 ± 0.2d	10.79 ± 0.0bcdef	0.28 ± 0.0ijk	1.10 ± 0.1f
		Fermented/ unadjusted pH	1.03 ± 0.0abc	9.94 ± 0.3cd	10.99 ± 0.1abcdef	0.33 ± 0.0ghij	1.39 ± 0.0d
	Barlow	Unfermented	0.93 ± 0.0defg	8.65 ± 0.4ef	12.33 ± 0.0abcd	0.52 ± 0.0ab	2.20 ± 0.0a
		Fermented/ adjusted pH	0.96 ± 0.0cdef	7.94 ± 0.0g	11.91 ± 0.0abcde	0.48 ± 0.0abcde	1.60 ± 0.0c
		Fermented/ unadjusted pH	1.07 ± 0.0a	8.73 ± 0.1e	12.35 ± 0.0abcd	0.52 ± 0.0abc	2.17 ± 0.0a
24	Emmer	Unfermented	0.91 ± 0.0efg	10.35 ± 0.0bc	13.0 ± 0.0a	0.37 ± 0.0fghi	1.12 ± 0.0f
		Fermented/ adjusted pH	0.92 ± 0.0defg	0.35 ± 0.1h	9.53 ± 0.0f	0.26 ± 0.0jk	ND
		Fermented/ unadjusted pH	1.06 ± 0.0ab	0.40 ± 0.0h	7.29 ± 0.0g	0.20 ± 0.0kl	ND
	Barlow	Unfermented	0.86 ± 0.0ghij	8.15 ± 0.0fg	12.73 ± 0.0ab	0.57 ± 0.0a	1.93 ± 0.0b
		Fermented/ adjusted pH	0.92 ± 0.0defg	0.29 ± 0.0h	11.41 ± 0.0abcdef	0.37 ± 0.0fghi	ND
		Fermented/ unadjusted pH	0.99 ± 0.0abcd	0.44 ± 0.0h	10.11 ± 0.0ef	0.14 ± 0.0l	ND
48	Emmer	Unfermented	0.79 ± 0.0jk	10.82 ± 0.0ab	12.84 ± 0.5a	0.38 ± 0.0efghi	0.97 ± 0.0g
		Fermented/ adjusted pH	0.90 ± 0.0efgh	0.06 ± 0.0h	10.10 ± 0.6ef	0.33 ± 0.0ghij	ND
		Fermented/ unadjusted pH	0.98 ± 0.0bcde	0.34 ± 0.0h	12.48 ± 0.3abc	0.41 ± 0.0defgh	ND

Table 9.1 Profile and content (µg/g DW) of individual phenolic compound determined using high-performance liquid chromatography (HPLC) (continued).

Time point (hours)	Sample extract[a]		Benzoic acid	Catechin	Gallic acid	Protocatechuic acid	Ferulic acid
	Barlow	Unfermented	0.81 ± 0.0ijk	7.89 ± 0.0g	12.27 ± 0.0abcd	0.56 ± 0.0a	1.21 ± 0.0e
		Fermented/ adjusted pH	0.87 ± 0.0ghij	0.10 ± 0.0h	10.48 ± 0.0cdef	0.43 ± 0.0bcdef	ND
		Fermented/ unadjusted pH	0.89 ± 0.0fghi	0.13 ± 0.0h	11.16 ± 0.1abcdef	0.49 ± 0.1abcd	ND
72	Emmer	Unfermented	0.74 ± 0.0k	10.94 ± 0.0a	12.62 ± 0.0ab	0.37 ± 0.0fghi	0.66 ± 0.0h
		Fermented/ adjusted pH	0.92 ± 0.0defg	0.08 ± 0.0h	9.79 ± 0.5f	0.37 ± 0.0fghi	ND
		Fermented/ unadjusted pH	0.94 ± 0.1defg	0.33 ± 0.0h	10.34 ± 1.5def	0.42 ± 0.0cdefg	ND
	Barlow	Unfermented	0.82 ± 0.0hijk	8.36 ± 0.0efg	12.47 ± 0.0abc	0.56 ± 0.0a	0.59 ± 0.0h
		Fermented/ adjusted pH	0.85 ± 0.0ghij	0.10 ± 0.0h	10.69 ± 0.0bcdef	0.42 ± 0.0defg	ND
		Fermented/ unadjusted pH	0.85 ± 0.0ghij	0.10 ± 0.0h	10.74 ± 0.0bcdef	0.52 ± 0.1abc	ND

[a] Mean value ± standard error. Different letters in each column represent statistically significant differences between sample extract × fermentation time-point interactions ($p < 0.05$).

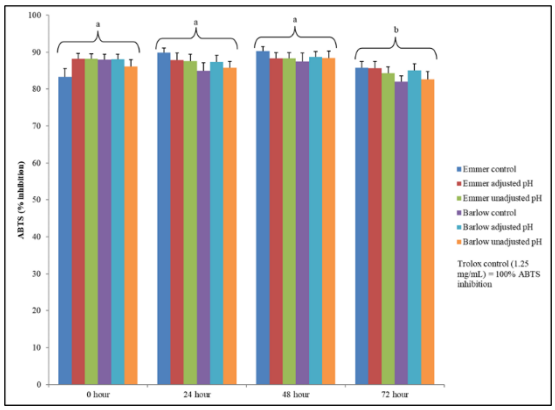
Among these phenolic compounds, higher concentrations of gallic acid (7.29 to 13 µg/g DW) and catechin (0.1 to 10.94 µg/g DW) were observed in both wheat extracts. The mean separation for all phenolic compounds was statistically significant between sample extracts, fermentation time points, and sample extract × fermentation time point interactions ($p < 0.05$). Overall, significantly higher benzoic acid content was observed in fermented wheat extracts when compared to the respective unfermented controls. Between sample extract × fermentation time point interactions, fermented Barlow without pH adjustment at 0–24 h had significantly higher benzoic acid content ($p < 0.05$). Among phenolic compounds, the catechin and ferulic acid content of both wheat samples declined significantly with fermentation, and ferulic acid content was not detected in the fermented samples at 24, 48, and 72 h (Table 9.1). In a previous study, whole grain, and milled fractions (flour, germ, and bran) of commercial soft and hard wheat varieties, were subjected to gastric conditions (low pH), and the vanillic and ferulic acid content was found to be higher in the treated wheat samples when compared to the untreated (control) samples (Liyana-Pathirana & Shahidi, 2005). In another study, whole wheat co-fermented with *L. plantarum* and *L. hammesii* displayed phenolic acid metabolism, in which ferulic acid was released from the bound form via the action of *L. hammesii*, and the free ferulic acid was converted to dihydroferulic acid and other volatile compounds via the action of *L. plantarum* (Ripari, Bai & Gänzle, 2019). Similarly, the reduction of ferulic acid to dihydroferulic acid by *L. plantarum* and *L. fermentum* was also reported in another study (Sánchez-Maldonado, Schieber & Gänzle, 2011). The metabolism of phenolic compounds by LAB mostly dictates the changes in the phenolic composition in fermented food matrices and in many cases is based on the physio-chemical properties of the substrate, as well as the bacterial species or strains used in fermentation (Antognoni, Mandrioli, Potente, Saa & Gianotti, 2019). Additionally, changes in

the ratio of soluble and insoluble dietary fiber during fermentation also influence the composition of free, bound, and conjugated phenolics in the fermented food matrix (Arora, Jood & Khetarpaul, 2010). In the current study, unfermented Emmer at 24, 48, and 72 h fermentation time points had significantly higher gallic acid content, which declined slightly following fermentation ($p < 0.05$). Unfermented Barlow had higher protocatechuic acid content, but it was statistically at par with fermented extracts (unadjusted pH), while in Emmer, higher protocatechuic acid was found in fermented extracts (without pH adjustment) at 48 and 72 h fermentation time points. The results of this study indicate that the fermentation of wheat with LAB can significantly alter the content and composition of phenolics, specifically due to changes in pH and/or based on the microbial enzymatic action that occurs during fermentation. However, the increase or decrease in the concentration of phenolics and their related bioactivity are largely based on the structure–function relationship of specific phenolic compounds that are present in the food substrates. Such changes also dictate phenolic-linked functional properties of fermented foods, such as antioxidant, antihyperglycemic, and antimicrobial properties, and should be accounted for when designing health-targeted functional foods and ingredients.

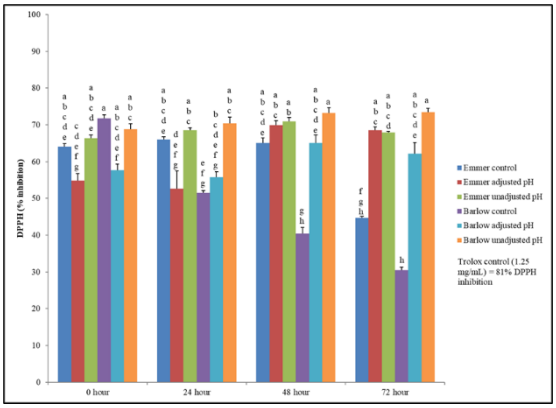
9.4.2. Total antioxidant activity

The antioxidant property of the phenolic compounds of plant-based food substrates is relevant for its value-added utilization in dietary support strategies to help manage chronic oxidative stress and associated non-communicable diseases. Therefore, in vitro antioxidant assays were carried out to understand the overall antioxidant potential of fermented wheat extracts and was investigated in the context of soluble phenolic content and phenolic profile and its subsequent relevance to specific health functional targets. The antioxidant activity of fermented and unfermented wheat extracts was determined based on ABTS and DPPH free

radical scavenging assays. Overall, higher mean antioxidant activity was observed with ABTS free radical scavenging assay, which ranged between 82–90% inhibition (similar range of antioxidant activity was also found in Trolox (1.25 mg/mL) (Figure 9.3 A).



(A)



(B)

Figure 9.3. (A) Total antioxidant activity (ABTS free radical scavenging assay) of unfermented (control) and fermented (adjusted pH and unadjusted pH) Emmer and Barlow wheat extracts (0.4 g/mL) at 0, 24, 48 and 72 h fermentation time points. Data is expressed as the mean ± standard error. Different letters indicate statistically significant differences between fermentation time points ($p < 0.05$) (the effect of sample extract × fermentation time-point interactions was not significant for ABTS-based antioxidant activity). (B) Total antioxidant activity (DPPH free radical scavenging assay) of unfermented (control) and fermented (adjusted pH and unadjusted pH) Emmer and Barlow wheat extracts (0.4g/mL) at 0, 24, 48, and 72 h fermentation time points. Data is expressed as the mean ± standard error. Different letters indicate statistically significant differences between sample extract × fermentation time point interactions ($p < 0.05$).

Statistically significant differences in ABTS scavenging activity were observed only between fermentation time points and not between sample extracts or sample extract ×

fermentation time point interactions (Figure 9.3A). Between fermentation time points, higher ABTS-based antioxidant activity was observed at 24 and 48 h, and it was statistically significant when compared to the antioxidant activity of the fermented and unfermented samples at 72 h ($p < 0.05$). The reason for not finding statistically significant differences in ABTS-based antioxidant activity among fermented and unfermented sample extracts might be due to the equally high saturated levels of mean antioxidant activity across all samples. In general, ABTS free radicals have a higher affinity towards hydrophilic antioxidants, and the results of this study indicate that fermented and unfermented aqueous extracts of wheat substrates might have equally high concentration of water-soluble antioxidants, which are amenable to the ABTS radical scavenging activity. The mean antioxidant activity of fermented and unfermented wheat extracts for the DPPH free radical scavenging assay was relatively lower (30–72% inhibition) when compared to the result of the ABTS assay (Figure 9.3 B).

The difference of results in antioxidant activity between the DPPH and ABTS radical scavenging activity could be due to the chemical structure of the synthetic radicals as DPPH being a more stable radical, takes a longer time to decolorize when compared to ABTS, and in addition, their differences in affinity towards hydrophilic and lipophilic antioxidants can also affect the inhibition response. However, statistically significant differences in DPPH scavenging activity were observed between sample extracts, fermentation time points, and sample extract × fermentation time point interactions ($p < 0.05$). At 0 h time point, unfermented Barlow had higher DPPH-based antioxidant activity, while at 24, 48, and 72 h time points, significantly ($p < 0.05$) higher antioxidant activity was observed in fermented sample extracts and specifically, in unadjusted pH (naturally acidic) extracts. Similarly, at 72 h time point, statistically higher DPPH-based antioxidant activity was also observed in fermented Emmer extracts (both with and

without pH adjustments) when compared to the antioxidant activity of unfermented Emmer. Overall, the antioxidant activity of unfermented wheat extracts declined significantly from 0 to 72 h. However, with LAB fermentation, antioxidant activity of both wheat extracts improved and was sustained at higher levels even after fermentation for 72 h. The values of the DPPH radical scavenging activity for the unfermented emmer and Barlow wheat extracts reported in this study was slightly higher when compared to the result of our previous study (Christopher et al, 2018b). As in the case of TSP content, the antioxidant activity of grains also varies between different crop years and locations, which is mostly because of different environmental factors contributing to the biosynthesis and distribution of stress-protective bioactive phenolic compounds and antioxidant enzymes at the pre-harvest production stages (Brandolini et al, 2013; Heimler et al, 2010; Reddivari et al, 2007).

