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Development of Kefir Products Using Aronia or Elderberries and the Impacts of Fermentation on the Health-promoting Characteristics of Aronia Polyphenols

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**DEVELOPMENT OF KEFIR PRODUCTS USING ARONIA OR ELDERBERRIES AND THE IMPACTS OF
FERMENTATION ON THE HEALTH-PROMOTING CHARACTERISTICS
OF ARONIA POLYPHENOLS**

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A DISSERTATION

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August 2018

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Dissertation Advisor: Dr. Angela D. Myracle

An Abstract of the Dissertation Presented
in Partial Fulfillment of the Requirements for the
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August 2018

Diabetes is a global health problem. The consumption of dietary polyphenols may help to decrease the risk of type 2 diabetes and slow the progression of diabetic complications. Aronia (*Aronia melanocarpa*) and elderberry (*Sambucus nigra* L. ssp. *canadensis*) fruits are rich in polyphenols and exhibit health-promoting properties, but they are underutilized. Aronia and elderberries are rarely consumed raw due to the astringent mouth feel. New food products are needed to increase their consumption. Kefir, a fermented dairy beverage, was chosen to be the matrix for incorporating berries due to: 1) the protein matrix can help mask the astringency; 2) an acidic environment is beneficial for the stability of phenolic compounds; 3) fermentative microorganisms may be able to increase the bioavailability of polyphenols.

The first objective of this research was to develop new palatable products using underutilized berries and different sweeteners (sucrose, stevia and monk fruit extracts). Sensory evaluations were conducted to assess consumer acceptability of berry-containing

kefirs. The results showed that aronia and elderberry kefirs sweetened with stevia or sucrose were all accepted by consumers where sucrose was the best-accepted sweetener. The second objective was to assess the health-promoting characteristics of the berry-containing kefirs. Aronia kefirs contained high levels of total phenolics and anthocyanins. Elderberry kefirs were moderate in total phenolics. All kefirs exhibited antioxidant capacity. The third objective was to evaluate the diabetes-beneficial properties of aronia kefir using an *in-vitro* digestion model. The impacts of fermentation on aronia polyphenols were also assessed. The results showed that the levels of bioaccessible polyphenols were elevated during digestion and the antioxidant capacity increased. Fermentation enhanced the inhibitory activity of aronia kefir on α -glucosidase but did not alter its weak inhibition on pancreatic α -amylase. Specific inhibition of α -glucosidase may decrease the absorption of carbohydrates and contribute to blood glucose control without side effects compared to pharmaceutical agents, such as acarbose.

In conclusion, new berry-containing kefirs were well-accepted by the consumers and the consumption of berry-containing kefirs may help to reduce oxidative stress and aid in blood glucose control. In addition, fermentation may be a good strategy to increase the bioavailability of dietary polyphenols.

DEDICATION

I would like to dedicate this dissertation to my grandmother, Xiuying Gao, who had always been there for me when I lose faith, and told me “don’t give up, the best is yet to come”.

I also want to dedicate this dissertation to my parents, Jige Du and Li Xue, for their support and for believing in me. To Yusen Zhai, my fiancé, who was tolerant of my bad temper and encouraged me when I doubted myself.

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LIST OF ABBREVIATIONS

AGE: Advanced glycation end product

Abs: Absorbance

CAT: Catalase

C3G: Cyanidin-3-glucoside

CBG: Cytosolic β -glucosidase

CDC: Centers for Disease Control and Prevention

COX: Cyclooxygenase

CVD: Cardiovascular disease

DM: Diabetes Mellitus

DPP IV: Dipeptidyl peptidase

DPPH: 2,2-diphenyl-1-picrylhydrazyl

DR: Diabetic Retinopathy

ETC: Electron-Transport Chain

FRAP: Ferric reducing antioxidant power

GAE: Gallic acid equivalent

GAPDH: Glyceraldehyde-3 phosphate dehydrogenase

GFAT: Glutamine fructose-6 phosphate amidotransferase

GIT: Gastrointestinal tract

GPx: Glutathione Peroxidase

GSH: Glutathione

HbA1C: Hemoglobin A1C

IL: Interleukin

LDL: Low-density lipoprotein

LPH: Lactate phlorizin hydrolase

LPS: Lipopolysaccharide

MCT: Monocarboxylic acids transporter

NADPH: Nicotinamide adenine dinucleotide phosphate

NO: Nitric Oxide

PG: Prostaglandin

PKC: Protein kinase C

RNS: Reactive Nitrogen Species

ROS: Reactive Oxygen Species

SGLT: Sodium-dependent glucose transporter

SOD: Superoxide Dismutase

STD: Streptozotocin

T1DM: Type 1 Diabetes Mellitus

T2DM: Type 2 Diabetes Mellitus

TA: Titratable acidity

TBARS: Thiobarbituric acid-reactive substances

TMA: Total monomeric anthocyanins

TNF- α : Tumor Necrosis Factor- α

TP: Total phenolics

TPA: 12-o-tetradecanoylphorbol-13-acetate

CHAPTER 1

LITERATURE REVIEW

The prevalence of diabetes has grown rapidly. In 2015, 9.4% of the United States population had diabetes [1]. Epidemiological studies demonstrate that the consumption of bioactive compounds from various fruits and vegetables may help to decrease the risk of chronic diseases, including diabetes [2, 3]. In addition, bioactive compounds obtained from the diet have been shown to be beneficial to prevent and delay secondary diabetic complications [4]. Aronia berries and elderberries are rich in bioactive compounds, but they lack palatability and are rarely consumed raw. New food products using aronia and elderberries were developed in this research. The hypotheses of this research were: 1) sucrose-sweetened berry kefirs will be better accepted by consumers compared to products sweetened with non-nutritive sweeteners; and 2) fermentation will improve the bioavailability of dietary polyphenols. In this literature review, the mechanism of diabetes, the health-promoting properties and the bioavailability of polyphenols will be introduced. In addition, current studies focusing on the health-beneficial properties of aronia berries, elderberry and kefir will be discussed in detail.

1.1 Oxidative Stress and Type 2 Diabetes

Oxidative stress is an underlying mechanism for the development and progression of type 2 Diabetes Mellitus (T2DM). In individuals with T2DM, hyperglycemia can increase the production of reactive oxygen/nitrogen species, which elevates cellular oxidative stress [4]. Strategies that decrease oxidative stress may help to decrease the risk of T2DM and its

secondary complications. The occurrence of oxidative stress and its relationship with T2DM will be explained in this section.

1.1.1 Oxidative Stress

Under aerobic conditions, oxygen participates in the energy production process in living organisms [5]. In eukaryotes, more than 90% of consumed oxygen is converted to water by cytochrome oxidase located in electron-transport chain (ETC) in mitochondria. Less than 10% of consumed oxygen is reduced to reactive oxygen species (ROS) [6]. ROS include hydrogen peroxide (H_2O_2), hydroxyl radicals ($\cdot OH$), and superoxide radical ($O_2^{\cdot -}$). ROS are important molecules involved in the normal metabolism of cells. They can directly interact with signaling molecules and participate in pathways that are critical to cell survival and proliferation [7, 8]. In addition to mitochondria, plasma membranes, endoplasmic reticulum and lysosomes also generate ROS [9]. Cells have an antioxidant defense system to scavenge ROS and maintain redox homeostasis. Antioxidant enzymes, such as superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT), are vital players in the free radical detoxifying process [10]. SOD can neutralize superoxide to less toxic hydrogen peroxide. Hydrogen peroxide can be converted to water and hydrogen by CAT and/or GPx [11]. Under normal conditions, the capacity of the antioxidant defense system is adequate to neutralize the ROS and no oxidative damage occurs to cells and tissues.

Oxidative stress is defined as an imbalance between the production of ROS and the elimination capacity of the antioxidant defense system where oxidative damages may occur to cellular proteins, lipids and DNA [12, 13]. Oxidative stress is associated with the overproduction of ROS and/or a decline in the antioxidant defense system. Oxidative stress can be triggered by

an unhealthy diet, such as high-fat and high-sugar diets. In addition, sedentary lifestyle and abnormal metabolic conditions including hyperglycemia and hyperlipidemia can aggravate oxidative stress [14]. Under oxidative stress conditions, excessive ROS can react with nitric oxide (NO) and form reactive nitrogen species (RNS), including peroxynitrite (ONOO-) and nitrogen dioxide (NO₂) [15]. RNS is another group of free radicals that can result in oxidative damages to cells. Excessive ROS/RNS can modify the structure of proteins and lipids, and impair their function [15]. Elevated ROS/RNS may lead to damages to the mitochondrial membrane and DNA, which can result in apoptosis [16]. Oxidative stress can intensify metabolic disorders and increase the risk of several diseases, such as cardiovascular disease, neurodegenerative diseases and diabetes mellitus. Additionally, oxidative stress can accelerate the development and progression of secondary complications of chronic diseases.

1.1.2 Oxidative Stress and Chronic Inflammation

Inflammation is part of the body's defense system against threats, such as infection and injury [17]. There are two stages of inflammation: acute and chronic [18]. Acute inflammation only persists for a short duration and benefits the host. If acute inflammation cannot eliminate these threats, the inflammatory response will continue and chronic inflammation will be developed [19]. Chronic inflammation is closely interrelated with oxidative stress [20]. ROS can activate pro-inflammatory pathways, such as NF- κ B and elevate the production of pro-inflammatory cytokines, such as tumor necrosis factor- α (TNF- α) and interleukin-6 (IL-6) [19]. Under chronic inflammatory conditions, the phagocytic cells can generate ROS/RNS and induce oxidative damage to tissues. In addition, the pro-inflammatory cytokines can also trigger the generation of ROS/RNS in non-phagocytic cells [21]. Thus, a vicious cycle between chronic

inflammation and oxidative stress is formed. In addition to oxidative stress, chronic inflammation is one of the underlying mechanism of the initiation and progression of chronic diseases [22].

1.1.3 Diabetes Mellitus

1.1.3.1 Introduction to Diabetes Mellitus

Diabetes Mellitus (DM) is a chronic metabolic disease that is characterized by high blood glucose levels (fasting blood glucose level ≥ 7 mmol/L) [23], which is associated with insulin deficiency and/or insulin resistance [24]. There are two major types of diabetes: type 1 diabetes mellitus (T1DM) and type 2 diabetes mellitus (T2DM). T1DM is also known as insulin-dependent diabetes and accounts for about 10% of diabetic cases [24]. T1DM is caused by the autoimmune destruction of β cells in the pancreatic islets. T1DM can be genetically linked and/or influenced by environmental factors [25]. T1DM typically develops in children and teenagers younger than 19 years old [26]. The destruction of β cells leads to diminished insulin production and eventually no production at all. For type 1 diabetic patients, lifelong exogenous insulin administration is necessary to control blood glucose [27]. T2DM is the more predominant type which accounts for approximately 90% of all diabetic cases [24]. T2DM is characterized by an impairment of insulin secretion and/or a decline in insulin sensitivity [28]. The onset of T2DM is strongly influenced by environmental factors, such as unhealthy eating habits, a sedentary lifestyle and obesity [29]. Healthy eating habits, regular exercise and pharmaceutical medication such as acarbose are used to assist blood glucose control in T2DM patients. Exogenous insulin administration may not be involved at the early stage but may be necessary when T2DM progresses over time [30].

From a global perspective in 2016, 422 million people were reported to have diabetes [31]. The United States Centers for Disease Control and Prevention (CDC) reported that 30.3 million (9.4%) of the United States population had diabetes in 2015 including 1.5 million newly diagnosed patients [1]. Diabetic patients need strict control of their blood glucose levels, otherwise complications may occur and deteriorate the individual's quality of life [32]. In the United States, the expense of diabetic health care is the highest among 155 disease conditions and health-related problems, including ischemic heart disease and cancer [33]. The annual expense of diabetic health care in 2013 was \$101.4 billion where 57.6% was spent on medication and 23.5% was spent on ambulatory care. From 1996 to 2013, the prevalence of diabetes in the United States increased from 3.0% to 6.5% and the diabetic health care cost increased \$64.4 billion in this time period [31, 33].

1.1.3.2 Diabetic Complications

Individuals with diabetes may suffer chronic or acute complications. Acute diabetic complications include ketoacidosis in individuals with T1DM and coma due to hypoglycemia [11]. Chronic diabetic complications are associated with damage to vascular endothelial cells caused by hyperglycemia. Vascular endothelial cells are susceptible to hyperglycemia due to lack of ability to down-regulate glucose uptake while extracellular glucose levels are elevated. [34]. Chronic diabetic complications are grouped into either microvascular (retinopathy, neuropathy and nephropathy) or macrovascular complications (diabetic cardiovascular diseases).