In a previous published study, whole grain, and milled fractions (flour, germ, and bran) of commercial soft and hard wheat varieties were subjected to gastric conditions (low pH), and the total antioxidant activity was found to be significantly higher in the treated samples, when compared to the untreated (control) samples ($p < 0.05$) (Liyana-Pathirana & Shahidi, 2005). The fermentation of whole grains (e.g., wheat, oats, barley, rye, sorghum, and millet) with LAB can potentially improve the total phenolic content and antioxidant activity of the grains through microbial enzymatic activity, which results in the breakdown of the cell wall of the grains and the release of bound bioactive compounds with antioxidant potential (Adebo & Medina-Meza, 2020). The improved antioxidant activity (DPPH-based) in LAB-fermented wheat extracts found in the current study has significant relevance for its wider health-targeted food and ingredient applications, especially to counter chronic oxidative stress and associated diseases, such as type 2 diabetes, cardiovascular diseases, and dyslipidemia. However, it is important to validate this

finding by conducting *in vivo* model-based studies and by investigating other functional properties, such as the antihyperglycemic, antihypertensive, and antimicrobial functionalities of LAB-fermented wheat extracts.

9.4.3. Alpha-amylase and α-glucosidase enzyme inhibitory activity

Managing glucose homeostasis by slowing down the breakdown of soluble dietary carbohydrates and restricting subsequent glucose uptake into the bloodstream is critical to counter the chronic hyperglycemia commonly associated with early stages of type 2 diabetes. Therefore, many dietary and therapeutic strategies target the inhibition of key enzymes, like α-amylase and α-glucosidase, which are involved in carbohydrate metabolism, to slow down the release and absorption of glucose during the post-prandial phase. To understand these antihyperglycemic benefit-relevant metabolic targets, we determined the α-amylase and α-glucosidase enzyme inhibitory activities of fermented and unfermented wheat extracts using rapid *in vitro* assay models. High α-amylase enzyme inhibitory activity, which was equivalent to the result of positive control -acarbose (10 mg/mL), was observed across all wheat sample extracts (0.4 g/mL) and at different fermentation time points (Table 9.2). Additionally, significant dose-dependent responses (undiluted, half, and one-fifth diluted) in α-amylase enzyme inhibitory activity were observed in all wheat sample extracts. The mean differences between sample extracts, fermentation time points, and sample extract × fermentation time point interactions for the undiluted, half, and one-fifth diluted extracts were also statistically significant ($p < 0.05$).

Table 9.2. Antihyperglycemic property relevant α-amylase and α-glucosidase (%) enzyme inhibitory activity of unfermented and fermented wheat extracts (0.4g/mL).

Time point (hours)	**Sample extract[a]**		**α-amylase inhibition**			**α-glucosidase inhibition**		
			Undiluted	1:2 diluted	1:5 diluted	Undiluted	1:2 diluted	1:5 diluted
0	Emmer	Unfermented	90.9 ± 1.4a	79.5 ± 1.1abcd	75.2 ± 0.8ab	34.7 ± 1.5efgh	23.2 ± 1.1defg	11.9 ± 0.8defg
		Fermented/ adjusted pH	94.4 ± 2.1a	79.4 ± 1.1abcd	75.6 ± 1.5a	26.3 ± 1.3hij	14.2 ± 1.2hi	7.4 ± 0.7hijk
		Fermented/ unadjusted pH	91.8 ± 1.2a	84.6 ± 0.8ab	80.9 ± 1.7a	37.6 ± 5.9efg	21.0 ± 0.9efg	9.4 ± 0.6fghij
	Barlow	Unfermented	93.2 ± 1.5a	89.9 ± 1.0a	79.9 ± 1.7a	29.1 ± 0.9fghi	20.7 ± 0.5fg	9.9 ± 0.4efghi
		Fermented/ adjusted pH	92.9 ± 2.9a	83.1 ± 1.6abc	79.7 ± 1.2a	20.9 ± 0.8ijk	13.5 ± 0.8hi	5.6 ± 0.8ijklm
		Fermented/ unadjusted pH	94.7 ± 1.2a	88.3 ± 1.6ab	82.0 ± 1.9a	28.1 ± 0.9ghi	16.9 ± 0.9gh	7.0 ± 0.8hijkl
24	Emmer	Unfermented	85.2 ± 1.9ab	79.2 ± 1.7abcd	66.7 ± 1.8abcd	41.2 ± 0.7def	27.3 ± 0.8cde	16.9 ± 0.6bc
		Fermented/ adjusted pH	68.6 ± 1.0cd	60.6 ± 0.3cdefg	51.6 ± 0.7defgh	15.6 ± 0.7kl	8.8 ± 0.6ijk	4.2 ± 0.4klmn
		Fermented/ unadjusted pH	91.2 ± 0.8a	53.2 ± 0.7efgh	49.2 ± 2.2efgh	48.6 ± 1.2bcd	24.1 ± 0.9def	9.6 ± 0.5fghi
	Barlow	Unfermented	91.4 ± 1.7a	80.9 ± 1.2abcd	71.5 ± 1.3abc	33.4 ± 0.7fgh	22.2 ± 0.4defg	11.9 ± 0.5defg
		Fermented/ adjusted pH	62.0 ± 0.8de	47.2 ± 0.8fgh	35.8 ± 1.4hij	9.2 ± 0.5l	4.8 ± 0.4jk	0.8 ± 0.3n
		Fermented/ unadjusted pH	85.3 ± 0.5ab	59.8 ± 0.9defg	39.5 ± 1.0ghi	40.8 ± 1.3def	19.5 ± 0.5fgh	4.4 ± 0.9klmn
48	Emmer	Unfermented	87.3 ± 2.4ab	74.9 ± 0.9abcde	58.4 ± 3.5bcdef	43.7 ± 1.7cde	33.9 ± 0.9ab	20.2 ± 0.6ab

Table 9.2. Antihyperglycemic property relevant α-amylase and α-glucosidase (%) enzyme inhibitory activity of unfermented and fermented wheat extracts (0.4g/mL) (continued).

Time point (hours)	**Sample extract[a]**		**α-amylase inhibition**			**α-glucosidase inhibition**		
			Undiluted	1:2 diluted	1:5 diluted	Undiluted	1:2 diluted	1:5 diluted
	Emmer	Fermented/ adjusted pH	24.4 ± 2.7g	14.2 ± 1.9ij	2.5 ± 0.9k	13.7 ± 0.4kl	5.2 ± 0.9jk	3.3 ± 0.5klmn
		Fermented/ unadjusted pH	92.9 ± 0.9a	78.1 ± 1.0abcd	2.6 ± 1.2k	57.2 ± 1.9ab	31.8 ± 0.8abc	11.4 ± 0.7defgh
	Barlow	Unfermented	85.5 ± 1.4ab	73.3 ± 2.9abcde	67.6 ± 2.8abcd	36.9 ± 0.7efg	24.7 ± 0.7def	14.0 ± 0.8cde
		Fermented/ adjusted pH	54.9 ± 1.4def	40.8 ± 1.3gh	28.3 ± 1.4ij	9.2 ± 0.4l	3.1 ± 0.6k	1.5 ± 0.3mn
		Fermented/ unadjusted pH	90.2 ± 1.2ab	66.5 ± 1.0bcdef	41.7 ± 0.5fghi	53.3 ± 0.9bc	28.2 ± 0.8bcd	12.2 ± 1.1defg
72	Emmer	Unfermented	86.8 ± 2.3ab	72.1 ± 1.7abcde	54.5 ± 6.2cdefg	49.3 ± 0.5bcd	36.9 ± 0.5a	22.4 ± 0.5a
		Fermented/ adjusted pH	9.6 ± 0.9h	1.8 ± 0.7j	0.2 ± 0.2k	16.3 ± 0.6jkl	9.4 ± 0.5ij	5.1 ± 0.5jklmn
		Fermented/ unadjusted pH	91.8 ± 0.8a	82.8 ± 0.6abc	2.6 ± 0.6k	64.5 ± 1.1a	34.9 ± 0.6a	13.8 ± 0.9cdef
	Barlow	Unfermented	78.1 ± 1.7bc	70.7 ± 9.6abcde	66.4 ± 3.4abcde	41.6 ± 0.9def	28.4 ± 0.9bcd	15.4 ± 0.5cd
		Fermented/ adjusted pH	49.2 ± 1.7f	34.4 ± 2.3hi	21.6 ± 1.1j	11.5 ± 0.7kl	5.8 ± 0.7jk	2.9 ± 0.6lmn
		Fermented/ unadjusted pH	91.5 ± 1.1a	75.5 ± 1.3abcde	40.0 ± 1.4ghi	58.2 ± 0.9ab	27.7 ± 1.1bcd	9.2 ± 1.1ghij
	Acarbose control	10 mg/mL	89			63		

[a] Mean value ± standard error. Different letters in each column represent statistically significant differences between sample extract × fermentation time-point interactions ($p < 0.05$)

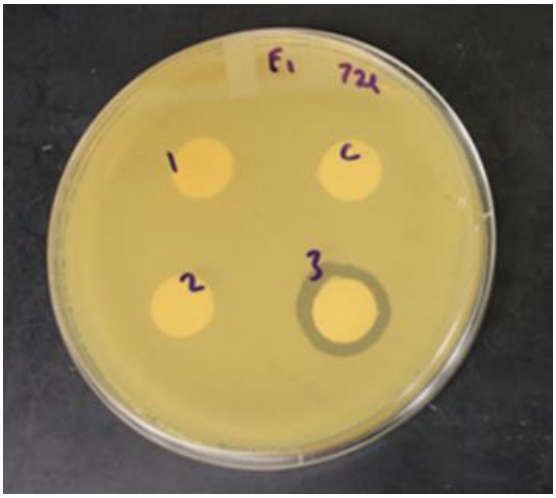
The undiluted extracts, fermented Barlow, and Emmer without pH adjustment at different fermentation time points had higher α-amylase enzyme inhibitory activity, when compared to the fermented wheat extracts with pH adjustment ($p < 0.05$). However, the value of the α-amylase enzyme inhibitory activity of fermented extracts without pH adjustment was statistically at par with unfermented wheat extracts. This similar trend and statistically significant differences in α-amylase enzyme inhibitory activity between sample extract × fermentation time point interactions were also observed for the half and one-fifth diluted extracts in a dose-dependent manner ($p < 0.05$). The results indicate that the acidic pH of the fermented wheat extracts directly interfered with the in vitro assay mechanism, which resulted in higher α-amylase enzyme inhibitory activity in pH-unadjusted wheat extracts. However, at 0 and 24 h time points, fermented wheat extracts with adjusted pH also had moderate to high α-amylase enzyme inhibitory activity relevant for dietary interventions targeting antihyperglycemic benefits.

Like the results of α-amylase enzyme inhibitory activity, a significant effect of acidic pH on α-glucosidase enzyme inhibitory activity was also observed. Overall, low to moderate α-glucosidase enzyme inhibitory activity was observed in fermented and unfermented wheat extracts, which was equivalent to positive control acarbose (10 mg/mL) (Table 9.2). The variations in α-glucosidase enzyme inhibitory activity between sample extracts, fermentation time points, and sample extract × fermentation time point interactions were statistically significant for the undiluted, half, and one-fifth diluted extracts ($p < 0.05$). In the undiluted extracts, fermented Emmer (without pH adjustment) at the 72 h time point had significantly higher α-glucosidase inhibitory activity when compared to the unfermented control ($p < 0.05$). Like the results of Emmer, higher α-glucosidase enzyme inhibitory activity was also observed in undiluted extracts of fermented Barlow (without pH adjustment) when compared to the

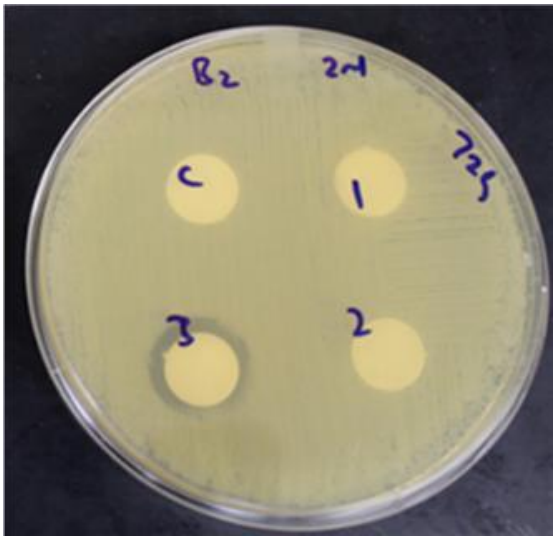
unfermented and fermented (with pH adjustment) extracts at 24, 48, and 72 h time points. The results of mean α-amylase and α-glucosidase enzyme inhibitory activity of wheat extracts were slightly higher than the results obtained in our previous study (Christopher et al, 2018b). Overall, the results of the current study indicated that, at the natural acidic pH, LAB-fermented wheat extracts are good dietary sources that can be integrated and targeted in dietary interventions to manage post-prandial blood glucose level. The release of bioactive compounds, such as phenolics in a fermented food matrix and changes in their functional properties under certain physio-chemical condition (acidic environment), might have greater influence on their overall functional properties including antihyperglycemic function. However, further studies with different wheat-based substrate sources (from multiple locations, different crop years, and genetic backgrounds) and different LAB species and strains are needed to optimize and develop wheat-based fermented foods and functional ingredients targeting type 2 diabetes benefits.