Diabetic retinopathy (DR) is diagnosed by the lesions in the retinal vascular system. The development of DR is associated with increased vascular permeability. This is caused by hyperglycemia via damage to the blood-retinal barrier, inducing edema and loss of pericytes [35, 36]. DR is the leading cause of vision loss worldwide among middle-aged and elderly people [37] and the vision loss is irreversible. The prevalence of diabetic retinopathy is estimated to reach 247.3 million by 2030 [38]. Almost all T1DM patients will develop retinopathy after twenty years of being diagnosed with diabetes [37]. More than 80% of insulin-treated and 50% of non-insulin-treated T2DM patients will develop some degree of retinopathy after having diabetes for two decades. Early detection and tight blood glucose control are beneficial to delay the progression of diabetic retinopathy [39].

Diabetic neuropathy is associated with progressive degeneration of the nerve fibers or whole nerve cells. Glucose toxicity is the primary cause of the nerve damage [40]. Patients with neuropathy have impaired sensation in the limbs, suffer extreme pain or may be asymptomatic [40]. The risk of peripheral neuropathy of diabetes increases along with the duration of diabetes and it is estimated that more than half of diabetic patients develop neuropathy to some extent [41]. For most cases, diabetic peripheral neuropathy is not reversible [40, 42].

Patients with nephropathy are characterized with morphological and ultrastructural changes in the kidney, such as the accumulation of extracellular matrix proteins, glomerular basement membrane thickening, podocyte injury, mesangial matrix expansion and tubulointerstitial damage [43-45]. Genetic predisposition and environmental factors, such as smoking and a sedentary lifestyle, contribute to the development of nephropathy [46, 47].

More than one-third of diabetic patients have hyperglycemia-induced renal changes [48]. Kidney transplant or dialysis may be necessary when nephropathy progresses to maintain the patient's life [43].

Cardiovascular disease (CVD) is a common macrovascular complication of diabetes mellitus, including accelerated atherosclerosis, cardiomyopathy, and stroke [49]. CVD is the leading cause of mortality in diabetes where 80% of deaths are caused by myocardial infarction and stroke [24, 50]. Diabetic patients have an estimated three to ten-fold higher risk of developing cardiovascular diseases compared to the non-diabetic population [51]. Even with adequate glycemic control, diabetic patients can still develop diabetic cardiovascular diseases [52]. This phenomenon indicates that hyperglycemia is not the only factor in the development of diabetic cardiovascular diseases. Other factors, such as excessive ROS, may be more relevant [49]. A healthy lifestyle, such as healthy eating habits, frequent exercise and smoking cessation, is a necessary component to delay the development and progression of diabetic cardiovascular diseases [53, 54].

1.1.4 Vicious Cycle between Type 2 Diabetes and Oxidative Stress

Elevated oxidative stress is common in T2DM. High serum glucose levels can enhance ROS/RNS production in the mitochondrial ETC [55]. High glucose level can partially block the electron transportation in complex III, which is a multisubunit transmembrane protein that plays a critical role in ETC, via increasing the hyperpolarization of the inner mitochondrial membrane potential. The inhibited electron transportation leads to excessive electrons which are accumulated to coenzyme Q and eventually result in the incomplete reduction of O₂ and

form superoxide [5]. Increased superoxide production contributes to glucose-induced damages to cells by inhibiting glyceraldehyde-3 phosphate dehydrogenase (GAPDH). Hyperglycemia activates the following pathways: the polyol pathway, the protein kinase C (PKC) pathway, the hexosamine pathway and the advanced glycation end product (AGE) pathway [56]. The activation of these pathways will contribute to oxidative stress. In the polyol pathway, an increased amount of glucose triggers aldose reductase to convert glucose to poly-alcohol. Nicotinamide adenine dinucleotide phosphate (NADPH) is consumed in this process. The decreased NADPH leads to a reduced regeneration of glutathione (GSH), which is a ROS scavenger, and eventually increases the ROS level [5]. Endothelial hyperglycemia activates the PKC pathway by increasing diacylglycerol synthesis. The activated PKC pathway results in the activation of NADPH oxidase and generates more ROS [5]. In the hexosamine pathway, fructose-6-phosphate is converted to uridine di-phosphate by glutamine fructose-6 phosphate amidotransferase (GFAT). Hyperglycemia can increase hexosamine pathway flux and lead to elevated GFAT activity. Increased GFAT activity causes more modification of proteins, including the modified transcription factor SP1, transforming growth factor β 1 and plasminogen activator inhibitor-1, which are harmful to blood vessels [5, 56]. AGEs are formed by non-enzymatic reactions between extracellular proteins and glucose. In a hyperglycemic condition, the inhibition of GAPDH leads to an increased production of the AGE precursor – methylglyoxal that is formed from glyceraldehyde-3 phosphate [56]. The increased AGE levels can bind their receptors and activate NADPH oxidase. The activation of NADPH oxidase favors the production of ROS [5]. In summary, hyperglycemia can contribute to oxidative stress in diabetic patients.

Oxidative stress can aggravate the dysfunction of β -cells and insulin resistance which can then accelerate the progression of type 2 diabetes.

1.1.5 Oxidative Stress and Diabetic Complications

1.1.5.1 Oxidative Stress and Diabetic Microvascular Complications

Under oxidative stress conditions, the excessive activation of PKC pathways can cause and accelerate diabetic complications by inducing blood-flow abnormalities & capillary occlusions and increasing vascular permeability [57]. In addition, oxidative stress can increase the production of AGE [58]. The intracellular accumulated AGE levels may alter cytoplasmic and nuclear factors, such as the alteration of gene transcription [58, 59]. In addition, the AGE can cross-link with structural proteins, such as collagen, and result in microvascular structural and functional changes. These changes include membrane thickening with reduced elasticity and sensitivity to protein clearance [36, 58]. Another mechanism behind increased AGE levels the diabetic microvascular complications is the interaction between AGE and their receptors which are located on the plasma membrane. This interaction can alter intracellular signaling, gene expression, release pro-inflammatory cytokines and free radicals, which can, in turn, increase the production of AGEs and result in a vicious cycle [58].

1.1.5.2 Oxidative Stress and Diabetic Macrovascular Complications

The development and progression of diabetic cardiovascular disease is associated with chronic inflammation which is linked to oxidative stress. Excessive free radicals in the system can increase the oxidation of low-density lipoprotein (LDL). The oxidized LDL will increase vascular inflammation via augmentation of the intimal macrophage infiltration and the

formation of foam-cells [60]. The reaction between AGE and AGE receptors also contributes to vascular inflammation by activating nuclear factor (NF)- κ B signaling [58]. Chronic vascular inflammation contributes to the atherogenesis and the formation of arterial thrombus [61]. In addition, ROS can damage contractile function[60] and induce cardiomyocyte apoptosis [62, 63]. Thus, the development and progression of diabetic macrovascular complications can be accelerated by low-grade inflammation and the action of ROS.

1.1.6 Dietary Strategies to Decrease Oxidative Stress

Nutrition plays an important role in the status of redox homeostasis. It is reported that diets containing high-sugar and high-fat content can cause oxidative stress [64]. Thus, these unhealthy diets can increase the risk of metabolic syndrome and chronic diseases. A decreased consumption of fat and sugar may reduce the production of ROS. The consumption of dietary antioxidants has been suggested as an effective strategy to decrease oxidative stress due to their capacity to eliminate ROS and/or boost the antioxidant defense system. Dietary antioxidants, include but are not limited to polyphenols, antioxidant vitamins (such as vitamin C and E), carotenoids and oil lecithin [65]. Some minerals, such as selenium and zinc, are also considered dietary antioxidants due to their essential roles as cofactors of endogenous antioxidant enzymes. The next section will focus on polyphenols and their impacts on T2DM.

1.2 Polyphenols

1.2.1 Introduction

Polyphenols are secondary, plant-based metabolites that are directly involved in the defense system of the plant to overcome abiotic stress [66, 67]. They are the largest group of phytochemicals [68]. Polyphenols are categorized by the number of aromatic rings and the basic structural elements that bind to the rings. Polyphenols are generally classified into two groups: non-flavonoids and flavonoids. Non-flavonoids are sub-classified as phenolic acids, lignans and stilbenes [68, 69]. Phenolic acids include hydroxyderivatives of benzoic acid and cinnamic acid and their esters. Stilbenes, such as resveratrol, have a double bond between the phenolic rings. Lignans are characterized by 2-phenylpropane units [70]. Flavonoids consist of more than 6000 phenolic compounds and account for 60% of total dietary polyphenols [70, 71]. Flavonoids are further sub-categorized as flavones, flavonols, isoflavones, anthocyanins, flavanols, tannins and flavanones [72]. The chemical structures of some polyphenols are shown in Figure 1.1.

Polyphenols are found in fruits, vegetables and cereal grains [72]. Berries, citrus fruits and broccoli are naturally rich in polyphenols. The amount of polyphenols in plants is affected by environmental factors, such as sun exposure and rainfall [68]. Polyphenol levels in plants may increase due to a response to stressful environmental conditions [66]. In addition, the degree of ripeness influences the amount of polyphenols in a fruit. During ripening, the phenolic acid content in the fruit decreases while the anthocyanin content usually increases [66]. The phenolic compound levels of fruits and vegetables may decrease during processing or storage [73]. The stability of individual phenolic compounds is related to structure. For instance,

catechin and ellagic acid are susceptible to heat due to its highly hydroxylated structure [73]. Cyanidins and delphinidins are less stable than pelargonidins during storage due to catechol groups [74].

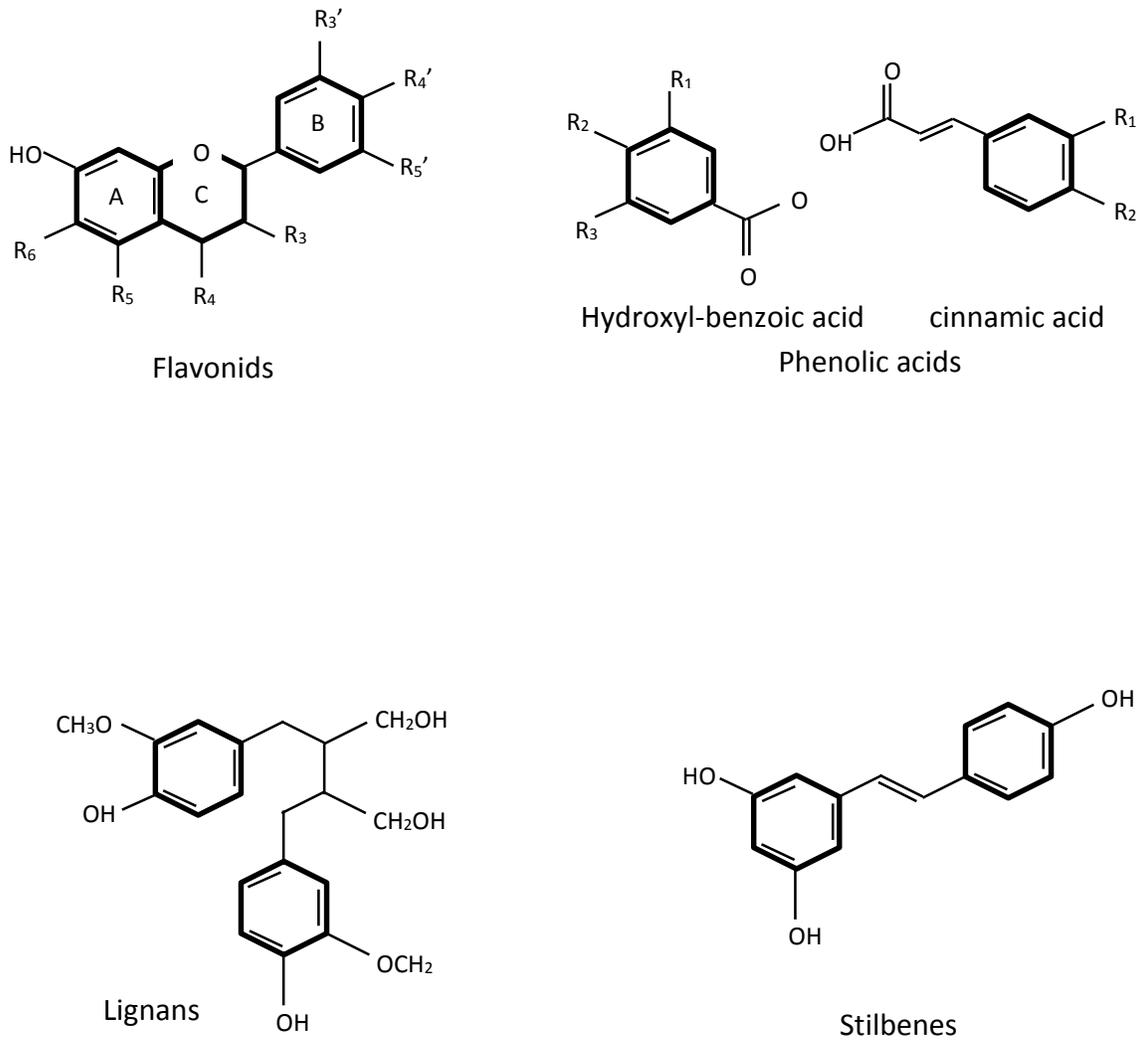


Figure 1.1 Structure of polyphenols

Polyphenols, especially flavanol polymers, are associated with bitter flavors and an astringent sensation [75, 76]. Astringency is defined as a puckering, rough and/or dry-mouth feel [75]. Bitter and astringency are generally considered as unappealing sensory properties to consumers. The plant-based foods that are rich in polyphenols, especially those that are rich in flavanols, may lack palatability due to astringency [76]. Sweeteners, like sucrose, are used to minimize bitterness and astringency in polyphenol-rich beverages, but beverages with added sugar may be less attractive to health-conscious consumers [77]. In order to better capitalize on the potential health benefits offered by polyphenols, effective strategies are needed to mask the less pleasant flavor and sensation.

1.2.1.1 Anthocyanins

Anthocyanins are a subclass of flavonoids that belong to the polyphenol group. They act as pollinator attractants and phytoprotective agents. Anthocyanins occur in all parts of plant: the leaves, stems, roots, flowers and fruits [78]. Anthocyanins are odorless and almost flavorless, but as other phenolic compounds, they contribute to the astringent. Structurally, anthocyanins have two benzene rings (A and B rings) and a heterocyclic ring (C ring). Most anthocyanins are derived from six common aglycones (anthocyanidins): malvidin, petunidin, delphinidin, peonidin, pelargonidin and cyanidin [79]. The structure of these aglycones is shown in Figure 1.2. In plants, anthocyanins are commonly found in the form of glycosides. A sugar moiety normally bonds to form the aglycone at position 3 of the C ring [79]. Sugar moieties that are frequently linked to anthocyanidins are glucose, galactose, arabinose and rutinose [79]. Anthocyanins are strong antioxidants. The antioxidant capacity of anthocyanins depends on their structure [80]. The ability of anthocyanins to neutralize free radicals depends on the

delocalization of the π -electron system of aromatic rings. The free radical scavenging capacity of anthocyanins is also related to the position of the hydroxyl groups [70].

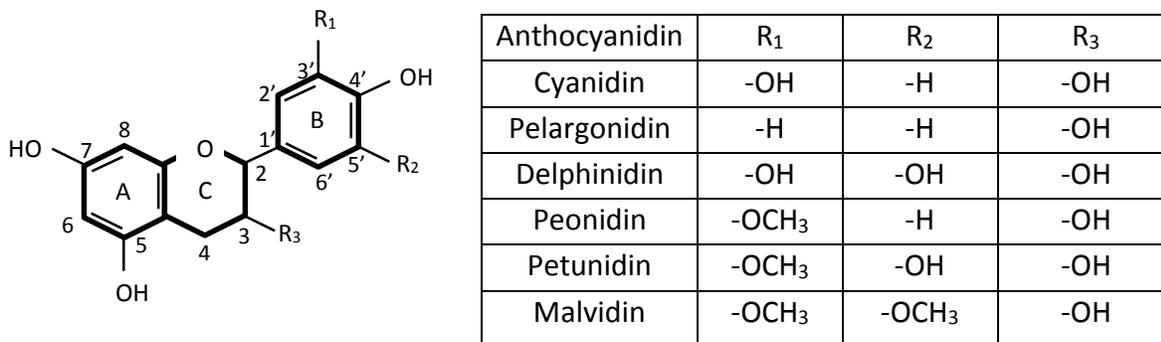


Figure 1.2 Structures of most common anthocyanidins [79]

Anthocyanins are also natural pigments that are responsible for the blue, red and purple color in plants [78]. In the food industry, they are used as natural dyes [78]. The color of anthocyanins is depended on the pH of the environment. When the environmental pH level shifts from a strong acid to a mild alkaline, the structure of anthocyanins shifts from a flavylum cation to a quinonoid (Figure 1.3) and the color changes from red to blue [81]. Anthocyanins are colorless at a pH level of 4.5 [82]. Anthocyanins are labile to pH of the environment and an acidic environment (pH 1-4) is beneficial to their stability, as they can easily degrade in alkaline environments [83]. Many factors can accelerate the degradation of anthocyanins, such as strong light and heat exposure [81]. The instability of anthocyanins restricts their utilization in the food industry and negatively affects their bioavailability.

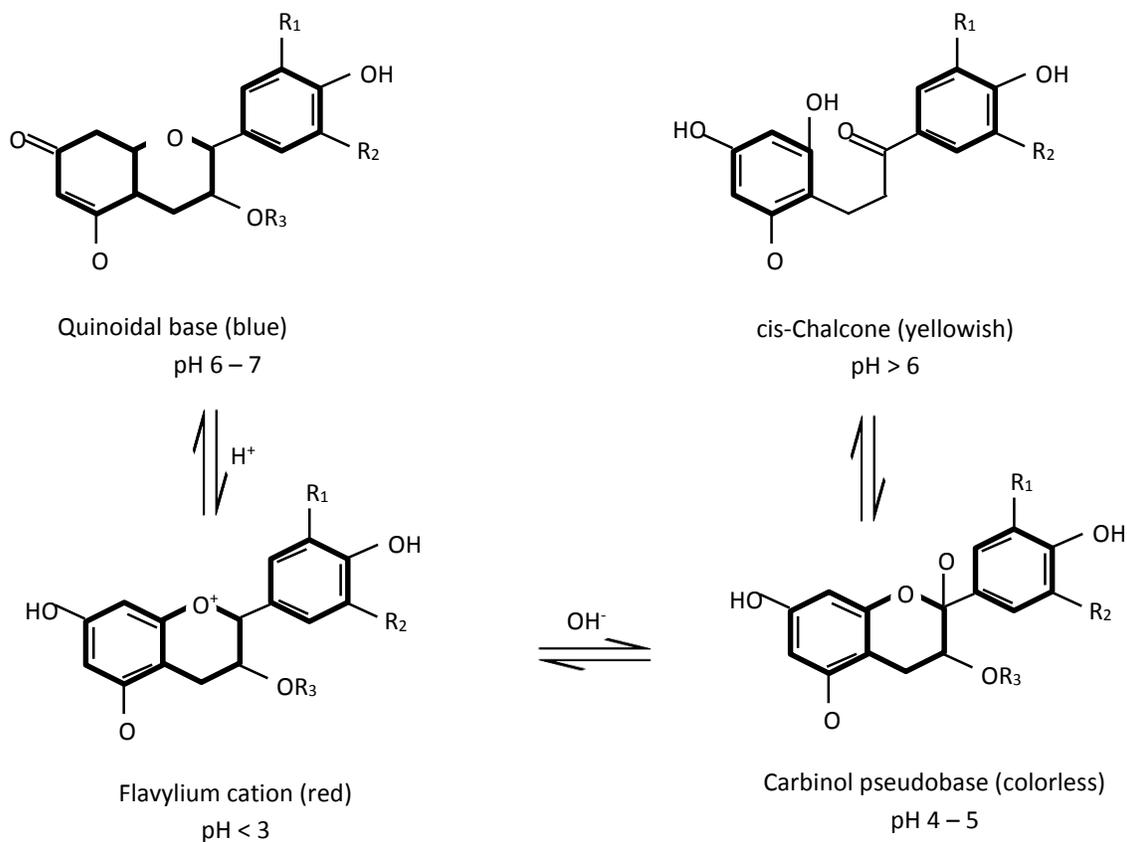


Figure 1.3 Molecular structure of anthocyanins in different environmental pH levels [84]

1.2.1.2 Proanthocyanins

Proanthocyanins are oligomers or polymers of polyhydroxyflavans linked by the C-C bonds, the polymerization of proanthocyanins can be up to 200 monomeric flavanol units [85]. Proanthocyanins are a subcategory of tannins which are also referred to as condensed tannins. Proanthocyanins can break down to anthocyanidins when they are heated under acidic conditions [86]. Proanthocyanins are widely present in plant-based food, such as tea, wine and cereals [87]. They are strong antioxidants but relatively labile to thermal treatment and UV exposure. Proanthocyanins are soluble in water, short-chain alcohols and acetone. At high concentrations (40-45%), proanthocyanins may yield to viscous colloids in water at room

temperature [88]. Proanthocyanins have a strong ability to bind to proteins and metal ions due to their multiple phenolic hydroxyl groups [89]. The interaction between proanthocyanins and salivary proteins causes an astringent sensation in the mouth. The intensity of this astringency/bitterness is associated with the structure of proanthocyanins, especially the degree of polymerization [90]. The levels of proanthocyanins decrease during the ripening process of berries due to oxidation/degradation [90]. Food products using berries at their full ripeness can help to reduce the unpalatable astringent sensation to some extent.

1.2.1.3 Phenolic Acids

Phenolic acids exist naturally in plants and are subcategorized as benzoic and cinnamic acids depending on the number of carbon atoms (Figure 1.1). Benzoic acids contain seven carbon atoms, such as gallic acid, salicylic acid, protocatechuic acid and ellagic acid. Cinnamic acids contain nine carbon atoms, which include caffeic acid, ferulic acid, chlorogenic acid, p-coumaric acid, etc. [91]. Compared to hydroxybenzoic acids, hydroxycinnamic acids are more commonly found in plant-based foods [92]. Phenolic acids are antioxidants and their antioxidant capacity is related to its structure. Based on their antioxidant activity, phenolic acids are utilized in the food industry as food preservatives and/or are used to prevent enzymatic browning [93].

1.2.2 Potential Health-Promoting Characteristics of Polyphenols

1.2.2.1 Polyphenols and Oxidative Stress

The consumption of polyphenol-rich foods can increase the antioxidant capacity of the human serum. Plaza *et al.* observed an enhanced serum antioxidant capacity in healthy volunteers after consumption of jaboticaba peel (containing 1.25 g total phenolics with

anthocyanins as the predominant phenolic compound) [94]. The ability of polyphenols to increase serum antioxidant capacity may be achieved by improving the activity of endogenous antioxidant enzymes, such as GPx, SOD and CAT. Noratto *et al.* demonstrated that the GPx activity in obese diabetic mice fed with raspberry powder (containing 963 mg of extracted gallic acid equivalent phenolics) was higher than the control group (no consumption of raspberry) [95]. Wu *et al.* reported that the consumption of anthocyanin-rich mulberry extract and cherry extract (200 mg/kg body weight) increased the activity of SOD and GPx in obese mice [96]. Shen *et al.* conducted an experiment using mice fed with a high-fat diet and demonstrated that supplementation with a polyphenol extract (containing flavonols and phenolic acids) from black highland barley (600 mg/kg body weight) increased the levels of CAT, SOD and GPx in the liver [97]. Increases in the gene expression of these antioxidant enzymes were also observed [97]. The improvement in the activity of antioxidant enzymes may enhance the antioxidant defense system and be beneficial to reduce oxidative stress. In summary, polyphenol intake shows promise to enhance the antioxidant capacity of the blood serum and decrease oxidative stress by improving the activity and gene expression of endogenous antioxidant enzymes.

1.2.2.2 Polyphenols and Chronic Inflammation

Polyphenols can attenuate chronic inflammation by down-regulating the production of pro-inflammatory cytokines and/or up-regulating the generation of anti-inflammatory molecules. Lee *et al.* reported that after an eight-week supplementation with blueberries (10g freeze-dried blueberry powder/100g diet), levels of pro-inflammatory cytokines (TNF- α and IL-1 β) in rats were lower compared to rats without blueberry supplementation [98]. Roth *et al.* demonstrated that treatment of ingesting anthocyanin-rich bilberry extract reduced

inflammation in patients with ulcerative colitis [99]. Compared to baseline levels (before treatment), the levels of pro-inflammatory cytokines (TNF- α and interferon- γ) were decreased and the levels of anti-inflammatory cytokines (IL-10) were increased [99]. The cytokine regulatory activity of polyphenols may be related to their interaction with cellular proteins, such as signaling molecules, receptors and transcription factors, and may influence the signaling pathways [100, 101]. In addition, polyphenols may interact with nucleic acids and alter the protein expression of inflammation-related molecules [100, 102].

1.2.2.3 Polyphenols and Type 2 Diabetes

Epidemiological studies reveal that higher consumption of polyphenols (mostly flavonoids) is correlated with a lower risk of T2DM [2]. Wedick *et al.* investigated the consumption of flavonoids and the risk of T2DM, the results showed that a higher intake of anthocyanins was related to a lower risk of developing the disease. No relationship was found between the consumption of other flavonoids and T2DM [3]. In addition to epidemiologic studies, the beneficial properties of polyphenols are well documented in experimental studies. Polyphenol intake can decrease cellular oxidative stress generated by metabolism and potentially reduce the risk for the initiation and/or delay the progression of T2DM. In addition, the consumption of polyphenols can increase insulin sensitivity and/or secretion, inhibit the production of AGE and improve glucose homeostasis by decreasing glucose absorption and/or increasing the cellular glucose uptake [103-106].