9.4.4. Antimicrobial and prebiotic activity

In addition to the antihyperglycemic functional benefits, potential antibacterial activity of fermented and unfermented wheat extracts against gastric ulcer causing pathogenic bacteria *H. pylori* was investigated using agar disc diffusion assay. The antibacterial activity was measured and expressed in millimeters based on the width of the zone of no growth around the discs. In this study, zones of inhibition were observed with fermented Emmer and Barlow (unadjusted pH) at the 72 h time point, and the width of the zones of inhibition ranged between 2 to 4 mm (Figure 9.4 A, B).



(A)



(B)

Figure 9.4. Antibacterial activity of wheat extracts against *H. pylori*. (A) Unfermented (control) and fermented (adjusted pH and unadjusted pH) Emmer wheat extracts (0.4 mg/mL) at the 72 h fermentation time point. (B) Unfermented (control) and fermented (adjusted pH and unadjusted pH) Barlow wheat extracts at 72 h fermentation time point. Zone of inhibition was measured in millimeters. C- water (control), 1- unfermented, 2- fermented (adjusted pH), 3- fermented (unadjusted pH).

LAB produces bacteriocins, which, in combination with weak organic acids (lactic acid) and a low pH, can contribute to the inhibition of non-acidogenic or non-aciduric bacterial pathogens (Cotter & Hill, 2003). These bacteriocins permeabilize the bacterial cell membrane leading to cellular leakage, while uncharged lipophilic weak organic acids produced during LAB

growth can translocate into the cell, where it disassociates into protons and anions. The protons lead to the acidification of the cell cytoplasm, causing the denaturation of proteins and bacterial DNA, while the anions cause osmotic stress to the bacterial cell (Cleveland, Montville, Nes & Chikindas, 2001; Hirshfield, Terzulli & O'Byrne, 2003; Rosenquist & Hansen, 1998). Since the *L. plantarum* strain used in this study is not reported to produce bacteriocins and antimicrobial activity was observed only for the fermented extracts (without pH adjustment), the results indicate that the conditions of a low pH (acidic environment) in combination with weak organic acids like lactic acid, combined with phenolic compounds, could have contributed to the inhibition of *H. pylori*. However, *H. pylori* can survive in the acidic environment of its natural habitat, the gastric mucosa, due the secretion of the bacterial enzyme urease, which hydrolyzes the urea that is present under physiological conditions in acidic medium (Bonifácio, dos Santos Ramos, Da Silva & Bauab, 2014). The ammonia produced during hydrolysis can act as a receptor for H+ ions, consequently generating a neutral pH, which is required for the survival of the bacterium ((Bonifácio et al, 2014). Apart from low pH and weak organic acid production, another explanation for the anti-*H. pylori* activity of the fermented extracts in the current study could be the antimicrobial activity of phenolic compounds present in the extracts, which could potentially regulate the cellular redox response through the proline-associated pentose phosphate pathway (Lin, Kwon, Labbe & Shetty, 2005; Sarkar, Ankolekar, Pinto & Shetty, 2015). In general, plant secondary metabolites, such as phenolic compounds, display potential antimicrobial activity against bacterial pathogens through a variety of mechanisms, which include the inhibition of DNA or protein synthesis, the inhibition of important microbial biosynthetic pathways (e.g., folic acid biosynthesis), the loss of cell-membrane integrity, leading to cell lysis, and a reduction in the availability of micronutrients needed for microbial growth

(Chibane et al, 2019; Cowan, 1999; Daglia, 2012; , Jayaraman et al, 2010a, 2010b; Radulovic et al, 2013). Furthermore, phenolic compounds such as flavonoids can inhibit the activity of the urease enzyme and other virulence factors involved in the pathogenesis of *H. pylori* (Bonifácio et al, 2014). In a previous study, gallic acid and catechin were found to inhibit the growth of two *H. pylori* strains in a dose-dependent manner, with gallic acid showing a stronger inhibitory activity than catechin, and a partial additive growth inhibitory effect was observed when gallic and catechin were used in combination (Díaz-Gómez, López-Solís, Obreque-Slier & Toledo-Araya, 2013). It is possible that in the current study, the low pH and production of weak organic acids during LAB fermentation enhanced the antimicrobial activity of these phenolic compounds that were present in wheat extracts against *H. pylori*. This is supported by the observation that only the fermented Emmer and Barlow extracts (without pH adjustment) displayed anti-*H. pylori* activity, while the unfermented and fermented (with pH adjustment) extracts did not any show antimicrobial activity. Additionally, there was no substantial change in TSP content during the fermentation period, indicating the possible role of phenolic compounds in the anti-*H. pylori* activity. Therefore, 72 h fermented Emmer and Barlow with natural acidic pH is relevant to potentially counter *H. pylori*-induced gastric ulcer, a common prevalent gut infection around the globe.

To target designed bioactive-enriched food substrates for human gut health benefits, it is also important to understand whether examples such as fermented wheat extracts in this study have any negative impact on beneficial gut microbes or not. To evaluate this potential, the growth of *B. longum* with unfermented and fermented wheat extracts was investigated and compared using a LAB proliferation prebiotic assay. The growth of *B. longum* ranged from 4.9 to 5.5 log CFU/mL, 4.4 to 6.8 log CFU/mL, 7.7 to 8.5 log CFU/mL, 8.9 to 9.5 log CFU/mL, and

8.9 to 9.6 log CFU/mL at the respective 0, 6, 12, 24, and 48 h time points of the proliferation assay (Table 9.3).

No statistically significant differences in growth were observed between the sample extracts and prebiotic assay control (water), and between sample extract × fermentation time point interactions. However, at the 0 h assay time point, 24 h-fermented wheat extracts had significantly higher log CFU/mL when compared to the 0 h-fermented wheat extracts and were statistically at par with the 48 h- and 72 h-fermented extracts ($p < 0.05$). These results indicate that the fermented wheat extracts had no negative effect on the growth of *B. longum* and can function as suitable substrates to support the growth of *B. longum*. Therefore, the LAB-based fermentation of wheat can be advanced as a safe dietary strategy to target and potentially manage pathogenic bacteria such as *H. pylori* and associated infections, without having any harmful side effects on beneficial gut microbes.

Table 9.3. Prebiotic activity (log CFU/ mL) of unfermented and fermented wheat extracts (0.4 mg/mL) for growth of *Bifidobacterium longum*.

Time point (hours)	Sample extract[a]		0-hour[b]	6-hour[b]	12-hour[b]	24-hour[b]	48-hour[b]
0	Assay control		4.9 ± 0.3	4.7 ± 1.6	8.1 ± 0.2	9.5 ± 0.0	9.5 ± 0.2
	Emmer	Unfermented	5.1 ± 0.2	6.3 ± 0.2	8.5 ± 0.0	9.5 ± 0.0	9.4 ± 0.1
		Fermented/ adjusted pH	5.4 ± 0.1	4.7 ± 1.6	8.4 ± 0.1	9.2 ± 0.1	9.2 ± 0.2
		Fermented/ unadjusted pH	5.4 ± 0.1	5.9 ± 0.3	8.4 ± 0.1	9.2 ± 0.2	9.6 ± 0.1
	Barlow	Unfermented	4.9 ± 0.3	6.5 ± 0.1	8.2 ± 0.1	9.5 ± 0.0	9.6 ± 0.0
		Fermented/ adjusted pH	5.3 ± 0.2	6.0 ± 0.2	8.2 ± 0.2	9.4 ± 0.1	9.3 ± 0.3
		Fermented/ unadjusted pH	5.4 ± 0.1	4.4 ± 1.5	8.3 ± 0.1	9.4 ± 0.1	9.4 ± 0.1
24	Assay control		5.1 ± 0.3	6.5 ± 0.1	8.3 ± 0.0	9.0 ± 0.1	9.1 ± 0.1
	Emmer	Unfermented	5.5 ± 0.0	6.6 ± 0.1	8.3 ± 0.1	9.2 ± 0.1	9.0 ± 0.1
		Fermented/ adjusted pH	5.5 ± 0.1	6.8 ± 0.1	8.2 ± 0.1	8.9 ± 0.0	9.1 ± 0.2
		Fermented/ unadjusted pH	5.6 ± 0.1	6.6 ± 0.1	8.2 ± 0.1	9.2 ± 0.2	8.9 ± 0.4
	Barlow	Unfermented	5.5 ± 0.1	6.5 ± 0.2	8.4 ± 0.1	9.1 ± 0.1	8.9 ± 0.1
		Fermented/ adjusted pH	5.5 ± 0.1	6.4 ± 0.2	8.3 ± 0.1	9.4 ± 0.1	9.2 ± 0.1
		Fermented/ unadjusted pH	5.5 ± 0.1	6.3 ± 0.2	8.3 ± 0.1	9.2 ± 0.1	9.6 ± 0.0
48	Assay control		5.5 ± 0.1	6.5 ± 0.1	8.3 ± 0.1	9.2 ± 0.1	9.2 ± 0.2

Table 9.3. Prebiotic activity (log CFU/ mL) of unfermented and fermented wheat extracts (0.4 mg/mL) for growth of *Bifidobacterium longum* (continued).

Time point (hours)	Sample extract[a]		0-hour[b]	6-hour[b]	12-hour[b]	24-hour[b]	48-hour[b]
	Emmer	Unfermented	5.1 ± 0.1	5.9 ± 0.1	8.1 ± 0.3	9.4 ± 0.1	9.3 ± 0.0
		Fermented/ adjusted pH	5.4 ± 0.1	5.9 ± 0.4	7.9 ± 0.4	9.4 ± 0.1	9.4 ± 0.1
		Fermented/ unadjusted pH	5.2 ± 0.2	4.8 ± 1.6	8.4 ± 0.1	9.3 ± 0.2	9.0 ± 0.3
	Barlow	Unfermented	5.4 ± 0.1	6.6 ± 0.1	8.4 ±0.0	9.3 ± 0.2	9.4 ± 0.1
		Fermented/ adjusted pH	5.5 ± 0.1	4.8 ± 1.6	8.3 ± 0.1	9.2 ± 0.1	9.3 ± 0.1
		Fermented/ unadjusted pH	5.5 ± 0.1	6.4 ± 0.1	8.5 ± 0.1	9.1 ± 0.2	9.3 ± 0.1
72	Assay control		5.1 ± 0.2	6.1 ± 0.2	7.7 ± 0.5	9.3 ± 0.1	9.5 ± 0.1
	Emmer	Unfermented	5.4 ± 0.1	6.0 ± 0.3	8.0 ± 0.4	9.4 ± 0.1	9.5 ± 0.1
		Fermented/ adjusted pH	5.5 ± 0.2	5.9 ± 0.2	8.4 ± 0.2	9.3 ± 0.1	9.5 ± 0.2
		Fermented/ unadjusted pH	5.5 ± 0.1	6.6 ± 0.0	8.4 ± 0.1	9.3 ± 0.1	9.5 ± 0.2
	Barlow	Unfermented	5.5 ± 0.1	6.4 ± 0.2	8.2 ± 0.2	9.3 ± 0.1	9.2 ± 0.2
		Fermented/ adjusted pH	5.3 ± 0.2	4.5 ± 1.6	8.3 ± 0.1	9.3 ± 0.1	9.4 ± 0.2
		Fermented/ unadjusted pH	5.3 ± 0.1	6.4 ± 0.1	8.2 ± 0.2	9.3 ± 0.1	9.3 ± 0.2

[a] Mean value ± standard error. [b] Prebiotic assay time point.