1.2.2.3.1 Polyphenols and insulin sensitivity. Insulin is the key hormone for maintaining glucose homeostasis. Insulin resistance commonly occurs in T2DM patients where the insulin signaling and/or action on the target tissues is impaired [107]. Insulin resistance leads to a lowered glucose uptake in non-hepatic peripheral tissues, a decreased suppression of hepatic glucose production and an elevated level of plasma glucose. Polyphenol consumption can attenuate insulin resistance. Oral administration of apple procyanidins ameliorated the hepatic insulin resistance in diabetic mice by suppressing hepatic inflammation [108]. The administration of anthocyanin-rich mulberry extracts improved insulin sensitivity in diabetic mice via activation of AMP-activated protein kinase [109]. Insulin sensitivity was increased in insulin-resistant-non-diabetic human subjects by consuming 333 mg/day of strawberry and cranberry polyphenol mixtures for six weeks [110]. Increased insulin sensitivity may help to decrease the hyperproduction of insulin and then restrain the β -cell failure [107, 111]. In summary, polyphenol consumption may help to enhance insulin sensitivity and delay the progression of T2DM.

1.2.2.3.2 Polyphenols and insulin-independent blood glucose control. Polyphenol intake can aid in blood glucose control by delaying carbohydrate absorption. The inhibitory activity of phenolic compounds on carbohydrate-hydrolyzing enzymes, such as pancreatic α -amylase and α -glucosidase, has been well documented [112-114]. Pancreatic α -amylase is responsible for hydrolyzing the α -bonds of large polysaccharides, such as starch and glycogen. Alpha-glucosidase can catalyze the breaking down of 1, 4 - α bonds in oligosaccharides and disaccharides. The inhibition of these digestive enzymes delays the digestion of complex carbohydrates and reduces the proportion of absorbed glucose [103]. In addition, the

consumption of polyphenols can increase the glucose uptake in skeletal muscle cells and help to maintain blood glucose homeostasis. This effect may be related to the altered expression and activity of GLUT4 through the activation of AMPK and/or PPAR gamma pathways [71, 115-117].

1.2.2.1.3 Polyphenols and AGE production. The consumption of dietary polyphenols can help to suppress the formation of AGEs under hyperglycemic conditions and delay the progression of diabetic complications [118]. The anti-glycation activity has been investigated by several *in-vivo* studies. Umadevi *et al.* stated that gallic acid had a protective effect on AGE-induced cardiac fibrosis in rats [119]. Chao *et al.* observed that the consumption of caffeic acid and ellagic acid decreased the formation of glycation products in the kidney of diabetic mice [120]. The anti-glycation property of polyphenols is related to their free radical scavenging capacity, metal chelating activity and their capacity to trap reactive carbonyl species, such as methylglyoxal [121].

In addition to the beneficial effects of phenolic compounds on diabetes, polyphenols are reported to have beneficial activity toward other health-related problems, such as cardiovascular disease [2], cancer [122], obesity [123], osteoporosis [124] and Alzheimer's disease [125, 126]. In order to better utilize the health-promoting potential of dietary polyphenols, the bioavailability of dietary polyphenols from various sources needs to be investigated.

1.3 Bioavailability of Phenolic Compounds

Bioavailability is defined as the proportion of ingested food compounds that enter metabolic circulation and reach specific sites in the body to exhibit biological function [127]. The bioavailability of different phenolic compounds varies, not only due to structural variations in polyphenols, but also because of individual physiological and biochemical differences of consumers. Generally, the bioavailability of phenolic compounds is relatively low due to low absorption rates, instability of these compounds in the gastrointestinal tract (GIT) and quick elimination [128]. Several steps are crucial for food constituents to be bioavailable: liberation, absorption, distribution, metabolism and elimination [129]. Bioaccessibility is an important factor to evaluate the potential of food compounds to be bioavailable and it is related to the liberation process. Bioaccessibility is defined as the amount of food compounds that are released in the GIT and have the potential to pass the intestinal barrier to be absorbed [127]. *In-vitro* digestion models are commonly used to evaluate the bioaccessibility of food components. The metabolism and bioavailability of proanthocyanins, anthocyanins and phenolic acid will be discussed in further detail in this section.

1.3.1 Bioavailability of Proanthocyanins

The bioavailability of proanthocyanins (condensed tannins) is generally low due to the large molecular size of these compounds. Proanthocyanins are polymerized flavan-3-ols and/or flavan-3,4-diols [85, 130]. Due to the large molecular size of proanthocyanins, it is unlikely for them to be absorbed intact. In the stomach, *in-vitro* studies showed that some proanthocyanins could be hydrolyzed into monomers due to the acidic environment [85], and then monomers

can be absorbed in the small intestine. However, *in-vivo* studies suggested that the depolymerization of proanthocyanins in the upper GIT (stomach and small intestine) was minimal [85, 131, 132]. The absorption characteristics of proanthocyanins in the small intestine are largely affected by the degree of polymerization. Dimeric and trimeric proanthocyanins are relatively easily absorbed because the permeability coefficients of proanthocyanins are similar to a paracellular transport marker – mannitol [133]. The absorbed dimeric and trimeric proanthocyanins reach the liver via the portal vein. The absorbed dimers/trimers conjugate in the liver. The conjugated derivatives may be exported into the bile and go back to the intestinal lumen or be released into the systemic circulation and then distributed to the organs. The metabolites are excreted in the urine. Most large proanthocyanins pass the upper GIT without alteration and reach the colon in their original form or as complexes with macronutrients. Proanthocyanins that are not absorbed may be metabolized by the colonic microflora in the GIT and excreted in the feces [134].

It has been suggested that proanthocyanins can exhibit bioactivity without being absorbed by altering the gut microflora profile [135]. A study conducted with rats showed that consuming proanthocyanins for three weeks caused a shift towards increasing gram-negative bacteria in the GIT [136]. In addition, proanthocyanin-rich grape seed extract (38.5%, w/w) increased the population of probiotics (*Bifidobacterium*) in healthy adults [137]. Gut microbiota plays a vital role in human health, such as synthesizing vitamins and protecting the host from potentially harmful invasive bacteria [128]. Thus, ingested proanthocyanins may affect human health by modulating the composition and catabolic activity of the gut microbiota [138, 139].

More studies are needed to better understand the impacts of proanthocyanins on gut microflora and to better utilize the potential benefits of proanthocyanins.

1.3.2 Bioavailability of Anthocyanins

Anthocyanins are relatively easily absorbed compared to proanthocyanins. The stomach is a major site for the liberation of anthocyanins from food matrices. The stomach pH and body temperature helps to hydrolyze and/or release anthocyanins. The major absorption site of anthocyanins is the small intestine [140]. Anthocyanin aglycones can pass the cellular membrane of enterocytes via passive diffusion. The absorption of anthocyanin glycosides involves enzymes or transporters [128, 141], such as lactate phlorizin hydrolase (LPH). LPH is located in the enterocyte brush border and LPH can cleave the glycosidic moieties of anthocyanin glycosides in the small intestinal lumen. The released aglycones can then enter the enterocytes via passive diffusion [128]. In addition, anthocyanin glycosides can be transported into the enterocytes in their glycoside form by using sodium-dependent glucose transporters (SGLT) [141]. The anthocyanin glycosides are hydrolyzed within the epithelial cells by a cytosolic β -glucosidase (CBG) and release the aglycones [140]. The absorption efficiency of anthocyanins varies depending on their structure. Yi *et al.* demonstrated that the transport efficiency of cyanidin glucoside was higher than cyanidin galactosides [142]. The authors also concluded that fewer free hydroxy groups and more methoxy groups contributed to a higher absorption efficiency [142]. The absorbed anthocyanin aglycones are transported via the portal vein to the liver, where anthocyanins undergo phase II transformations including conjugations to methyl, glucuronic acid and/or sulfate groups [141, 143]. After being metabolized in the liver, anthocyanins can either re-enter the enteric system through bile or can be distributed to

organs, such as the eyes, bladder, kidney, and brain [144-149] and then be excreted in urine [141].

One restricting factor for the bioavailability of anthocyanins is their instability in an alkaline environment. The small intestine is a mild alkaline environment and anthocyanins may degrade in the small intestine before being absorbed [143]. The instability of anthocyanins in the small intestine was observed by many studies using *in-vitro* models. Liu *et al.* reported that 42% of total anthocyanins from blueberries degraded during intestinal digestion [150]. Strategies that can increase the stability of anthocyanins in the small intestine may help to enhance their bioavailability.

1.3.3 Bioavailability of Phenolic Acids

The absorption of phenolic acids can occur in the stomach, small intestine and colon [151]. Absorption in the stomach provides the lowest phenolic absorption levels and may be achieved by passive diffusion [152]. The small intestine, specifically the jejunum is the major absorption site for phenolic acids [153]. The intestinal absorption of phenolic acids can occur via monocarboxylic acids transporters (MCT) and the absorption rate is affected by the affinity between phenolic acids and MCT [154]. Free phenolic acids are absorbed rapidly in the stomach and in the small intestine [155, 156], but the majority of phenolic acids (approximately 80%) in plant-based foods are bound to cell wall polymers by covalent cross-linkages [157] and are inaccessible for absorption. Bound phenolic acids are insoluble and the human endogenous enzymes cannot release them [155]. The microflora in the colon may produce enzymes that are able to liberate the bound phenolic acids, such as xylanases and esterases [158]. The absorption

of the released phenolic acids in the colon is low due to: 1) poor absorption capacity of the colon and 2) break down of the aglycones by colon microflora [155]. Thus, the bound phenolic acids can be released and absorbed in the colon to some extent. However, the bioavailability of bound phenolic acids is lower than free phenolic acids.

After absorption, phenolic acids are distributed to organs and undergo several biotransformations in the intestine, liver and kidney where the liver is the major site [151]. The biotransformation of phenolic acids includes dehydroxylation, demethylation, dehydrogenation, hydrogenation, O-methylation, sulphation, glucuronization, glycation and/or GSH conjugation [151]. Phenolic acids and the conjugates may re-enter the intestinal lumen via bile when the circulating levels are high. Phenolic acids are mainly excreted in the urine in both the intact and biotransformed form [159].

1.3.4 Factors Affecting the Bioavailability of Phenolic Compounds

Many factors affect the bioaccessibility and bioavailability of phenolic compounds. First, processing methods such as thermal treatments and storage may either increase the bioavailability by enhancing the release of polyphenols from the food matrices [160] or decrease the bioavailability by accelerating degradation [161]. Second, the interaction with other ingested dietary ingredients such as proteins may alter the liberation process of phenolic compounds and in most cases negatively affect the bioaccessibility of phenolic compounds [162]. Third, the molecular structures of polyphenols have a vital impact on their bioavailability. Larger phenolic compounds like proanthocyanins are difficult to absorb while the absorption rates of smaller phenolics like phenolic acids are much higher. Fourth, digestion related factors, such as the intestinal transit time of polyphenols and enzyme activity, may positively or

negatively affect the release of phenolic compounds. Longer retention times in the small intestine leads to more degradation of anthocyanins but longer retention times in the stomach may cause more liberation [161]. Additionally, the microbiota in the lower GIT may break down some larger phenolic compounds and increase their bioavailability [163, 164]. Fifth, the frequency of polyphenol consumption may alter the expression of transport proteins and/or metabolizing enzymes that associated with polyphenol metabolism in human [166, 167]. Thus, consuming polyphenol-rich foods may affect their bioavailability by altering the individual's ability to absorb and metabolize phenolic compounds [165].

1.3.5 Potential Methods to Improve Bioavailability of Phenolic Compounds

1.3.5.1 Thermal Processing

Thermal processing methods are frequently used to heat treat food ingredients. Thermal treatments can decrease the bioavailability of polyphenols in food by accelerating the degradation process. Some phenolic compounds, such as anthocyanins, are labile to heat [168]. However, some thermal processing methods are found to be beneficial to enhance the bioavailability of phenolic compounds in certain foods. For example, roasting nuts can increase the amount of free phenolic acids which are more bioavailable compared to bound phenolic acids [73, 169]. Rossi *et al.* reported that steam blanching blueberries inactivated the polyphenol oxidase and resulted in an increase in extractable polyphenols [170]. Arkoub-Djermoune *et al.* demonstrated that some thermal processing methods, such as grilling and baking, enhanced the total phenolic compounds content and flavonol content in eggplant but decreased the flavonoid and anthocyanin levels [171]. In summary, thermal processing may

increase the bioavailability of some phenolic compounds in certain food matrices, but the effects vary and need to be investigated on a case by case basis.