9.5. Conclusion

Fermentation with LAB is an effective and safe bioprocessing strategy to improve the phenolic bioactive-linked functional properties of wheat, hence concurrently enhancing and advancing its role as a functional food or food ingredient for human-health-targeted benefits. Due to the acidic conditions generated during the growth of LAB and through potential microbial enzymatic action, the fermentation of wheat with LAB can alter its phenolic profile and content, as well as related bioactivity, such as its antioxidant, antihyperglycemic and antimicrobial activity against bacterial pathogens. An acidic pH can affect the stability of phenolic compounds present in plant-based food substrates such as wheat, as different phenolic bioactive compounds have different sensitivities to low pH, and the acidic conditions generated during fermentation can release bound or esterified phenolics, which in turn enhance phenolic-linked bioactivity. In this study, the improvement in benzoic acid content and enhanced antioxidant activity (DPPH-based) were observed in fermented wheat extracts, while soluble phenolic content remained at a constant level even at the 72 h fermentation timepoint. Higher antihyperglycemic function-relevant α-amylase and α-glucosidase enzyme inhibitory activities were also observed in fermented wheat extracts due to the effect of acidic condition generated during LAB growth. Additionally, a combination of low pH, weak organic acid production, and the antimicrobial activity of phenolic compounds during wheat fermentation resulted in the inhibition of the gastric-ulcer-causing bacterium *H. pylori*, which indicates the potential human gut health benefits of fermented wheat. Furthermore, ancient and modern wheat varieties can serve as a suitable substrate source for the growth of probiotic and beneficial LAB, which can be utilized in designing functional foods and food ingredients. In conclusion, the results of this study provide important insights on the benefits of a LAB-based fermentation strategy, which can be advanced

as an effective bioprocessing tool to improve the health-protective functional benefits of underutilized (Emmer) and conventional (Barlow) wheat for wider health-targeted food applications.

CHAPTER 10. CORN DISTILLERS DRIED GRAINS WITH SOLUBLES (DDGS) AS A TARGET FOR FERMENTATION TO IMPROVE BIOACTIVE FUNCTIONALITY FOR ANIMAL FEED AND AS A SOURCE FOR NOVEL MICROORGANISM WITH ANTIBACTERIAL PROPERTY

10.1. Abstract

Plant-Based bioenergy by-products such as corn distillers dried grains with solubles (DDGS) are widely utilized as animal feed sources and feed ingredients due to their balanced nutritional profile and animal health protective functional qualities. Bioprocessing of this bioenergy by-product using beneficial lactic acid bacteria (LAB) based fermentation strategy to improve animal-health targeted functional qualities has wider relevance for animal feed applications. In this study, liquid extracts of corn DDGS were fermented with *Lactiplantibacillus plantarum* and *Lactobacillus helveticus.* The unfermented and fermented extracts were then analyzed (at 0, 24, 48 and 72-h) for their total soluble phenolic content (TSP), phenolic profile, antioxidant activity via ABTS and DPPH radical scavenging activity, and antimicrobial activity against the gut pathogen *Helicobacter pylori* using *in vitro* assay models. Statistical differences in antioxidant activity and phenolic content were observed among the unfermented and fermented extracts. Major phenolic compounds detected in corn-DDGS were gallic, dihydroxybenzoic, *p*-coumaric, caffeic and ferulic acid, and catechin. Antimicrobial activity against *H. pylori* was observed for the unfermented extracts and the antimicrobial activity was attributed to the growth of a corn-DDGS-endemic culture in the unfermented extract. The culture was isolated, sequenced and identified as *Bacillus amyloliquefaciens*. These results indicated that processing strategies of by-products such as LAB- based fermentation of corn-DDGS can affect its bioactive-linked functional qualities due to microbial interaction with the phytochemicals.

Furthermore, plant by-products can serve as novel sources of beneficial microflora that have relevance in wider agriculture, food safety and therapeutic applications.

Keywords: Antioxidant, Antimicrobial, Corn DDGS, Fermentation, Lactic acid bacteria

10.2. Introduction

Distillers dried grains with solubles (DDGS) is a corn bioenergy by-product from the dry grinding process during ethanol production. In 2021, over 15 billion gallons of ethanol were produced from corn in the United States with 44 million metric tons of DDGS being generated in the process (U.S. Grains Council, 2022). Corn DDGS is commonly used as a livestock feed in the dairy and beef cattle industry and used as an animal feed and ingredient source for poultry, swine, sheep, goat, and horses, as well as in aquaculture diets for fish and shrimp (U.S. Grains Council, 2018). The nutrient composition and digestibility of DDGS can vary among corn sources with ash, fiber, fat, lysine, tryptophan, and phosphorus content having high variation among the different DDGS sources (U.S. Grains Council, 2018). Feed rations supplemented with corn DDGS were found to alter the intestinal microbiota of the broiler chickens, and DDGS was negatively correlated with the genera *Faecalibacterium* and *Streptococcus* while *Turicibacter* was positively associated with corn DDGS supplemented broiler feed (Abudabos et al., 2017). Egg-laying hens fed on a diet supplemented with corn DDGS resulted in the egg yolk having a lower proportion of saturated fatty acids and a higher proportion of unsaturated fatty acids, while DDGS supplemented up to 10% in the diet had no adverse effect on egg-laying performance (Jiang et al., 2013). Commercial pork production operations in the U.S. have reduced the cost of swine feed by adding corn DDGS to the diet. Due to favorable economics, many pork producers have been feeding diets containing as much as 30 % DDGS and have achieved excellent growth performance and carcass lean percentages (Shurson, 2011).

There is an increasing interest in microbial fermentation of corn DDGS as a value-added strategy to improve its role as an animal feed. The carbohydrate component of corn DDGS can be converted to other compounds including succinic acid, acetone, butanol, ethanol, and lactic acid (Iram et al., 2020). In addition to improving the nutritional value through microbial fermentation, corn DDGS is a rich source of phytochemical compounds that may provide antioxidant and wider animal health benefits in addition to the macronutrient and micronutrient composition (Shin et al., 2018). Protein hydrolysates of corn DDGS were found to have potential use as naturally derived antioxidants in food, pet food, and feed systems with good protection against lipid oxidation, which is relevant for the improvement of stability of the product during storage (Hu et al., 2020). Hence in this regard, microbial fermentation as a value-added strategy can potentially improve the health protective benefits of corn DDGS by altering its phytochemical content and composition.

Apart from the nutritional and health benefits of corn DDGS which can be utilized in animal feed to improve animal health, the native microflora associated with such by-products often have diverse biotechnological applications due to the production of useful enzymes or proteins. Therefore, it is important to screen and bioprocess bioactive enriched corn DDGS to improve their antioxidant and animal health-relevant properties. Additionally, isolating and characterizing the associated microflora of corn DDGS for potential biotechnological applications in agricultural, industrial, or pharmaceutical industries has wider relevance. Therefore, the aim of this study was to advance the biotransformation of corn DDGS using lactic acid bacteria (LAB)-based fermentation strategy to improve the phenolic phytochemical-linked functional qualities such as antioxidant activity and antimicrobial property against the gut pathogen *Helicobacter pylori*. Targeting *H. pylori* as a model antibacterial screening is based on

the rationale that it shares similar environmental or growth conditions with bacterial pathogens such as *Campylobacter jejuni*, which is a major poultry-related food-borne pathogen, and hence can be subsequently targeted in poultry food safety applications. Furthermore, an unknown microbial culture isolated from unfermented corn DDGS was identified and the antimicrobial activity against *H. pylori* was characterized.

10.3. Materials and Methods

10.3.1. Preparation of corn-DDGS extracts

The Corn-DDGS sample was obtained from Tharaldson ethanol plant (Casselton, North Dakota, USA) and was extracted in duplicate based on the cold-water extraction protocol as described in a previous study (Christopher et al., 2018). For this study, the corn-DDGS sample was blended with cold distilled water in a 1:4 ratio using a waring blender set at medium speed for 5 minutes. The extracts were then centrifuged at 8,500 rpm for 20 min and the supernatant was collected and re-centrifuged at 8,500 rpm for 15 min. The extracts were then pasteurized in a water bath set at 70 °C for 15 min, after which the extracts were immediately cooled down using an ice bath, followed by storage at 4 °C prior to advancing LAB fermentation.

10.3.2. Bacterial strains

The bacterial strains used in this study were *Lactiplantibacillus plantarum* (ATCC 8014), *Lactobacillus helveticus* (ATCC 15009), and *Helicobacter pylori* (ATCC 43579). The *L. plantarum* and *L. helveticus* cultures were stored as frozen stocks in MRS broth (Difco) containing 20 % glycerol as the cryoprotectant. The *H. pylori* culture was stored as frozen stocks in *H. pylori* special peptone broth (HPSP) containing 10 g L^{-1} special peptone (Oxoid, Basingstoke, UK), 5 g L^{-1} sodium chloride (Fisher Scientific, MA, USA), 5 g L^{-1} yeast extract (Difco), and 5 g L^{-1} beef extract (Difco), with 20 % glycerol as the cryoprotectant. The MRS and

HPSP agar plates were prepared by the addition of 15 g L^{-1} of granulated agar (Difco) to the respective broths. All media were autoclaved prior to use. For the revival of frozen bacterial stocks, 100 µL of thawed *L. plantarum*, *L. helveticus*, and *H. pylori* stocks were inoculated in 10 mL of the respective MRS or HPSP broth and incubated at 37 °C for 24 h. Then 100 µL of the 24- h culture was re-inoculated into 10 mL sterile MRS or HPSP broth and incubated at 37 °C for another 24 h. The revived cultures were used in the respective fermentation and *in-vitro* antimicrobial analysis.

10.3.3. Fermentation of corn-DDGS extracts

Liquid fermentation was carried out based on the protocol as described previously (Ankolekar et al, 2012), in which 10 mL of the revived *L. plantarum* and *L. helveticus* cultures were added to the respective sterile flasks containing 90 mL of the pasteurized extracts. For the control or unfermented extracts, 10 mL of sterile MRS broth was added instead of the culture. The extracts were then incubated in duplicate at 37 °C for a period of 72 h and samples were collected at 0, 24, 48 and 72-h time points of fermentation for *in vitro* analysis. The growth of *L. plantarum* and *L. helveticus* was measured at each time point by serially diluting the fermented extracts followed by plating onto MRS agar plates. The plates were then incubated anaerobically at 37 °C for 48 h in BBL GasPak jars (Becton, Dickinson & Co) containing the BD GasPak EZ anaerobe container system sachets (Becton, Dickinson & Co). The number of colonies were counted and expressed in log CFU mL^{-1}. The unfermented and fermented extracts collected at each time point were centrifuged at 8,500 rpm for 15 min after which the supernatant was collected and the pH of one the duplicates of the fermented extracts was adjusted using 1M NaOH to match the pH of the unfermented extracts (pH 6.3 to 6.6). An equal amount of distilled water was added to the other duplicate of the fermented extract (without pH adjustment) to

maintain equal volume. The unfermented and fermented extracts (with and without pH adjustment) were analyzed for their TSP content and antioxidant activity at the 0, 24, and 48 and 72-h time points of the fermentation period. The unfermented and fermented extracts (with and without pH adjustment) at each time point were also filtered through sterile 0.22 µm syringe filters (Millipore Corp, MA, USA) and stored at -20 °C for later analysis of the phenolic profile and antimicrobial activity of the extracts.

10.3.4. Total soluble phenolic (TSP) content

The TSP content of the unfermented and fermented corn-DDGS extracts were determined using the Folin-Ciocalteu method based on the protocol as described previously (Shetty et al, 1995). For this assay, 0.5 mL aliquots of the unfermented and fermented extracts were taken into the respective glass tubes, after which 1 mL of 95% ethanol, 0.5 mL of 50% (v/v) Folin-Ciocalteu reagent, and 1 mL of 5% sodium carbonate were added sequentially to the extracts. The tubes were then mixed using a vortex machine and incubated for 1 h under dark condition. The absorbance values were then measured at 725 nm with a UV-visible spectrophotometer (Genesys 10S UV-VIS spectrophotometer, Thermo Scientific, NY, USA). Using a standard curve of different concentrations of gallic acid in 95 % ethanol, the absorbance values of the extracts were converted into the TSP content, which was expressed in milligram gallic acid equivalents per gram dry weight (mg GAE g^{-1}).

10.3.5. High performance liquid chromatography analysis of phenolic profile

The profile of the phenolic compounds was determined using high performance liquid chromatography (HPLC) method, in which 5 µL of the unfermented and fermented corn-DDGS extracts were injected using an Agilent ALS 1200 auto-extractor into an Agilent 1260 series (Agilent Technologies, Palo Alto, CA) HPLC equipped with a D1100 CE diode array detector.

The solvents used for gradient elution were 10 mM phosphoric acid (pH 2.5) and 100 % methanol. The methanol concentration was increased to 60 % for the first 8 min, then to 100 % over the next 7 min, then decreased to 0 % for the next 3 min and was maintained for 7 min with a total run time of 25 min per injected sample run. 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 0.7 mL min^{-1} at room temperature. The absorbance values were recorded at 214 nm, 230 nm, 260 nm, and 306 nm and the chromatogram was integrated using Agilent Chem station enhanced integrator. Pure standards of *p*-coumaric acid, gallic acid, dihydroxybenzoic acid, caffeic acid, ferulic acid and catechin in 100 % methanol were used to calibrate the respective standard curves and retention times. The phenolic compounds detected in the extracts were expressed in micro gram per gram dry weight (μg g^{-1}).

10.3.6. Antioxidant activity

The antioxidant activity of the unfermented and fermented corn extracts was measured by their scavenging activity against the free radicals 2, 2-Dipheny-1-Picryl hydrazyl (DPPH) (D9132-5G, Sigma-Aldrich), and 2, 2-Azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) (A1888-5G, Sigma-Aldrich) respectively. The DPPH scavenging assay was based on the protocol as described previously (Kwon et al, 2006) in which 0.25 mL of the extract was added to 1.25 mL of 60 mM DPPH (prepared in 95 % ethanol) while the control had 0.25 mL of 95 % ethanol instead of the extract. After 5 min of incubation, the extracts and their corresponding controls were centrifuged at 13,000 rpm for 1 min and the absorbance values of the supernatant was measured at 517 nm using a UV-visible spectrophotometer (Genesys 10S UV-VIS spectrophotometer, Thermo Scientific, NY). The ABTS scavenging assay was based on the protocol as described previously (Re et al, 1999) in which 0.05 mL of the extract was added to 1

mL of ABTS (prepared in 95 % ethanol) while the control had 0.05 mL of 95 % ethanol instead of the extract. After 2 min of incubation, the extracts and their corresponding controls were centrifuged at 13,000 rpm for 1 min and the absorbance values of the supernatant was measured at 734 nm using a UV-visible spectrophotometer (Genesys 10S UV-VIS spectrophotometer, Thermo Scientific, NY). The absorbance values from the DPPH and ABTS radical scavenging assays were used to calculate the percentage of antioxidant activity of the extracts using the following formula:

$$\% \text{ Antioxidant activity} = \frac{\text{Control}^{\text{absorbance}} - \text{Extract}^{\text{absorbance}}}{\text{Control}^{\text{absorbance}}} \times 100$$

10.3.7. Antimicrobial activity

The *in vitro* antimicrobial activity of the unfermented and fermented corn-DDGS extracts against *H. pylori* was measured using the agar diffusion method based on the protocol described previously (Ranilla et al, 2017). The frozen *H. pylori* culture was revived and streaked onto HPSP agar plates with the help of sterile cotton swabs. Then sterile 12.7 mm paper discs (BBL Taxo, Becton, Dickinson & Co) were placed on the HPSP agar plates and 100 µL of the filter sterilized extracts from the 0, 24, 48 and 72-h fermentation time points were added to their respective paper discs with each plate having a control disc of sterile water. The plates were incubated at 37 °C for 48 h in BBL GasPak jars (Becton, Dickinson & Co) containing BD GasPak Campy container system sachets (Becton, Dickinson & Co) to help maintain a microaerophilic environment. After incubation, the plates were examined for any zones of inhibition (no growth) around the discs, and the diameter of the zones of inhibition was expressed in millimeters. To determine if the antimicrobial activity was due to the bacterial or fungal growth present on the surface of the pasteurized corn-DDGS extracts, the corn-DDGS

extracts were untreated, pasteurized (70 °C for 15 min), or autoclaved (121 °C for 20 min) respectively, followed by incubation at 37 °C for 72 h. At the 0, 24, 48 & 72-h time points of incubation, samples were collected and centrifuged at 8,500 rpm for 15 min, after which the supernatant was collected and filter-sterilized for analysis of *in vitro* antimicrobial activity against *H. pylori*.

10.3.8. Isolation of microorganism from pasteurized corn-DDGS extract

The pasteurized corn-DDGS extracts were incubated at 37 °C for up to 72 h under aerobic conditions. After 24 h of incubation, the microorganism growth present on the surface of the extracts was streaked onto nutrient agar plates (Difco, Becton Dickinson & Co) using an inoculation loop, and the plates were incubated at 37 °C for 24 h. The isolated colonies were then sub-cultured in 10 mL nutrient broth (Difco, Becton Dickinson & Co) for another 24 h at 37 °C after which the culture broth was serially diluted, and the higher dilutions were streaked onto nutrient agar plates and incubated for another 24 h at 37 °C. The plates containing the isolated colonies were used as the stock plates. The isolated colonies from the stock plates were inoculated in nutrient broth and incubated at 37 °C for a period of 72 h. At the 0, 24, 48 and 72-hour incubation time points, samples of the culture broth were collected aseptically and centrifuged at 8,500 rpm for 15 min. The cell-free supernatant (CFS) was collected and filter-sterilized using 0.20 µm syringe filters (Millipore Corp, MA, USA), followed by storage at -20° C for later analysis of *in vitro* antimicrobial activity against *H. pylori*.

10.3.9. Characterization of antimicrobial activity

The effect of pH and temperature on the antimicrobial activity of the CFS collected at 0, 24, 48, and 72-h time points of incubation were analyzed. To determine the effect of pH on antimicrobial activity, the CFS was adjusted to acidic (pH 3.0), neutral (pH7.0), or alkaline (pH

9.0) pH respectively using 1N HCl or 1N NaOH, while CFS without pH adjustment (native pH) was used as control treatment. To determine the effect of temperature, the CFS was autoclaved at 121 °C for 20, 30, and 45 min respectively, while the CFS without heat treatment was used as control. The pH-adjusted and heat-treated CFS along with their corresponding controls were filter-sterilized and stored at -20 °C for later analysis of *in vitro* antimicrobial activity against *H. pylori*. The antimicrobial activity of CFS collected at 0, 24, 48 and 72-h time points of incubation was also compared to Nisin, which is an antibacterial peptide. Nisin was prepared to a working concentration of 1000 IU/mL in 0.02 N HCl and was filter sterilized and stored at -20 °C prior to analysis in the *in vitro* antimicrobial assay.

10.3.10. PCR amplification with bacterial and fungal-specific primers

Under aseptic conditions, a loopful of the culture isolated from pasteurized corn-DDGS extracts was used in DNA extraction with the Qiagen DNeasy Plant Mini Kit (Qiagen, Cat. # 69106), and the quality of extracted DNA was determined with a UV-Vis spectrophotometer (NanoDrop^{TM} 2000, ThermoFisher). To determine if the growth isolated from pasteurized corn-DDGS extract (unfermented) was prokaryotic or eukaryotic, conventional PCR (C1000 Touch thermal cycler, BioRad) was performed using bacterial-specific 16s rRNA universal primers and fungal-specific ITS4/5 primers. The PCR was performed in 50 µL reaction volumes containing 1X Green GoTaq reaction buffer with 1.5 mM $MgCl_2$ (Promega, Madison, WI), 0.2 mM of each dNTP, 0.4 µM of each primer, 1.25u of GoTaq DNA polymerase (Promega, Madison, WI), 0.5 µg of template DNA, and nuclease-free water. For the ITS4/5 primers, the following thermocycler parameters were used: initial denaturation at 95 °C for 3 min; 31 cycles of 94 °C for 90 s, 60 °C for 90 s, and 72 °C for 2 min, followed by a final extension at 72 °C for 10 min. For the 16s rRNA primers, the following thermocycler parameters were used: initial denaturation

at 95 °C for 10 min; 36 cycles of 95 °C for 30 s, 55 °C for 1 min, and 72 °C for 2 min, followed by a final extension at 72 °C for 5 min. For both PCR runs, DNA isolated from *Colletotrichum coccodes* and *Streptomyces scabies*, both laboratory isolates, were used as the respective fungal and bacterial positive controls while sterile water was used as the negative control. The PCR product was purified using a PCR clean up kit (MidSci IBI Gel Extraction Kit, item #ASDNARNAKIT6) and was sent to MCLAB (www.mclab.com/DNA-Sequencing-Services.html) for sequencing. The sequence data was analyzed using a consensus tool (https://www.geneious.com/) and the resulting consensus sequence was compared to the nucleotide database using the Nucleotide BLAST tool at NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

10.3.11. PCR amplification with Bacillus specific primer and construction of the phylogenetic tree

The DNA extracted from the bacterial isolate was subjected to conventional PCR using the *Bacillus* specific forward primer BacF (5'- GGGAAACCGGGGCTAATACCGGAT- 3') and the universal bacterial 16S rDNA reverse primer R1378 (5'-CGGTGTGTACAAGGCCCGGGAACG-3') as described earlier (Garbeva, Van Veen & Van Elsas, 2003). The PCR was performed in 50 µL reaction volumes containing 1X Green GoTaq reaction buffer with 1.5 mM $MgCl_2$ (Promega, Madison, WI), 0.2 mM of each dNTP, 0.4 µM of each primer, 1.25u of GoTaq DNA polymerase (Promega, Madison, WI), 0.5 µg of template DNA, and nuclease-free water. The thermocycler parameters used were as follows: initial denaturation at 95 °C for 15 min; forty cycles of 95 °C for 60 s, 63 °C for 30 s, and 72 °C for 60 s, followed by a final extension at 72 °C for 8 min. DNA isolated from *Bacillus subtilis* (ATCC 6051) and *Helicobacter pylori* (ATCC 43579) were used as the respective positive and negative

controls. The PCR product was purified and sent to Eurofins (https://www.eurofins.com/genomic-services/our-services/custom-dna-sequencing/) for sequencing. From the resulting sequence data, a consensus sequence was generated using MultAlign (http://multalin.toulouse.inra.fr/multalin/) (Corpet, 1988). A pairwise sequence alignment of the forward sequence with a reverse complement of the reverse sequence was done using the Smith-Waterman algorithm (Smith & Waterman, 1981), and the missing base pairs were filled with the help of complementary base-pairing from the aligned sequences. This step was done to ensure that the final 16s rRNA sequence had the best base coverage. The final consensus sequence was submitted to the GenBank (https://submit.ncbi.nlm.nih.gov/) and the accession number ON553411 was assigned to the 16S rRNA sequence (https://submit.ncbi.nlm.nih.gov/subs/?search=SUB11502195). Nucleotide BLAST tool at NCBI was used to compare ON553411 against the database with the taxonomy ID- 1386 (*Bacillus* species) in the organism category. A phylogenetic analysis of ON553411 was done with MEGA X software (Kumar et al., 2018) in which the sequence was first aligned using MUSCLE algorithm followed by phylogenetic analysis via the neighbor-joining, maximum-parsimony, and maximum-likelihood method, each performed with 100 bootstrap replications.

10.3.12. Statistical analysis

Fermentation, phenolic profile characterization, and antioxidant and antimicrobial assays were repeated two times with repeat extractions and duplicate samples. The means and standard error were calculated from 12 (n) data points using Microsoft Excel XP software. The analysis of covariance was determined using Statistical Analysis Software (SAS version 9.4, SAS Institute, Cary, NC). Statistical mean separation among extracts, fermentation time points, and extract × fermentation time point interactions were determined using Tukey's test at 0.05 probability level.

10.4. Results and Discussion

10.4.1. Total soluble phenolic content (TSP) and phenolic profile

The TSP content of the corn-DDGS extracts (unfermented and fermented) ranged from 2.49 to 2.85 mg GAE g^{-1} (Table 10.1). Statistical differences in TSP content were observed among main effects of extracts and fermentation time points individually ($p<0.05$), but not among extract × fermentation time point interactions. Overall, unfermented extracts had higher TSP content and the mean TSP content increased after 24 h of fermentation.

Individual phenolic compounds detected through HPLC analysis of the unfermented and fermented corn-DDGS were gallic acid, dihydroxybenzoic acid, *p*- coumaric acid, ferulic acid, caffeic acid, and catechin, with their respective concentrations ranging from 1.5 to 6.9, 1.48 to 9.53, 10.18 to 16.7, 0.27 to 1.65, 0.51 to 1.63, and 2.35 to 4.44 µg g^{-1} (Table 10.2). In a previous study, corn-DDGS samples collected from different ethanol production plants in the US were analyzed for their phytochemical content and the study found that corn DDGS had a higher content of tocopherols, tocotrienols, lutein and ferulic acid (free and bound) when compared to yellow corn (Shin et al, 2018). The same study found the free ferulic acid content to be 3 times higher in the corn DDGS samples when compared to yellow corn (Shin et al., 2018). In another study, corn DDGS samples from three different ethanol production plants were found to have the phenolic compounds- vanillic, caffeic, *p*-coumaric, ferulic, and sinapic acids, with ferulic and *p*-coumaric acid accounting for 80% of the total phenolic acid content (Luthria et al, 2012). Also, the same study found the total phenolic acid content to be 3 times higher in the corn DDGS samples when compared to yellow corn samples (Luthria et al, 2012).