1.3.5.2 Fermentation

Fermentation has been widely utilized to process polyphenol-rich foods for centuries, as in the making of wine. Fermentation not only adds flavor to the food but also modifies the food components including phenolic compounds. Lactic acid bacteria are commonly utilized in fermentation [172]. Some lactic acid bacteria, such as *Lactobacillus plantarum*, can metabolize phenolic compounds and the metabolites may be more bioavailable [173]. Frediansyah *et al.* demonstrated that black grape juice fermented with *Lactobacillus plantarum* exhibited higher α -glucosidase inhibitory activity than non-fermented black grape juice [174]. Hole *et al.* observed that fermentation with lactic acid bacteria increased the level of free phenolic acids in whole barley grains and improved their bioavailability [175]. These increases may be caused by the liberation of bound phenolic acids due to the esterase activity of lactic acid bacteria [175]. Similarly, Curiel *et al.* reported that due to the esterase activity, *L. plantarum* fermentation increased the levels of gallic acid, ellagic acid, myricetin and quercetin in *Myrtus communis* berries and increased radical scavenging capacity [176]. In addition to lactic acid bacteria, other fermentative microorganisms may have the capacity to alter the bioavailability of phenolic compounds. Wang *et al.* demonstrated that fermentation with yeast (*Saccharomyces cerevisiae bayanus* EC 118) enhanced the anti-inflammatory activity of maqui berry juice by improving the capacity to inhibit the expression of iNOS and COX-2 in a macrophage cell line (RAW264.7) [177]. The authors concluded that this enhancement might be related to the increase of gallic acid due to yeast fermentation [177]. In summary, fermentation may increase the

bioavailability of dietary polyphenols by altering the structure, generating more bioavailable metabolites and/or releasing the bound phenolic compounds. Studies in regard to the impacts of fermentation on the bioavailability of phenolic compounds are limited. More research involving various microorganisms and different food matrices is needed.

In addition to the above-listed processing methods (thermal processing and fermentation), other methods, such as milling, high-pressure processing and encapsulation may have positive impacts on the bioavailability of phenolic compounds [160, 178]. The effects of processing on the bioavailability of phenolics depend on not only the processing methods, but also the phenolic compounds structure and the food matrix. Currently, research studies are investigating impacts of a variety of processing methods on the bioavailability of phenolic compounds from different food matrices, but more processing studies are needed to efficiently utilize the potential health benefits of certain phenolic compounds.

1.4 Aronia Berries

1.4.1 Botany and Current Commercial Utilization of Aronia Berries

Aronia shrubs are native to North America [179]. The genus *Aronia* (belongs to the *Rosaceae* family and *Maloideae* subfamily) includes two species: *Aronia melanocarpa* and *Aronia arbutifolia*. *A. melanocarpa* fruits are black, also known as the “black chokeberry”, “wild gooseberry”, “chokepear” or “dogberry”. *A. arbutifolia* fruits are red and are known as the “red chokeberry”. A third controversial species is called *A. prunifolia* (purple chokeberry), which is considered a hybrid of *A. melanocarpa* and *A. arbutifolia* [180]. Most research has been conducted on the black aronia – *A. melanocarpa*. In this thesis, the word “aronia” is used to indicate the black aronia, specifically. Aronia are relatively easy to grow and they are not

susceptible to severe diseases and pests [179]. Birds do not consume the fruit which may be due to its astringent taste [179, 181, 182]. Mature aronia shrubs can be 2-3 meters in height and yield up to 1.2 kg fruit/m² in five years [183]. Aronia has been traditionally used as an alternative medicine to treat colds among Native Americans [179]. Aronia gained popularity in the Soviet Union and Eastern Europe in the 20th century, and it was used as a treatment for hypertension, atherosclerosis, hemorrhoids and achlorhydria [179]. Currently, aronia extracts are used as natural food colorants to provide dark purple or blue color [184]. Some food products made with aronia, such as juice, jam, jelly and tea are commercially available in the United States, but these products are limited. More products need to be developed with aronia berries to increase their consumption [185].

1.4.2 Nutritional Value and Bioactive Compounds of Aronia Berries

The content of nutrients and bioactive compounds in aronia berries are affected by several factors, such as the shrub cultivar, the maturation of the berries, the variety and frequency of fertilizers used and the growth location/climate [186, 187]. Fresh aronia berries contain 5.6% dietary fiber which includes pectin [188, 189]. The seedless fraction of aronia pomace contains 70% dietary fiber on a dry weight basis [190]. Total sugar content in fresh aronia is approximately 620 mmol/kg including 3.5% glucose, 2.8% fructose and 0.41% sucrose [191]. Though the reported individual sugar content varies, glucose and fructose are the dominant sugars in aronia berries [192, 193]. Aronia berries contain a relatively high amount of sorbitol (46.2 g/kg), which is a sugar alcohol [192]. Malic acid is the predominant organic acid in aronia berries, which is approximately 1.22 g/100 g in fresh berries, followed by citric acid (0.13 g/100 g). Tartaric acid, fumaric acid and shikimic acid are detected in aronia berries but these

amounts are low [191]. Fresh aronia berries contain small amounts of fat (0.14 g/100g) and up to 0.7 g/100g protein [183]. The predominant fatty acid is linoleic acid which is contained in its seeds [194]. Aronia berries have a relatively high concentration of potassium (0.22 g/100g fresh weight). Vitamins B₁ (17-19 µg/100g), B₂ (17-27 µg/100g), B₆ (24-29 µg/100g), B₉ (2-4 µg/100g), C (4.0-19.3 mg/100g), niacin (27-34 µg/100g) and pantothenic acid (225-382 µg/100g) have also been identified in aronia berries [183, 195].

Phenolic compounds are the most important bioactive compounds in aronia berries. They are potentially responsible for the pharmaceutical effects of aronia, such as anti-influenza activity [183]. Aronia berries are rich in phenolic compounds ranging from 1013 to 2010 mg gallic acid equivalents/100 g fresh berries, which is higher than that in blueberries, bilberries, blackberries, strawberries, blackcurrants and cranberries [191, 196 - 198]. The major type of phenolic compounds in aronia are proanthocyanidins [199]. Procyanidins are the only form of proanthocyanidins found in aronia berries. Procyanidins are a subcategory of proanthocyanidins that consist of (epi)catechin subunits and aronia berries have a high concentration of procyanidins [198]. The degree of polymerization of aronia procyanidins ranges from 2 to over 30 where large polymers are dominant. The major linkages between the subunits of aronia procyanidins are C4-C6 and C4-C8 bonds [179]. The content of procyanidins in dried aronia berries is above 5 g/100 g which is higher than the proanthocyanidins in other berries, such as blueberries [200]. Anthocyanins are the second most dominant phenolic group in aronia berries [179]. Aronia is one of the berries that contains the largest concentration of anthocyanins [79]. Wu *et al.* reported that the total anthocyanin content in fresh aronia berries was 1480 mg/100 g, which is higher than that in black raspberry (687 mg/100 g), wild blueberry

(486.5 mg/100 g), blackcurrant (476 mg/100 g), blackberry (245 mg/100 g), cranberry (140 mg/100g) , and strawberry (21.2 mg/100 g) [201]. There are four major anthocyanins in aronia berries: cyanidin-3-galactoside, cyanidin-3-glucoside, cyanidin-3-xyloside and cyanidin-3-arabinoside where cyanidin-3-galactoside is the dominant anthocyanin followed by cyanidin-3-arabinoside [199, 202]. Other anthocyanins were occasionally detected at low concentrations, such as pelargonidin-3-arabinoside [198]. The major phenolic acids in aronia berries are chlorogenic acid and neo-chlorogenic acid, the contents of which range from 16.3 to 301.8 and from 92.3 to 291 mg/100 g in dried aronia berries, respectively [199, 203]. Other phenolic acids, such as caffeic and ferulic acids were only occasionally identified in aronia berries [204].

1.4.3 Health-promoting Characteristics of Aronia Berries

1.4.3.1 Antioxidant Capacity of Aronia Berries

Antioxidant capacity is an important parameter to evaluate the potential health-beneficial properties of a food ingredient. Aronia berries exhibit strong antioxidant capacity due to the large amounts of phenolic compounds [196]. In addition, other compounds, such as vitamin C and β -carotene, contribute to its antioxidant activity [205]. The consumption of aronia berries can help to reduce oxidative stress and decrease cellular oxidative damage. Aronia intake can increase the antioxidant capacity of the plasma and scavenge the excessive free radicals in the human body. Nowak *et al.* observed an increase in plasma antioxidant capacity in healthy people after one-week consumption of aronia juice [206]. In a double-blind study with rowing athletes, Skarpanska-Stejnborn *et al.* stated that the antioxidant capacity of plasma in athletes supplemented with aronia juice consumption (150 mL/day) for eight weeks was higher than the placebo group [207]. Pilaczynska-Szczesniak *et al.* observed that daily

intake of 150 mL aronia juice (containing 34.5 mg anthocyanins) reduced the levels of thiobarbituric acid-reactive substances (TBARS) in the blood and concluded that an increase in anthocyanin consumption could decrease exercise-induced oxidative damage to red blood cells in athletes [208]. Lee *et al.* reported that aronia extracts reduced ROS production in HT22 mouse hippocampal cells and protected the cells against the glutamate-induced oxidative stress [209]. The consumption of aronia berries can also improve the antioxidant defense system by boosting the activity of endogenous antioxidative enzymes. Kardum *et al.* observed that after consuming aronia juice for three months, the SOD and GPx activity in healthy women was increased significantly [210]. Faff *et al.* observed that the reduction of GSH caused by exercise was alleviated in aronia-fed rats [211]. A study conducted by Francik *et al.* showed that aronia juice administration increased the activity of plasma CAT in rats fed a high-fat diet [212]. Kondeva-Burdina *et al.* stated that aronia juice prevented GSH depletion caused by tert-butyl hydroperoxide-induced oxidative stress in rat hepatocytes and increased cell viability [213]. The positive impacts of aronia on oxidative stress are potentially beneficial to prevent chronic diseases and slow their progression.

1.4.3.2 Anti-inflammatory Activity of Aronia Berries

Chronic low-grade inflammation is one of the underlying factors of chronic diseases. A reduction in low-grade inflammation may benefit the diabetic individuals by dealying the progression of diabetic complications. Additionally, decreased low-grade inflammation may reduce the risk of T2DM in individuals with metabolic syndroms. The anti-inflammatory effects of aronia berries have been documented in *in-vitro* studies using cell models for understanding the mechanisms. Appeal *et al.* stated that aronia juice concentrate activated the NF-kB pathway

in RAW264.7 macrophages and inhibited the production of pro-inflammatory cytokines (TNF- α , IL-6 and IL-8) in human peripheral monocytes which were isolated from a healthy human volunteer [214]. Martin *et al.* reported that aronia berries inhibited IL-6 production in lipopolysaccharide (LPS)-simulated mice splenocytes and that cyanidin-3-arabinoside might be the major effective anthocyanin [215]. These studies demonstrate that aronia berries exhibit anti-inflammatory activity by suppressing the production of pro-inflammatory cytokines.

In addition to *in-vitro* studies, the anti-inflammatory activity of aronia berries has been tested in animal models. Ohgami *et al.* reported that aronia extract decreased the levels of NO, TNF- α and prostaglandin (PG)-E2 in rats with endotoxin-induced uveitis [216]. Goh *et al.* observed that aronia concentrate suppressed the formation of 12-o-tetradecanoylphorbol-13-acetate (TPA)-induced ear edema in mice and decreased the production of pro-inflammatory cytokines (TNF- α , IL-1 β and IL-6) [217]. Kang *et al.* concluded that the consumption of aronia berries ameliorated the symptoms of dextran sulfate sodium-induced ulcerative colitis (a type of inflammatory bowel disease) in mice, and decreased the production of NO, IL-6 and TNF- α [218]. Loo *et al.* carried out an intervention with individuals with slightly high blood pressure levels and demonstrated that the consumption of dried aronia powder or aronia juice (equivalent to about 336 g fresh berries per day) decreased low-grade inflammation, while reductions in TNF- α were observed [219]. Low-grade chronic inflammation increases the risk of T2DM and CVD. The anti-inflammatory activity of aronia berries can aid in the prevention and treatment of chronic diseases. More *in-vivo*, especially clinical research studies, are needed to establish recommended dosages.

1.4.3.3 Aronia and Diabetes

The potential benefits of aronia berries for diabetes were suggested by several *in-vitro* studies. Rugina *et al.* demonstrated that aronia extract helped to protect mouse pancreatic β -cells (β TC3 cell line) from high glucose-induced cytotoxicity. The authors also observed that the insulin secretion increased in the aronia extract-treated β TC3 cells [220]. Aronia extracts may benefit insulin secretion by inhibiting the activity of dipeptidyl peptidase IV (DPP IV). DPP IV is a serine peptidase which can reduce insulin secretion by inactivating incretin. The inhibition of DPP IV can increase the secretion of insulin and be beneficial to diabetic individuals. Kozuka *et al.* investigated the DPP IV inhibitory activity of aronia juice *in-vitro* and concluded that the cyanidin 3,5-diglucoside was more efficient than cyanidin or cyanidin-3-glucoside to inhibit DPP IV [221]. Aronia consumption may aid in modulating blood glucose levels by inhibiting enzymes involved in carbohydrate absorption, such as α -glucosidase [222]. Wangenstein *et al.* investigated the inhibitory activity of aronia extracts against α -glucosidase and demonstrated that the inhibitory activity of aronia extracts was stronger than acarbose, which is a therapeutic agent for diabetes [200]. *In-vitro* studies suggest that aronia may benefit diabetic individuals by increasing the secretion of insulin and inhibiting carbohydrate-hydrolyzing enzymes.