Table 10.1. Total soluble phenolic content (mg GAE g^{-1}) and antioxidant activity (% inhibition) of unfermented and fermented corn-DDGS extracts.

Fermentation time point (h)	**Extracts**	**TSP[1,2,3]**	**ABTS[1,4,5]**	**DPPH[1,5,6]**
0	Unfermented	2.85 ± 0.1aAB	77.2 ± 2.3bc	76.5 ± 0.4abc
	L. plantarum fermented/ adjusted pH	2.76 ± 0.0bcAB	74.0 ± 3.0c	76.1 ± 2.2abc
	L. plantarum fermented/ unadjusted pH	2.65 ± 0.1dAB	83.9 ± 1.8a	70.6 ± 0.5cdef
	L. helveticus fermented/ adjusted pH	2.78 ± 0.0abAB	82.2 ± 1.8a	77.9 ± 2.4ab
	L. helveticus fermented/ unadjusted pH	2.64 ± 0.0cdAB	81.9 ± 1.7ab	73.0 ± 0.9bcd
24	Unfermented	2.83 ± 0.0aA	81.0 ± 2.1ab	66.2 ± 2.2efgh
	L. plantarum fermented/ adjusted pH	2.76 ± 0.0bcA	82.2 ± 1.9a	80.6 ± 0.9a
	L. plantarum fermented/ unadjusted pH	2.68 ± 0.1dA	82.9 ± 1.7a	65.9 ± 1.2fgh
	L. helveticus fermented/ adjusted pH	2.79 ± 0.0abA	82.6 ± 1.6a	81.8 ± 0.8a
	L. helveticus fermented/ unadjusted pH	2.73 ± 0.1cdA	83.0 ± 1.8a	68.6 ± 0.9defg
48	Unfermented	2.77 ± 0.1aB	82.8 ± 1.7a	79.0 ± 0.6ab
	L. plantarum fermented/ adjusted pH	2.75 ± 0.0bcB	82.7 ± 2.1a	78.4 ± 0.3ab
	L. plantarum fermented/ unadjusted pH	2.56 ± 0.0dB	83.0 ± 1.7a	60.2 ± 0.4hi
	L. helveticus fermented/ adjusted pH	2.73 ± 0.0abB	81.4 ± 2.0ab	78.9 ± 0.3ab
	L. helveticus fermented/ unadjusted pH	2.56 ± 0.0cdB	81.4 ± 2.0ab	62.9 ± 0.5ghi

Table 10.1. Total soluble phenolic content (mg GAE g-1) and antioxidant activity (% inhibition) of unfermented and fermented corn-DDGS extracts (continued).

Fermentation time point (h)	Extracts	TSP[1,2,3]	ABTS[1,4,5]	DPPH[1,5,6]
72	Unfermented	2.74 ± 0.1aC	82.7 ± 1.7a	72.7 ± 3.6bcde
	L. plantarum fermented/ adjusted pH	2.52 ± 0.1bcC	81.5 ± 1.8ab	75.3 ± 0.3abcd
	L. plantarum fermented/ unadjusted pH	2.49 ± 0.0dC	83.8 ± 1.7a	57.3 ± 0.6i
	L. helveticus fermented/ adjusted pH	2.66 ± 0.1abC	83.2 ± 2.2a	76.1 ± 0.2abc
	L. helveticus fermented/ unadjusted pH	2.52 ± 0.1cdC	81.0 ± 2.3ab	62.0 ± 1.1ghi

[1] Mean ± standard error
[2] Different lowercase letters in first column represent statistical differences in TSP content between unfermented and fermented extracts (p<0.05).
[3] Different uppercase letters in first column represent statistical differences in TSP content between fermentation time points (p<0.05).
[4]2, 2-Azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) radical scavenging activity expressed as % inhibition.
[5] Different lowercase letters in each column represent significant differences between extract × fermentation time point interactions (p<0.05).
[6] 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity expressed as % inhibition.

In the current study, *p*-coumaric acid was the dominant phenolic acid while ferulic acid was present in smaller quantities (Table 10.2). Dihydroxybenzoic acid was detected in unfermented and *L. helveticus* fermented extracts and not in the *L. plantarum* fermented extracts, thereby indicating possible microbial metabolism of dihydroxybenzoic acid by *L. plantarum*. Also, gallic acid was significantly higher in the unfermented extracts when compared to the fermented extracts at the 24 and 48-h time points, thereby indicating possible microbial metabolism of gallic acid by *L. helveticus* and *L. plantarum*. Furthermore, the low pH generated during fermentation due to growth of LAB can also affect the stability of phenolic compounds, as a pH close to neutral favors a better stability of these water-soluble phenolics (Friedman & Jürgens, 2000). These results indicate that fermentation of corn-DDGS with LAB did not

drastically alter the TSP content, however the profile and concentration of individual phenolic compounds can be altered depending upon the type of LAB used for fermentation and total duration of fermentation.

Table 10.2. Phenolic profile of unfermented and fermented corn-DDGS extracts (µg/g).

Fermentation time point (h)	**Extracts**	**Phenolic profile[1,2]**					
		Gallic acid[3]	Dihydroxybenzoic acid[3]	*p*-coumaric acid[3]	Ferulic acid	Caffeic acid[3]	Catechin[4]
0	Unfermented	5.49 ± 0.8b	3.72 ± 0.1	13.76 ± 0.0def	ND	1.27 ± 0.0g	2.37 ± 0.0B
	L. plantarum fermented/ adjusted pH	5.14 ± 0.1bc	1.48 ± 0.3	14.26 ± 0.0def	0.27 ± 0.0	1.29 ± 0.0fg	2.35 ± 0.1B
	L. plantarum fermented/ unadjusted pH	5.03 ± 0.1bcd	1.88 ± 0.0	16.35 ± 0.2a	0.68 ± 0.0	1.42 ± 0.0cd	2.69 ± 0.0B
	L. helveticus fermented/ adjusted pH	5.32 ± 0.0bc	4.04 ± 0.0	14.25 ± 0.1def	0.33 ± 0.0	1.32 ± 0.0ef	2.48 ± 0.4B
	L. helveticus fermented/ unadjusted pH	5.05 ± 0.1bcd	2.05 ± 0.0	16.16 ± 0.1abc	0.72 ± 0.0	1.39 ± 0.0d	2.47 ± 0.0B
24	Unfermented	5.56 ± 0.1b	2.00 ± 0.0	16.27 ± 0.0ab	0.79 ± 0.0	1.43 ± 0.0c	3.11 ± 0.0A
	L. plantarum fermented/ adjusted pH	1.78 ± 0.0ij	ND	14.39 ± 0.0def	0.39 ± 0.01	1.49 ± 0.0b	3.55 ± 0.1A
	L. plantarum fermented/ unadjusted pH	1.51 ± 0.0j	ND	13.44 ± 0.0efgh	0.80 ± 0.0	1.33 ± 0.0e	3.77 ± 0.0A
	L. helveticus fermented/ adjusted pH	2.90 ± 0.0ghi	4.11 ± 0.0	14.55 ± 0.0cdef	0.45 ± 0.0	1.30 ± 0.0fg	3.00 ± 0.1A
	L. helveticus fermented/ unadjusted pH	1.96 ± 0.0ij	4.21 ± 0.0	14.70 ± 0.0bcde	0.72 ± 0.0	1.27 ± 0.0g	4.38 ± 0.9A
48	Unfermented	6.97 ± 0.1a	9.53 ± 0.1	12.20 ± 1.2gh	0.59 ± 0.0	0.92 ± 0.0i	3.72 ± 0.8A
	L. plantarum fermented/ adjusted pH	4.45 ± 0.3bcdef	ND	11.92 ± 0.0hi	1.65 ± 0.0	1.19 ± 0.0h	3.79 ± 0.1A
	L. plantarum fermented/ unadjusted pH	3.86 ± 0.0efg	ND	13.32 ± 0.0efgh	0.72 ± 0.1	1.28 ± 0.0g	4.21 ± 0.2A
	L. helveticus fermented/ adjusted pH	4.99 ± 0.1bcde	3.47 ± 0.0	13.02 ± 0.0fgh	0.66 ± 0.0	1.20 ± 0.0h	3.09 ± 0.0A
	L. helveticus fermented/ unadjusted pH	4.71 ± 0.0bcde	4.47 ± 0.0	16.70 ± 0.0a	0.79 ± 0.0	1.49 ± 0.0b	3.67 ± 0.0A

Table 10.2. Phenolic profile of unfermented and fermented corn-DDGS extracts (µg/g) (continued).

Fermentation time point (h)	**Extracts**	**Phenolic profile[1,2]**					
		Gallic acid[3]	Dihydroxybenzoic acid[3]	*p*-coumaric acid[3]	Ferulic acid	Caffeic acid[3]	Catechin[4]
72	Unfermented	4.48 ± 0.1bcdef	2.68 ± 0.9	10.18 ± 0.0j	ND	0.70 ± 0.0j	3.92 ± 0.0A
	L. plantarum fermented/ adjusted pH	3.92 ± 0.1defg	ND	12.95 ± 0.1fgh	0.96 ± 0.1	1.19 ± 0.0h	4.44 ± 0.0A
	L. plantarum fermented/ unadjusted pH	3.34 ± 0.1fgh	ND	10.40 ± 0.0ij	0.51 ± 0.0	0.51 ± 0.0k	3.78 ± 0.0A
	L. helveticus fermented/ adjusted pH	4.27 ± 0.2cdef	3.40 ± 0.0	13.37 ± 0.0efgh	ND	1.51 ± 0.0b	3.33 ± 0.0A
	L. helveticus fermented/ unadjusted pH	2.68 ± 0.01hi	4.02 ± 0.0	15.47 ± 0.1abcd	0.86 ± 0.0	1.63 ± 0.0a	3.65 ± 0.0A

[1] Mean value ± standard error.
[2] Concentration expressed in microgram per gram dry weight (µg g^{-1} D.W).
[3] Different lowercase letters in each column represent statistically significant differences between extract × fermentation time point interactions ($p < 0.05$).
[4] Different uppercase letters in last column represent statistically significant differences in catechin content between fermentation time points ($p < 0.05$).

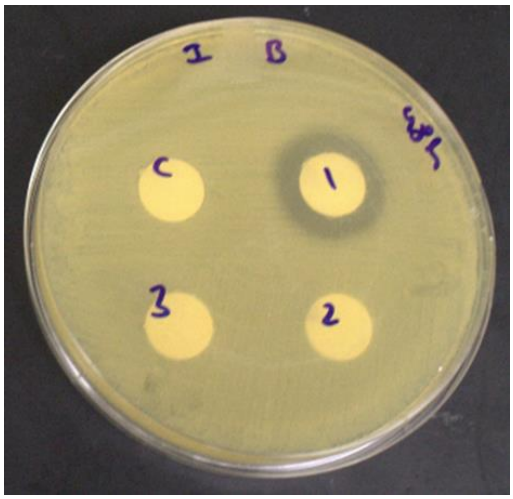
10.4.2. Antioxidant activity

The ABTS and DPPH radical scavenging activity of corn DDGS extracts (unfermented and fermented) ranged from 74 % to 83.9 % and 57.3 % to 81.8 % respectively (Table 10.1). Statistical differences in ABTS and DPPH radical scavenging activity were observed among extracts, fermentation time points, and extract × fermentation time point interactions ($p<0.05$). Overall, the unfermented and fermented corn-DDGS extracts had higher ABTS scavenging activity when compared to DPPH scavenging activity. This difference in activity between two free radical scavenging assays is due to the chemical nature of the radicals in which DPPH being a more stable radical, requires stronger antioxidant activity when compared to ABTS. In a previous study, corn-DDGS samples collected from different ethanol production plants in the US were analyzed for their antioxidant activity via DPPH radical scavenging activity, and the activity was found to vary greatly among the corn-DDGS samples, with activity being 3 times higher in the corn-DDGS samples when compared to yellow corn (Shin et al, 2018). Similarly in another study, the antioxidant activity of corn-DDGS samples from three different ethanol production plants was found to be 2.5 times higher when compared to yellow corn (Luthria et al, 2012). In the current study, LAB fermentation resulted into improvement in antioxidant activity (based on DPPH results) after 24 h fermentation. The results of the current study indicated that fermentation of corn-DDGS, specifically for 24 h can be a potential strategy to improve the stability of phenolic phytochemicals and associated antioxidant activity targeting wider animal feed and health benefits.

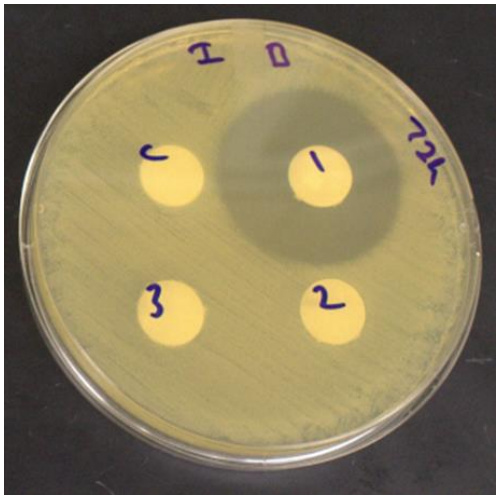
10.4.3. Antimicrobial activity

Antimicrobial activity against *H. pylori* was observed only among the unfermented extracts at the 48- and 72-h fermentation time points, while no antimicrobial activity was

observed for the extracts fermented with *L. plantarum* and *L. helveticus* (with and without pH adjustment) (Figure 10.1 A, B & Figure 10.2 A, B). The zones of inhibition for the unfermented extracts ranged from 2 to 4 mm and 11 to 12 mm at the 48- and 72-h fermentation time-points respectively.

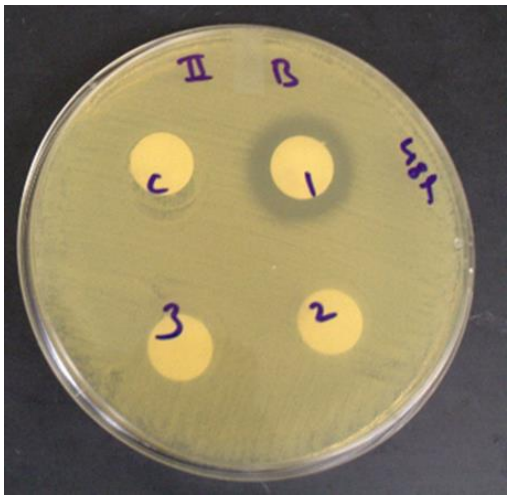


A



B

Figure 10.1. Antimicrobial activity of unfermented and *L. plantarum* fermented (with and without pH adjustment) corn-DDGS extracts against *H. pylori* at (A) 48-and (B) 72-h fermentation time points. C- control (sterile water), 1- unfermented extract, 2-fermented extract (adjusted pH), and 3- fermented extract (unadjusted pH). Zone of inhibition measured in millimeters (mm).



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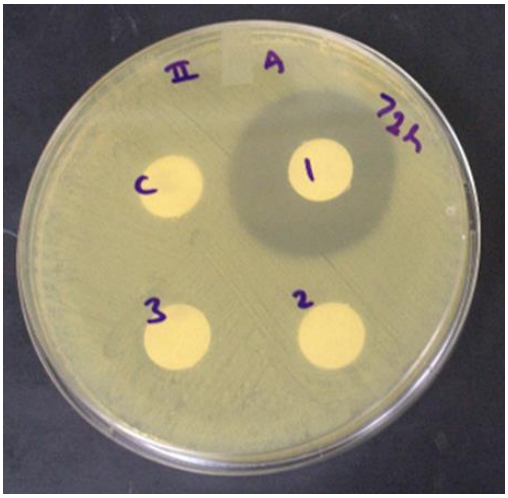


B

Figure 10.2. Antimicrobial activity of unfermented and L. heveticus fermented (with and without pH adjustment) corn DDGS extracts against H. pylori at (A) 48-and (B) 72-h fermentation time points. C- control (sterile water), 1- unfermented extract, 2- fermented extract (adjusted pH), and 3- fermented extract (unadjusted pH). Zone of inhibition measured in millimeters (mm).

Growth of an unknown microorganism was observed at the surface of the unfermented pasteurized corn-DDGS extracts after 24 h of incubation at 37 °C. To confirm if the antimicrobial activity was due to the microorganism growth, the corn-DDGS extracts were untreated, pasteurized, or autoclaved, followed by incubation at 37 °C for 72 h. A microbial lawn appeared at the surface of the pasteurized corn-DDGS extracts after 24 h of incubation, while

some turbidity was observed for the untreated extracts which could be due to microbial growth, and no turbidity or growth was observed in the autoclaved extracts (Figure 10.3).

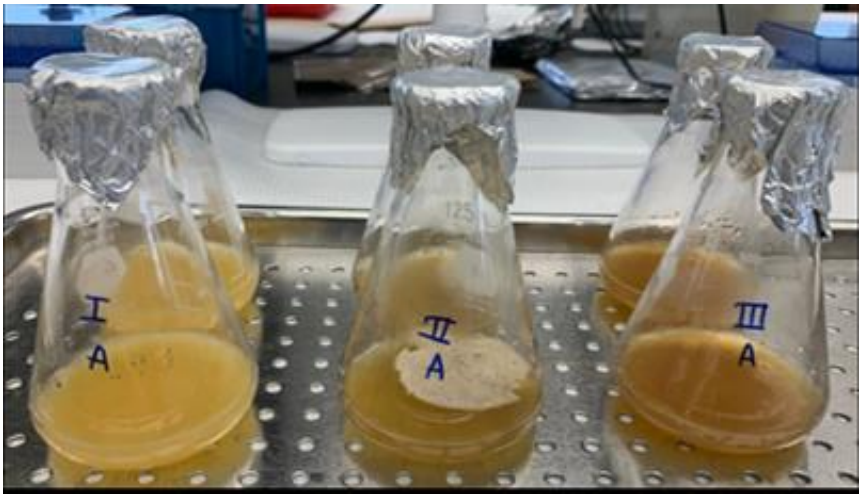
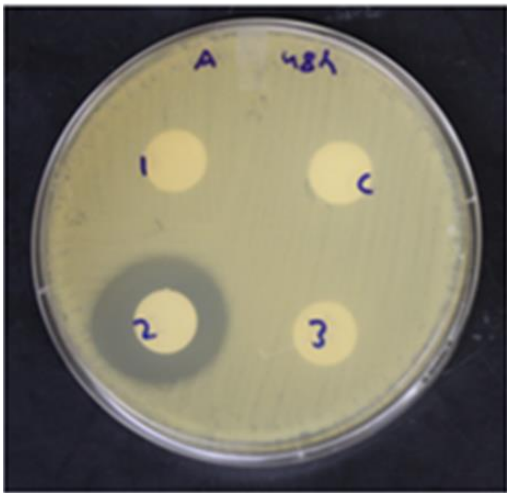
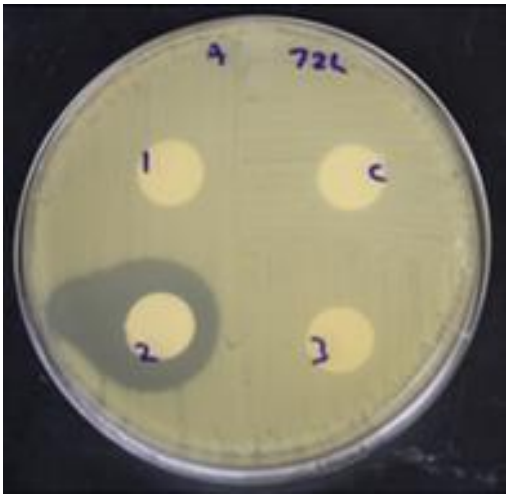


Figure 10.3. Presence of microorganism growth at the surface of pasteurized corn-DDGS extracts. I- untreated extract, II- pasteurized extract (70 °C for 15 min), and III- autoclaved extract (121 °C for 20 min).

Antimicrobial activity was observed only for the pasteurized extract at the 48- and 72-h incubation time points, with zones of inhibition ranging from 6 to 10 mm and 7 to 14 mm, respectively (Figure 10.4 A, B). These results indicated that the pasteurization of the corn-DDGS extract was responsible for the growth of the microorganism endemic to the corn-DDGS sample, and pasteurization led to the activation of bacterial spores, which later was confirmed belonging spore producing genus of *Bacillus.* Furthermore, the microorganism growth which was present only in the pasteurized corn-DDGS extracts was responsible for exhibiting antimicrobial activity against *H. pylori*.



A



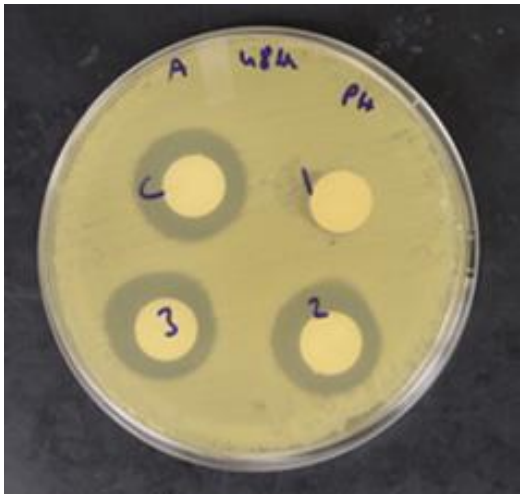
B

Figure 10.4. Antimicrobial activity of untreated, pasteurized, and autoclaved corn DDGS extracts against *H. pylori* at the (A) 48-h and (B) 72-h incubation time points. C- control (sterile water), 1- untreated extract, 2- pasteurized extract, and 3- autoclaved extract. Zone of inhibition measured in millimeters (mm).

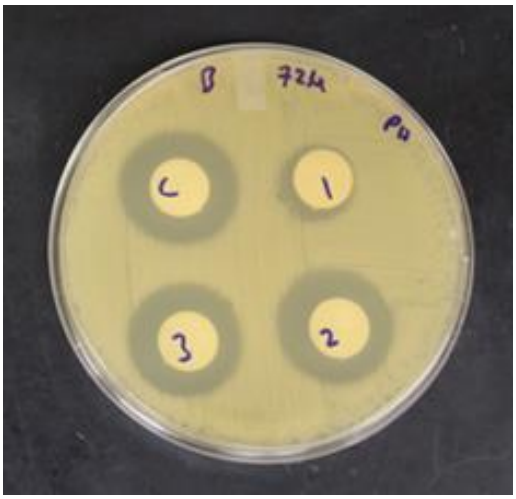
10.4.4. Characterization of antimicrobial activity

The antimicrobial activity was affected by pH and high temperature. With regard to the effect of pH, at the 48-h incubation time point, no antimicrobial activity was observed for CFS adjusted to acidic pH (pH 3.0) while the activity was unaffected at neutral (pH 7.0) and alkaline (pH 9.0) when compared to the control (native pH 5.0) (Figure 10.5 A). At the 72-h incubation

time point, mild antimicrobial activity was observed at acidic pH (pH 3.0) while the activity was unaffected at neutral and alkaline pH (Figure 10.5 B).



A

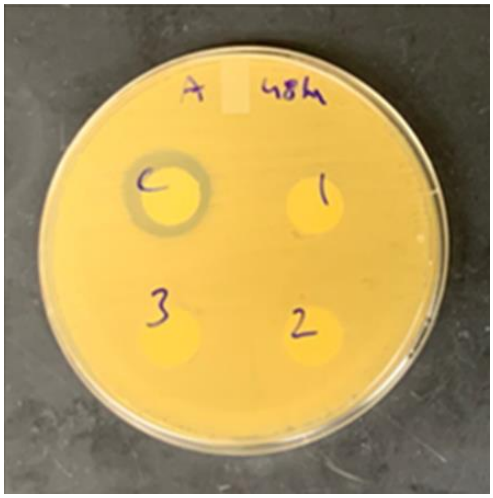


B

Figure 10.5. Antimicrobial activity of pH-adjusted cell free supernatant (CFS) against *H. pylori* at (A) 48- h and (B) 72-h incubation time points. C- control (native pH 5.0), 1- acidic (pH 3.0), 2- neutral (pH 7.0), and 3- alkaline (pH 9.0). Zone of inhibition measured in millimeters (mm).

In terms of the effect of temperature, at the 48-h incubation time point, no antimicrobial activity was observed for CFS treated at 121 °C for 20, 30, and 45 min respectively when compared to control (unadjusted pH) (Figure 10.6 A). However, at the 72-h incubation time

point, mild antimicrobial activity was observed for CFS treated at 121 °C for 20, 30, and 45 min respectively when compared to the control (untreated) (Figure 10.6 B).