The hypoglycemic effects of aronia have been documented by *in-vivo* studies using animal models. Valcheva-Kuzmanova *et al.* reported that the levels of plasma glucose levels in streptozotocin (STD)-induced diabetic rats were reduced after a six-week administration of aronia juice. The authors observed 44% and 42% decreases in blood glucose in diabetic rats fed with 10 and 20 mL/kg aronia juice, respectively [223]. Oprea *et al.* demonstrated that the consumption of aronia juice decreased the blood glucose levels of alloxan-induced diabetic rats

by 42.83% [224]. In a study conducted by Yamane *et al.*, the blood glucose levels of diabetic mice (KK-Ay mice) supplemented with aronia juice was lower than the control group (without the supplementation of aronia juice). The trend was consistent over the entire experiment (28 days) [225]. In the same study, aronia juice consumption led to an inhibition of α -glucosidase activity in the upper small intestine of diabetic mice. Hypoglycemic activity of aronia was also observed in animal models with metabolic syndrome but not diabetes. Qin and Anderson performed a study with Wistar rats fed a fructose-rich diet. They demonstrated that the administration of aronia berry extracts (100 or 200 mg/kg body weight) lowered the risk factors related to insulin resistance via a variety of possible mechanisms, such as enhancing mRNA levels of insulin receptor substrate 1 and 2, which may help to increase insulin sensitivity [226]. Takahashi *et al.* illustrated that four weeks of aronia extract consumption (17.4 g aronia polyphenols per kg diet) decreased the fasting blood glucose levels in high-fat diet-induced obese rats [227]. In a human intervention conducted by Simeonov *et al.*, three-month consumption of aronia juice (200 mL/day) lowered the fasting blood glucose in 21 patients with non-insulin dependent diabetes [228]. These studies demonstrate that aronia berries can help to normalize blood glucose levels not only in diabetic individuals, but also in individuals with metabolic syndrome. Clinical studies in regards to the impacts of aronia on diabetes are limited. More studies are needed to confirm the beneficial properties of aronia for T2DM and to establish effective dosages for human.

1.4.3.4 Aronia and Other Health Problems

In addition to the antioxidant, anti-inflammatory properties and potential benefits for diabetes, the consumption of aronia berries is also beneficial for other health problems. The anti-influenza activity of aronia was reported by Park *et al.* in both *in-vivo* and *in-vitro* experiments [229]. In this study, aronia extracts (0.125 mg/mL) exhibited more than 60% inhibition against all five tested viruses (H1/K09, H3/PE16, B/BR60, H1/K2785 and HPAI rH5/IS06) including oseltamivir-resistant strains (H1/K2785 and HPAI rH5/IS06). Oseltamivir is an antiviral drug used to treat and prevent influenza. The inhibitory activity of aronia extracts on the influenza virus was dose-dependent. Additionally, the authors observed that the lethal rate of rPR8-GFP virus-infected mice was reduced by aronia treatment (1 mg/kg, twice daily) [229]. In a long-term crossover intervention performed by Handeland *et al.*, the incidence of urinary tract infections (UTI) among nursing home residents was reduced after patients consumed aronia juice for three months (containing 715 mg/100 mL gallic acid equivalent total phenolics). The authors observed a 55% and a 38% decrease in UTIs among groups given 156 mL and 89 mL juice consumption, respectively [230]. Aronia consumption may have other potential beneficials in addition to the properties mentioned above, such as chemoprotective activity. More studies need to be performed to optimize the health-promoting properties of aronia and the effective dosages of aronia consumption should be established. Additionally, more mechanistic research should be carried out to further understand the mechanisms of how aronia can have positive effects on human health.

1.4.4 Challenge of Incorporating Aronia Berries in Diet

Regular consumption of aronia berries can increase the consumers' daily intake of phenolic compounds and potentially benefit their health. However, aronia berries are not commonly consumed because they are less palatable than other popular berries, such as blueberries. A major sensory attribute that negatively affects the flavor of aronia is the astringency caused by the high phenolic content, especially procyanidins. Duffy *et al.* conducted a sensory evaluation of aronia juice and concluded that the juice was not widely accepted due to the low sweetness and high astringency [77]. The consumer acceptance was increased when sweeteners were added to the juice [77]. Troszynska *et al.* observed that the addition of polysaccharides (guar, xanthan, arabic gums and carboxymethylcellulose) decreased the sensation of astringency in aronia extracts whereas carboxymethylcellulose was observed to have the best effects on lowering astringency compared to other gums [231]. Ares *et al.* investigated the ability of sucrose, sucralose, polydextrose and milk to mask the astringency of phenolic-rich extracts from *Achyrocline satureioides* and *Baccharis trimera* by conducting a sensory study [232]. Results of this study showed that milk was the most efficient in lowering the astringent sensation followed by sucrose. Astringency is caused by the interaction between polyphenols and salivary proteins. The authors stated that the milk proteins could complex with polyphenols and yielded less available polyphenols to form insoluble compounds with salivary proteins. Thus, the astringent mouth-feel was reduced [232]. One concern about using other food ingredients to reduce astringency is the possibility of lowering the bioavailability of aronia polyphenols. The liberation pattern of aronia polyphenols during digestion can be altered if they interact with other food matrices, which affects the absorption of polyphenols. Another

concern about incorporating aronia into other food matrices is the stability of aronia polyphenols may be lowered. For example, the addition of milk is able to decrease the astringency of the phenolic-rich extract [232]. The pH of milk is neutral to slightly alkaline, which negatively affects the stability of anthocyanins. Food products incorporated with aronia in a neutral food matrix may not be able to maintain the full potential of health-promoting properties during shelf life. In summary, in order to increase the consumption of aronia, products with reduced astringency need to be developed. Additionally, in regard to optimize the potential health benefits of aronia berries, the bioavailability of aronia polyphenols in the new developed food products should not be decreased.

1.5 Elderberries

1.5.1 Botany and Current Commercial Utilization of Elderberries

Black elderberries (*Sambucus nigra* L. Family *Adoxaceae*) are deciduous shrubs that can be located in shady, moist areas in Europe, northern Africa, west and central Asia, and North America [233]. There are two subspecies of black elderberries: European elderberries (*Sambucus nigra* L. ssp *Nigra*) and American elderberries (*Sambucus nigra* L. ssp. *canadensis*) [234]. European and American elderberries are similar in most botanical characteristics, except European elderberries are shrubs with a single or a few trunks while American elderberries usually have many canes and can spread by underground rhizomes [235]. This thesis will focus on the American elderberry, also known as the “American elder”.

American elderberry is native to eastern and central North America [236]. American elderberry plants are multi-stemmed shrubs that can reach up to 9 m in height [236]. The plants bloom in early summer showing umbrella-shaped clusters of small, white flowers, which develop into dark purplish-black berries in the late summer or early fall. A cluster of elderberry fruits can contain up to 2,000 berries (5-9 mm in diameter for an individual berry) [236]. Elderberries are a very attractive food for birds. After consuming the berries, birds will then disperse the seeds. Elderberry colonies are commonly located in areas where birds nest, such as along railways, roadways, forest edges and fence lines [235]. Second-year American elderberry shrubs can yield up to 3 kg per plant. At the fourth year, 8 kg average yields can be achieved [236]. American elderberries can produce high yields (about 6 kg per plant) in cold areas, such as Normandin, Quebec, Canada [237]. Traditionally, American elderberries and elderberry flowers are used as herbal remedies to treat colds, flus and inflammation [238]. Elderberry food products have limited availability in the United States. Elderberry jams, pies, wines and juices are the major types of food products on the market. However, the demand for elderberries has increased due to their health-promoting properties [239]. More market-available products using elderberries may encourage farmers to grow elderberries, develop value-added elderberry products and increase their income.

1.5.2. Nutritional Value and Bioactive Compounds of Elderberries

The nutrient level in elderberries is affected by many factors, such as the cultivar of the plant, the degree of fruit ripeness and environmental conditions [239]. Citric acid is the dominant acid in most cultivars of American elderberries followed by malic and succinic acid. Fructose is the dominant sugar in elderberries followed by glucose [240]. American elderberries

are a good source of vitamin C (36 mg/100 g fresh weight basis), potassium (280 mg/100 g fresh weight basis), phosphorus (39 mg/100 g fresh weight basis), calcium (38 mg/100 g fresh weight basis) and magnesium (5 mg/100 g fresh weight basis) [241].

American elderberries are rich in polyphenols. Phenolic compounds are the most important group of bioactive compounds in elderberries. The total phenolic content of fresh berries ranges from 277 to 532 mg gallic acid equivalents/100 g [235, 242, 243]. Elderberries are among the berries that contain the largest amount of anthocyanins [79]. The content of anthocyanins is up to 446.8 mg cyanidin-3-glucosides/100 g of fresh berries [242, 243]. Most anthocyanins in American elderberries are cyanidin-based. Cyanidin 3-(E)-p-coumaroyl-sambubioside-5-glucoside and cyanidin-3-sambubioside-5-glucoside are the most abundant anthocyanins. Cyanidin-3-glucoside, cyanidin-3,5-diglucoside and cyanidin-3-sambubioside have been discovered in relatively high amounts in American elderberries [235]. More than 60% of American elderberry anthocyanins are acylated anthocyanins. In addition to cyanidin-3-(E)-p-coumaroyl-sambubioside-5-glucoside, cyanidin-3-(Z)-(p-coumarin-sambubioside-5-glucoside, cyanidin-3-(p-coumarin)-glucoside and cyanidin-3-(p-coumarin)-sambubioside have also been identified [239]. The presence of these acylated anthocyanins may be used to distinguish American elderberries from European elderberries since acylated anthocyanins are not found in European elderberries [235, 244]. Anthocyanins in American elderberries are more stable than those in European elderberries when exposed to heat and light due to the presence of these acylated anthocyanins. This stability advantage allows American elderberries to be used as a source of industrial food colorants [244, 245]. Cinnamic acids, such as neo-chlorogenic acid, chlorogenic acid and crypto-chlorogenic acid have been identified in elderberries where

chlorogenic acid is dominant in most cultivars [235, 240]. Rutin (quercetin-3-rutinoside) is the predominant flavonol glycoside in elderberries; other flavonols, such as isoquercetin, kaempferol-3-rutinoside, isorhamnetin-3-rutinoside and isorhamnetin-3-glucoside have been identified, as well [235].

1.5.3 Health Promoting Characteristics of Elderberries

The health-beneficial properties of European elderberries are well documented. However, most studies involving American elderberries are in regard to cultivation. Research that investigates the impact of American elderberries on health are limited. In this section, European elderberry studies are included to introduce the potential bioactivity of American elderberries on human health since they are closely related.

1.5.3.1 Antioxidant Capacity of Elderberries

American elderberries have been shown to exhibit strong antioxidant capacity *in-vitro*. Ozgen *et al.* evaluated fourteen accessions of American elderberries by the ferric reducing antioxidant power (FRAP) and the 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging capacity [242]. All samples exhibited ferric reducing antioxidant power (13.4 – 31.7 μmol Trolox equivalents/ g on a fresh weight basis) and DPPH scavenging capacity (5.4 – 13.5 μmol Trolox equivalents/ g fresh weight basis). In the same study, authors observed a strong correlation between antioxidant capacity and the levels of total phenolic compounds [242]. In a study conducted by Simonyi *et al.*, anthocyanin-rich extracts of American elderberries inhibited the production of ROS in LPS or IFN- γ -simulated mouse microglial cells (bv-2), which means American elderberries may help to decrease the cellular oxidative stress [246]. Currently,

little to no research studies have documented the ability of American elderberries to decrease oxidative stress *in-vivo*. The benefits of European elderberries on oxidative stress have been observed in several animal studies. Dubey *et al.* demonstrated that the fish oil-induced oxidative stress in BioF1B hamsters was attenuated by consuming European elderberry extract [247]. Ciocoiu *et al.* reported that the consumption of European elderberry extract (0.05 g/kg body weight every two days for 16 weeks) decreased lipid peroxides and inhibited LDL oxidation in STD-induced diabetic rats [248]. In the same study, levels of reduced GSH and SOD were elevated in both healthy and STD-induced diabetic rats [248]. These results indicate that the consumption of European elderberries reduced oxidative stress in rats, which may be due to improving their antioxidant defense system. American elderberries may exhibit different antioxidant activity *in-vivo* compared to European elderberries due to the presence of acylated anthocyanins. More studies using rodent models or human subjects need to be conducted better to understand and utilize the potential health-promoting properties of American elderberries.

1.5.3.2 Anti-inflammatory Activity of Elderberries

The anti-inflammatory activity of polyphenol-rich fruits has been observed by both *in-vivo* and *in-vitro* studies [100, 249, 250]. However, studies on the anti-inflammatory activity of American elderberries are rare. Simonyi *et al.* observed that the NO production in LPS or IFN- γ -stimulated mouse microglial cells (bv-2) was reduced by American elderberry extracts, which means that the inflammatory response was decreased [246]. Seeram *et al.* demonstrated that anthocyanin-rich extracts from American elderberries inhibited the activity of cyclooxygenase-1 and cyclooxygenase-2 (COX-1 and COX-2) *in-vitro*. The COX inhibitory activity of American

elderberry anthocyanins was stronger than cranberries, blueberries and bilberries [251]. COX can catalyze the production of prostaglandins which are important compounds in the generation and promotion of the inflammation response [252]. Inhibition of COX activity can decrease the degree of inflammation. Farrell *et al.* conducted an *in-vivo* study where mice were fed European elderberry extracts for sixteen-weeks (20-40 and 200-400 mg/kg body weight). Researchers observed lower levels of inflammatory markers (TNF- α and chemoattractant protein-1) and decreased insulin resistance in obese mice compared to the control group [253]. However, the *in-vivo* anti-inflammatory activity of American elderberries has not been well documented. More research investigating the anti-inflammatory bioactivity of American elderberries needs to be conducted.

In addition to antioxidant and anti-inflammatory activities, American elderberries may have other health-promoting properties that have been shown in research studies with European elderberries, such as hypoglycemia [254, 255], anti-influenza virus [256, 257], cardiovascular protective [258, 259] and chemoprotective activities [260]. However, the composition of phenolic compounds in American elderberries are different than European elderberries. Thus, the health-promoting properties of American elderberries may be different, which provides justification for investigating the potential health benefits of consuming American elderberries.

1.6 Kefir

1.6.1 History and Production of Kefir

Kefir is a fermented dairy beverage that has a creamy texture and sour taste along with natural carbonation [261]. Kefir originated from the Caucasus Mountains in west Asia. The word “kefir” is derived from the Turkish word “keyif” which means “good feeling” [262]. Kefir was first discovered when the people of the Caucasus region stored fresh milk in leather pouches and the milk naturally fermented into an effervescent beverage [263]. Around the Caucasus region, kefir is commonly made with sheep milk. Currently, kefir is popular in European countries where cow milk is the typical base [263].

The major difference between kefir and other fermented dairy products is the presence of kefir grains and yeast. Kefir grains are white, cauliflower-shaped small granules (3-35 mm in diameter). These kefir grains are used in the production of kefir and are recovered after the fermentation process [264]. The composition of kefir grains varies due to many factors, such as the incubation conditions and the types of milk used [265]. Generally, they are mixtures of microflora, polysaccharides and proteins [261]. The microflora in kefir grains consists of up to 30 species of lactic acid bacteria, yeasts and sometimes acetic acid bacteria [261, 266]. The kefir culture used in the research for this dissertation was a commercial products (Yogourmet®, Canada) containing *Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris*, *Lactococcus lactis* subsp. *bv. diacetylactis*, *Lactobacillus acidophilus* and lactic yeasts. The polysaccharides in kefir grains are known as kefiran, which comprises glucose and galactose units [266], while the major proteins in kefir grains are caseins [261].

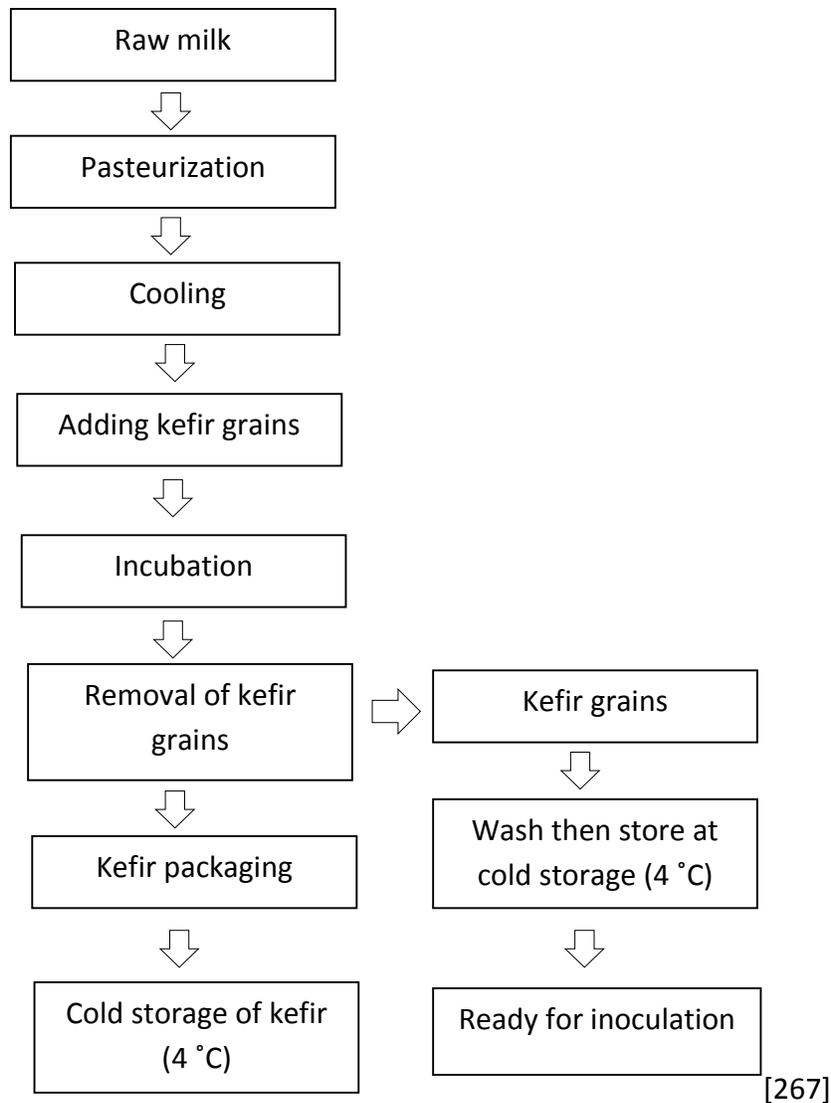


Figure 1.4 Brief flowchart of traditional kefir production

Traditionally, kefir fermentation is initiated by adding kefir grains to cooled pasteurized or unpasteurized milk. The fermentation process takes approximately twenty four hours at room temperature [267]. After fermentation, kefir grains are recovered and separated by filtering the kefir with sieves. The collected kefir grains are dried and stored at cold temperature (4°C) until the next inoculation [267]. However, lyophilized starters are typically used in industry instead of kefir grains. One major reason is the difficulty of separating the kefir

grains at the end of fermentation [266, 267]. In order to obtain consistent kefir products, cultures with a known composition of microorganisms isolated from kefir grains may be used [268]. A flowchart of traditional kefir production was illustrated by Guzel-Seydim *et al.* and is shown in Figure 1.4 [269].

1.6.2 Nutritional Value of Kefir

The nutritional profile of kefir is affected by the milk composition, kefir grain composition, fermentation conditions and storage conditions [270-272]. Sarkar reported that kefir typically contains 89-90% water, 0.2% lipid, 3% protein, 6% sugar and 0.7% ash [270]. Kefir is a source of complete protein, as the essential amino acid levels in kefir are reported to be: 376 mg/100 g lysine, 262 mg/100 g isoleucine, 231 mg/100 g phenylalanine, 220 mg/100 g valine, 183 mg/100 g threonine, 137 mg/100 g methionine and 70 mg/100 g tryptophan [273, 274]. Kefiran, a heteropolysaccharide (glucogalactan), is the major sugar in kefir [261]. During fermentation, the predominant sugar in milk – lactose, is hydrolyzed by β -galactosidase from bacteria in kefir grains and metabolized to glucose and galactose. Glucose is utilized by lactic acid bacteria which generates lactic acid. Lactic acid and/or galactose may be used as the carbon source for the yeasts in kefir grains [275]. Kefir is a suitable dairy beverage for lactose-intolerant individuals due to the activity of β -galactosidase [270, 273, 275, 276]. Kefir contains various vitamins, such as vitamins B₁, B₂, B₅, B₆, B₁₂, folic acid, A, C and K [270, 276]. The vitamin composition of kefir is influenced by both the type/composition of the milk used and the microbial profile of the kefir grains [270]. Kefir is a good source of potassium (1.65%), phosphorus (1.45%), calcium (0.86%) and magnesium (0.30%). Other minerals such as zinc, copper, iron, molybdenum and cobalt were also reported in kefir [274]. Kefir has also been

reported to have several bioactive compounds, such as bioactive peptides, exopolysaccharides (kefiran) and probiotics. These bioactive compounds may work independently or synergistically to enhance health-promoting activities [277]. The health-promoting properties of kefir will be introduced in the next section.

1.6.3 Health Promoting Characteristics of Kefir

1.6.3.1 Anti-inflammatory Activity of Kefir

The anti-inflammatory activity of kefir has been documented by several studies using animal models with metabolic syndrome. In a study performed by Rosa *et al.*, metabolic syndrome was induced in male SHR (Spontaneously Hypertensive Rats) by monosodium glutamate injection. After ten weeks of administering kefir to their diet (kefir group, 1 mL/day) or using water as placebo (control group), a reduction in pro-inflammatory cytokines (IL-1 β) and an increase in anti-inflammatory cytokine (IL-10) were observed in the kefir group compared to the control group. Beneficial effects on other biomarkers of metabolic syndrome, such as reductions in plasma triglycerides and insulin resistance, were also observed [278]. Hadisaputro *et al.* observed that the levels of pro-inflammatory cytokines (IL-1, IL-6) in STD-induced hyperglycemic rats were decreased after thirty days of plain kefir consumption (3.6 mL/day). Additionally, an increase in the anti-inflammatory cytokines (IL-10) was observed in the kefir-fed rats [279]. The anti-inflammatory activity of certain compounds in kefir was investigated. Kwon *et al.* reported that the administration of kefiran (50 mg/kg body weight) reduced the numbers of inflammatory cells in the lavage fluid and the lung tissues of female mice with ovalbumin-induced asthma. The levels of inflammatory cytokines (IL-4 and IL-5) in bronchoalveolar lavage fluid was reduced to normal after kefiran consumption [280]. Chen *et*

al. performed an experiment using kefir peptide and male mice challenged with high-fructose corn syrup [281]. The results revealed that the status of mice with fatty liver syndrome was improved by kefir peptides administration. Decreased levels of inflammatory cytokines (TNF- α , IL-6 and IL-1 β), hepatic triglycerides, cholesterol and serum alanine aminotransferase were observed in mice fed with kefir peptide compared to mice fed water [281]. In summary, regular consumption of kefir may help to ameliorate the symptoms of metabolic syndrome and decrease the risk of chronic diseases due to anti-inflammatory activities. Kefiran and bioactive peptides appear to contribute to the anti-inflammatory activities.

1.6.3.2 Kefir Benefits for Diabetes

The consumption of kefir may benefit diabetic individuals by aiding in blood glucose control. In a double-blind intervention conducted by Ostadrahimi *et al.*, sixty T2DM patients were divided into two groups and consumed 600 mL kefir per day (probiotic group) or 600 mL placebo per day (conventional fermented milk without probiotics). After an eight-week intervention, the fasting blood glucose levels and the glycated hemoglobin A1C (HbA1C) levels of the probiotic groups (6.40 ± 1.91) were significantly reduced compared to baseline (7.61 ± 1.22) [282]. These results indicated that regular consumption of kefir could aid in the blood glucose control of individuals with diabetes. Compared to the control group, the fasting glucose levels and HbA1C levels of the probiotic groups were lower. These results also showed that the probiotic microorganisms (*Lactobacillus casei*, *Lactobacillus acidophilus* and *Bifidobacteria*) played an important role in the hypoglycemic activity of kefir [282]. In a study using diabetic KKAy mice, Maeda *et al.* observed that a thirty-day feeding of kefir lowered the blood glucose in these mice [283]. Reductions in insulin resistance resulting from kefir

supplementation was observed by Rosa *et al.* in rats with metabolic syndrome [278]. Teruya *et al.* investigated the effects of kefir on glucose uptake in an insulin-responsive muscle cell line (L6 skeletal muscle cells). The authors reported that the glucose uptake ability of L6 cells was increased by a water-soluble fraction of kefir in the presence or absence of insulin [284]. Punaro *et al.* demonstrated that eight-weeks kefir consumption (1.8 mL/day) reduced glycogen accumulation in the renal tubules of STD-induced type 1 diabetic rats. This result indicates that kefir can slow the progression of renal injury in diabetic individuals [285]. Thus, kefir intake may be beneficial to diabetic individuals via modulating blood glucose levels, decreasing insulin resistance, increasing glucose uptake in muscle and reducing the progression of diabetic complications. The potential benefits of kefir for diabetes may be attributed to both the probiotic microorganisms and the microbial metabolites, such as kefiran.

In addition to anti-inflammatory properties and potential benefits to diabetes, kefir has been documented to have anti-allergic activities [286], anti-carcinogenic effects [287], cardiovascular protective activity [288, 289] and the ability to modulate gut microflora [286, 290]. The probiotic microorganisms in kefir contribute to their health-promoting properties. In addition, the cell-free fractions of kefir, such as kefiran and bioactive peptides, also exhibit health-promoting activities [264, 291].