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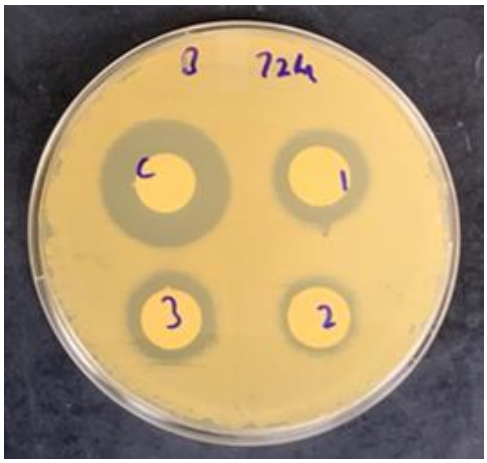

B

Figure 10.6. Antimicrobial activity of heat-treated (121°C) cell free supernatant (CFS) against *H. pylori* at (A) 48-h and (B) 72-h incubation time points. C- control (untreated), 1- 20 min, 2- 30 min, and 3- 45 min. Zone of inhibition measured in millimeters (mm).

The sensitivity of antimicrobial activity to pH and high temperature indicated that extracellular antimicrobial peptides produced by unknown bacterial/fungal growth are potentially responsible for antimicrobial activity against *H. pylori*. Furthermore, the mild antimicrobial activity observed for heat-treated CFS at the 72-h incubation time point indicated that sufficient concentration of these antimicrobial peptides was produced to show antimicrobial activity even

at a high temperature, thereby indicating that these antimicrobial peptides are generally heat stable. Finally, the CFS at the 48 and 72-h incubation time points had similar antimicrobial activity when compared to Nisin (Figure 10.7), thereby indicating that these peptides would have relevant applications for developing natural antibiotics with potential antimicrobial activity against other bacterial pathogens.

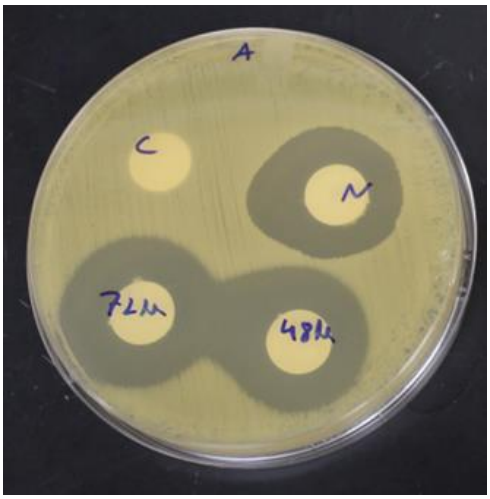

Figure 10.7. Antimicrobial activity of cell free supernatant (CFS) and Nisin against *H. pylori* at 48-h and 72-h incubation time points. C- control (sterile water), N- Nisin (1000 IU/ mL), 48 h- and 72 h- incubation time point.

10.4.5. Isolation and identification of microorganism growth

The isolated microbial growth on the stock plates appeared as dry wrinkled colonies on the nutrient agar plate which is the colony morphology often associated with certain *Bacillus* spp., (Figure 10.8) (Koneman, Allen, Janda, Schreckenberger & Winn Jr, 1997). The ability of *Bacillus* spp., to form a biofilm or lawn at the surface of the liquid medium (Figure 10.3) has also been reported in a previous study (Lu, Guo & Liu, 2018).

Figure 10.8. Colony morphology of unknown microbial growth isolated from pasteurized corn-DDGS extracts.

Species belonging to *Bacillus* are rod-shaped, endospore-forming, gram-positive bacteria that are abundant in the soil and can produce structurally diverse antimicrobial peptides that exhibit a wide spectrum of antibiotic activity (Sumi, Yang, Yeo & Hahm, 2015). The formation of endospores enables *Bacillus* spp., to survive long periods of adverse environmental conditions and the germination of endospores can occur due to pressure, chemical treatment, or sublethal heat treatment (Cronin & Wilkinson, 2008; Luu et al, 2015). In the current study, the pasteurization of the corn-DDGS extracts at 70 °C potentially led to the germination of endospores resulting in the growth of the *Bacillus* isolate, while the untreated and autoclaved (121 °C) extracts did not show any growth of the isolate (Figure 10.3).

10.4.6. PCR identification of microorganism growth and phylogenetic analysis

To identify whether the growth was bacterial or fungal, the isolated culture was subjected to PCR using bacterial-specific 16s rRNA primers and fungal-specific ITS4/5 primers. PCR with 16s rRNA primers gave an amplified product while no product was observed with the fungal-specific primers, therefore indicating that the isolated growth was bacterial (Figure 10.9).

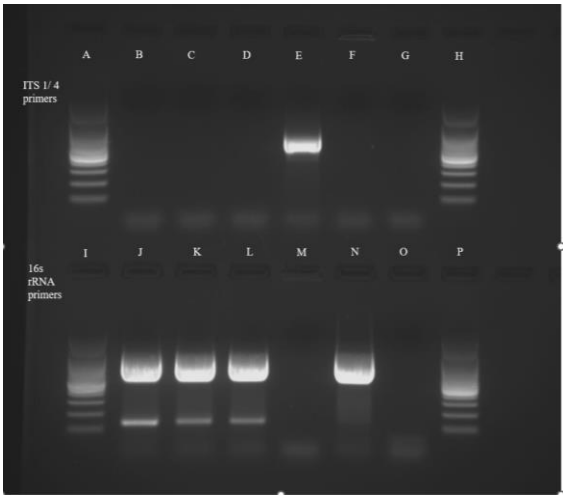

Figure 10.9. PCR with fungal-specific ITS 1/ 4 primers: A- 100 bp ladder, B- DNA isolate (1st replicate), C- DNA isolate (2nd replicate), D- DNA isolate (3rd replicate), E- fungal control (*Colletotrichum coccodes*), F- bacterial control (*Streptomyces scabies*), G- negative control (water), and H- 100 bp ladder. PCR with bacterial-specific 16s rRNA primers: I- 100 bp ladder, J- DNA isolate (1st replicate), K- DNA isolate (2nd replicate), L- DNA isolate (3rd replicate), M- fungal control (*C. coccodes*), N- bacterial control (*S. scabies*), O- negative control (water), P- 100 bp ladder.

Sequence analysis showed a close match with *Bacillus* spp., (99.51 % to 99.75 %). However, a higher degree of specificity could not be determined with the universal 16s rRNA primers. To improve the accuracy of the bacterial identification, PCR with *Bacillus*-specific 16S rRNA primers was performed (Figure 10.10), and sequence analysis of the PCR product (956 bp) (consensus sequence assigned as ON553411) showed a close match (99.9%) with *Bacillus amyloliquefaciens*, *B. velezensis*, *B. subtilis*, and *B. siamensis*. To determine the closest match among these candidates, a phylogenetic tree was constructed using neighbor joining method with 100 bootstrap replications and ON553411 was found to share a cluster with *B. amyloliquefaciens* strain B6 16S rRNA gene partial sequence (MN908674.1) (Figure 10.11).

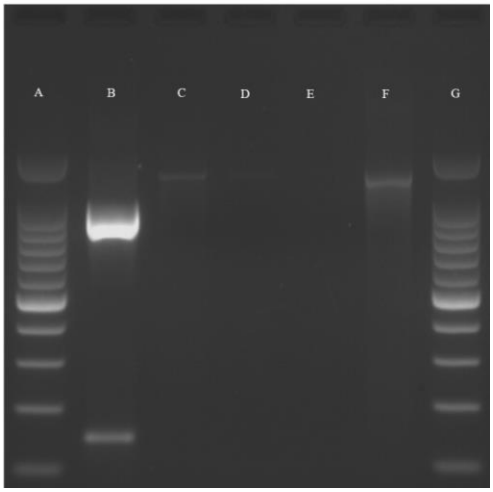

Figure 10.10. PCR with *Bacillus*-specific primers: A- 100 bp ladder, B- 16s rRNA PCR product, C- positive control (*B. subtilis*), D- negative control (*H. pylori*), E- negative control (water), F- DNA isolate (single replicate), G- 100 bp ladder.

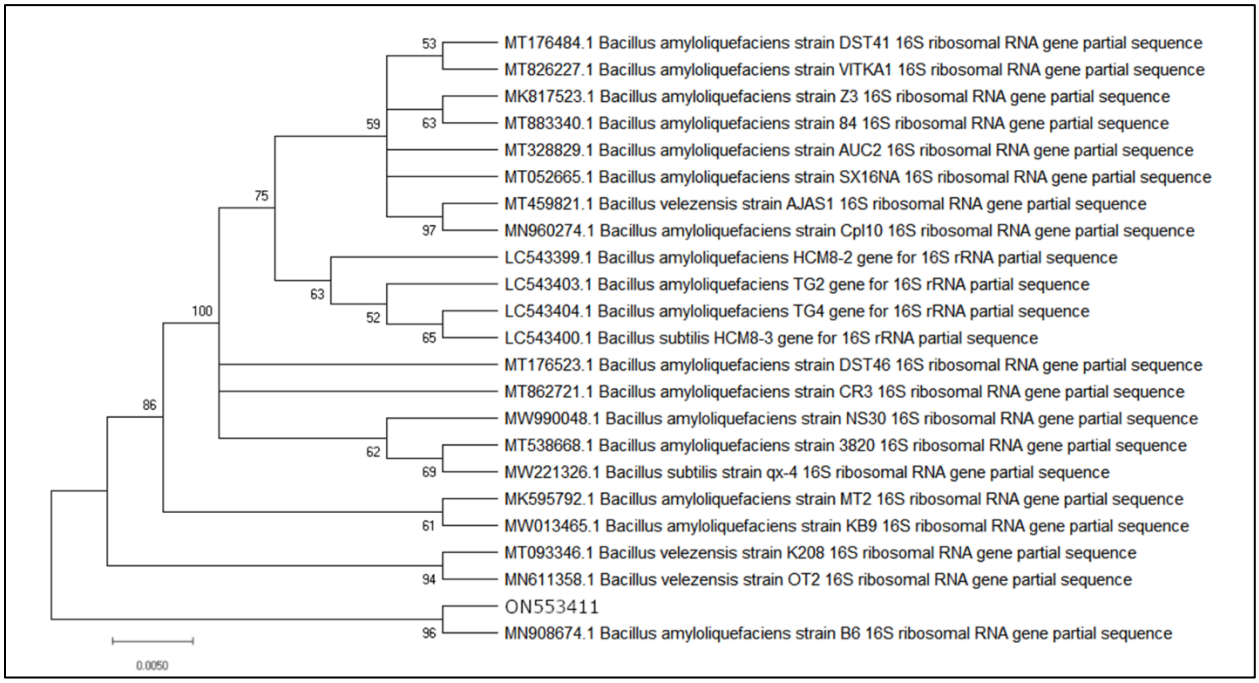

Figure 10.11. Phylogenetic analysis of ON553411 sequence based on 16SrRNA gene sequences of *Bacillus* strains. Neighbor joining method was used with 100 bootstrap replications and genetic distances were computed by Kimura's two-parameter model. Only bootstrap percentages above 50 % are shown. Bars, 0.005 substitutions per nucleotide position.

Although PCR based methods using 16S rRNA sequencing are used to discriminate between *Bacillus* spp., a high degree of sequence similarity can make it difficult to accurately

discriminate between the species. In a previous study, a *Bacillus* strain isolated from soil shared a high sequence similarity (95% to 99%) with 16S rRNA and *gyr*B sequences of *B. amyloliquefaciens* and *B. velezensis* (Wang, Lee, Tai & Kuo, 2008). In the same study, a phylogenetic analysis of the 16S rRNA sequences showed that the *Bacillus* strain isolated from the soil was in the same cluster as other *Bacillus* species including *B. velezensis*, *B. amyloliquefaciens*, and *B. vallismortis*, thereby making it difficult to accurately identify the isolated strain (Wang et al, 2008). Similarly in another study, sequence analysis of the primer annealing sites revealed no clear-cut differences in the variable region (V 1) of the 16S rRNA and *gyr*B gene among the *B. cereus* and *B. thuringiensis* strains that were tested (Chen & Tsen, 2002). These studies indicated that identification of *Bacillus* spp., can be challenging due to high sequence similarity, especially among strains of *B. amyloliquefaciens* and *B. velezensis*.

10.5. Conclusion

Plant-based by-products from biotechnological or industrial processing often have further applications as animal feed, due to their suitable calorific content and potential phytochemical-linked health protective benefits. Furthermore, bio-transformative strategies such as fermentation with LAB can promote value-added functional qualities to the animal feed in terms of altering or improving these associated health protective benefits of the feed. In addition to plant-based by-products being utilized as animal feed, these by-products can also serve as a source for novel native microflora that have suitable biotechnological, industrial, or pharmaceutical applications. Hence corn-DDGS can be a suitable substrate for fermentation strategies aimed at improving the phenolic-linked health protective benefits in animal feed, as well as a source for novel microorganisms that have biotechnological, industrial, or pharmaceutical applications.

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