1.7 Conclusions

The onset and progression of T2DM is associated with oxidative stress. The consumption of dietary antioxidants may help to reduce the risk of T2DM and slow the progression of secondary complications via decreasing oxidative stress. Dietary polyphenols are strong antioxidants. Higher consumption of polyphenols has been associated with many health

benefits. Aronia and American elderberries are polyphenol-rich fruits, and their health-promoting properties have been suggested by many studies. The health-promoting properties of phenolic compounds, aronia berries and elderberries are shown in Table 1.1. Aronia berries and elderberries are suitable to be grown in most areas of the United States, including Maine, but the consumption of these berries is low due to their unpalatable taste and limited types of commercial products. The development of new products that are acceptable to consumers may help to increase the consumption of these berries. One concern about developing food products using these berries is that processing and the addition of other food ingredients may lower the amount of bioavailable berry phenolics. Kefir, a fermented dairy beverage, may be a suitable food matrix for the incorporation of berries due to the following reasons: 1) kefir is acidic and the low pH environment can be beneficial to the stability of phenolic compounds; 2) kefir is rich in proteins, which may help to mask the astringency of polyphenols; 3) the fermentative microorganisms in kefir may increase the bioavailability of phenolic compounds. In order to ensure consumers will receive the optimal health benefits of these berries, the health-promoting properties of the newly developed products must be evaluated. Therefore, research studies in this dissertation focused on the development of berry-added kefirs and the evaluation of their bioavailability and health-beneficial characteristics.

Table 1.1 Health-promoting characteristics of phenolic compounds, aronia berry, elderberry and kefir

	Health-promoting characteristics
Phenolic compounds	Reduce oxidative stress <i>in-vivo</i>
	Attenuate chronic inflammation
	Beneficial to T2DM, including increase insulin sensitivity, aid in blood glucose control, and decrease AGE production
Aronia berry	Reduce oxidative stress <i>in-vivo</i>
	Anti-inflammatory activity
	Beneficial to T2DM, including increase insulin secretion and help to control postprandial blood glucose
	Others: anti-influenza activity, anti-infection, etc.
Elderberries (American)	Anti-oxidant capacity <i>in-vitro</i>
	Anti-inflammatory activity
Kefir	Anti-inflammatory activity
	Beneficial to diabetes, including decrease insulin resistance, aid in blood glucose control and reduce glycogen accumulation
	Others: anti-allergic activity, anti-carcinogenic effect, cardiovascular protective activity and gut microflora modulatory effect.

CHAPTER 2

DEVELOPMENT AND EVALUATION OF KEFIR PRODUCTS MADE WITH ARONIA OR ELDERBERRY

JUICE: SENSORY AND PHYTOCHEMICAL CHARACTERISTICS

This chapter is accepted by the International Food Research Journal and will be published in volume 25, issue 4.

2.1 Chapter Abstract

Aronia and elderberry are edible berries that are rich in anthocyanins and phenolic compounds. They are rarely consumed raw due to safety concerns and their unpalatable taste. Aronia and elderberry are not widely grown and they are not commonly used as food ingredients for commercial products, thus they are considered underutilized. Incorporating these berries into new products, such as kefir, provides diverse food choices and may increase the dietary consumption of these bioactive compounds. In this study, kefir containing either aronia or elderberry juice was developed using different sweeteners and levels of sweetness. Sensory tests were conducted to evaluate the consumer acceptability of the kefir products made with fresh aronia juice or commercial elderberry juice from Wyldewood Cellars® (Wichita, KS, U.S.). In the aronia kefir sensory test, the product sweetened with sucrose received the best overall acceptability (6.3) while the product sweetened with monk fruit extract was least favored (4.9). In the elderberry kefir sensory test, 5.7% sucrose-sweetened product was best accepted (6.6) followed by 4.3% sucrose-sweetened (6.1). Non-nutritive sweeteners (stevia and monk fruit extracts) were less accepted than sucrose in both tests. Phytochemical analyses

showed that aronia kefir contained high amounts of total phenolic compounds and anthocyanins. Kefir made with commercial elderberry juice had a moderate amount of total phenolics and a low amount of anthocyanins. Antioxidant capacity was observed in all products indicating that the consumption of berry-added kefir may benefit the decrease of oxidative stress. Kefir made with aronia or elderberry are acceptable functional foods which may contribute to the prevention of inflammation and chronic diseases when incorporated into a healthy diet.

2.2 Introduction

Epidemiological studies suggest that the consumption of anthocyanin-rich fruits may contribute to decreasing the risk of type 2 diabetes [3]. The protective impacts of phenolic compounds on oxidative-stress-related diseases are related to their high antioxidant capacity [292-294]. Rios *et al.* indicate that blueberry extracts rich in polyphenols could decrease the levels of oxidized DNA bases in human subjects and attenuate DNA damage induced by H₂O₂ [295]. Anthocyanin intake (two levels, 40 and 200mg/kg) is able to reduce high-fat-diet-induced oxidative stress in mice by boosting the activity of antioxidant enzymes (SOD and GPx). Systemic inflammation could be decreased with this range of intake by lowering the expression levels of inflammatory cytokines, such as IL-6 and TNF- α [296]. *In-vitro* studies revealed that anthocyanin-rich extracts could inhibit the formation of advanced glycation end products and consequently decrease the risk of diabetic complications [297].

Aronia melanocarpa (aronia) and *Sambucus nigra* L. ssp. *canadensis* (elderberry) are berries that contain high amount of anthocyanins and polyphenols [179, 239]. They are not widely commercial cultivated thus they are underutilized. These berries exhibit high antioxidant

capacity due to the phenolic compounds. Aronia and elderberry are known to reduce oxidative stress in humans [196, 298]. Aronia and elderberry are popular in Europe where they are utilized as functional food ingredients and color additives [179, 299]; however, a market for these berries in the United States has not been well developed.

Kefir is a fermented dairy beverage that originated in the Caucasus Mountains region over one hundred years ago [268, 300]. Kefir grains consist of complex microbial communities and contain up to 30 species [301] where lactic acid bacteria is usually predominant, followed by yeast and acetic acid bacteria [264]. Several studies suggest that kefir has anti-microbial, anti-inflammatory, and anti-carcinogenic activities [275, 302, 303]. Kefir is naturally lactose-free, this property makes kefir a good source of calcium and protein for lactose intolerant individuals [270]. The fermentative microorganisms may have the ability to increase the bioaccessibility and bioavailability of phenolic compounds due to the release of bound phenolic constituents by some lactic acid bacteria [315, 304]. The low acid environment of kefir is helpful to decrease the natural degradation of phenolic compounds [305]. Therefore, the combination of anthocyanin-rich berries and kefir may result in a value-added functional product. The objective of this study was to develop functional food products by incorporating either aronia or elderberry juice into a kefir beverage and evaluate their sensory and phytochemical characteristics. Different natural sweeteners (sucrose, stevia or monk fruit extract) were used to enhance the sweetness of the products. The consumer acceptability of the products was evaluated via sensory tests. The aronia sensory test focused on the impact of sweetener variety on consumer acceptability. The elderberry kefir sensory test assessed the influence of sweetness levels. Bioactive constituents of the kefir products were analyzed in the laboratory.

2.3 Material and Methods

2.3.1 Chemicals

Methanol, citric acid anhydrous, potassium chloride, hydrochloric acid, and sodium bicarbonate were obtained from Fisher's Scientific (Waltham, MA, U.S.). 2, 2-diphenyl-1-picrylhydrazyl (DPPH), Folin - Ciocalteu's phenol reagent and gallic acid were purchased from Sigma-Aldrich (St. Louis, MO, U.S.), and sodium acetate from Chem-Impex int'l inc. (Wood Dale, IL, U.S.). Ultrapure water was obtained from a Millipore water system (EMD Millipore, Billerica, MA, U.S.).

2.3.2 Dietary Material

Aronia (*Aronia melanocarpa*, variety 'Viking') were obtained from the University of Connecticut (Storrs, CT, U.S.) from the 2014 growing season. Elderberry juice was a gift of Dr. John Brewer from Wyldewood Cellars® (Wichita, KS, U.S.). Elderberries (*Sambucus nigra*, L. ssp. *canadensis*) used in making samples for fresh juice phytochemical analyses were collected from the experimental garden at the University of Maine (Orono, ME, U.S.) during the 2015 harvest season. Berries were harvested at full ripeness which was determined by a deep purple color and softness of the berry. They were de-stemmed and washed, then frozen at -20°C. The thawed berries were pasteurized (100°C, 5 minutes) in an aluminum sauce pan then juiced with a domestic juicer (Hamilton Beach, Southern Pines, NC, U.S.). Juice yield from 1 kg berries averaged 360 g. Fresh juice was used immediately to make the kefir products. Commercial kefir culture (Yogourmet®, Lachute, QC, Canada) was used as the starter for the kefir. The viability of the lactic bacteria in the starter was determined by inoculation on MRS agar (Nedgen, Lansing, MI, U.S.) at 40°C for 48 hours. The viability of the yeast in the starter was measured by plating

on antibiotic plate counting agar (Alpha Biosciences, Baltimore, MD, U.S.) at room temperature for 96 hours. Sucrose (Great Value®, Bentonville, AR, U.S.), stevia extract (Stevia in the Raw®, New York City, NY, U.S.), monk fruit extract (Monk Fruit in the Raw®, New York City, NY, U.S.) and 2% milk (Oakhurst®, Portland, ME, U.S.) were purchased from local supermarkets.

2.3.3 Kefir Manufacture and Formulas

All kefir products were prepared by the following method: 2% milk was heated to 82°C in a commercial size aluminum sauce pan, cooled to 26°C using an ice bath, and then transferred to a 4 L pyrex glass bowl. The commercial starter was added to the milk (5 g per quart), and the mixture was stirred for 5 minutes to ensure the starter was dissolved. Either aronia juice (~13%, w/w) or elderberry juice (~10%, w/w) was added to the mixture. The amount of non-nutritive sweetener used in each product was adjusted according to the instruction on the package to create equal sweetness to sucrose. After the addition of all ingredients, the mixture was covered with a cloth and left at room temperature to ferment for 24 hours. The kefir was homogenized with a Hamilton Beach immersion blender (Southern Pines, NC, U.S.). Each kefir product was divided into two containers with sealed lids and stored at 4°C. The kefir was allowed to chill at 4 °C for 2 hours prior the sensory tests. The sensory tests were completed within 36 hours. Formulas are shown in Table 2.1. Kefir products were only formulated with juice and sweetening agents (sucrose, stevia extract, or monk fruit extracts); no additional modifiers were used. Low sucrose levels were selected for reducing calorie content and to ensure that the predominant flavor was from the berry juice.

An aliquot of the kefir prepared for the sensory test was collected and stored at -20°C until laboratory analyses. The initial study design was to utilize fresh berry juices for the kefir products but due to crop failures in 2013 and 2014, fresh elderberries were not available. Commercial elderberry juice (shelf-stable product) was used in this study. To better understand the difference between commercial and fresh elderberry juice, an additional set of elderberry kefir products made with fresh juice was prepared and analyzed in the laboratory at a later date.

Table 2.1 Formulas of kefir products for sensory evaluation

Product	Sweetener	Milk with starter (g)	Commercial elderberry juice (g)	Fresh elderberry juice (g)	Fresh aronia juice (g)	Sucrose (g)	Stevia (g)	Monk fruit (g)
Aronia kefir	sucrose	83	----	----	13	4.0	----	----
	stevia	83	----	----	13	----	0.40	----
	monk fruit	83	----	----	13	----	----	0.80
Elderberry kefir (commercial juice)	low sucrose (4.3%)	90	10	----	----	4.5	----	----
	high sucrose (5.7%)	90	10	----	----	6.0	----	----
	low stevia (0.4%)	90	10	----	----	----	0.45	----
	high stevia (0.6%)	90	10	----	----	----	0.60	----
Elderberry kefir (Fresh juice)	low sucrose (4.3%)	90	----	10	----	4.5	----	----
	high sucrose (5.7%)	90	----	10	----	6.0	----	----
	low stevia (0.4%)	90	----	10	----	----	0.45	----
	high stevia (0.6%)	90	----	10	----	----	0.60	----

Note: ---- indicates the ingredient was not used in the formula

2.3.4 Sensory Analyses

All sensory tests were performed in the Sensory Testing Center at the University of Maine, Orono campus. Sensory tests were approved by the Institutional Review Board for the Protection of Human Subjects at the University of Maine (IRB). Two tests were conducted and 100 healthy participants for each test were recruited from the community. Demographic information was collected, such as age and gender. Consumer familiarity with the berries and kefir was assessed. Consumer attitude toward purchasing healthy food was asked. Color, flavor, sweetness, texture and overall acceptability of all samples were evaluated using a 9-point hedonic scale (1=dislike extremely, 5=neither like nor dislike, 9=like extremely). Products received random 3-digit codes and samples were presented to the consumers in a randomized sequence. Samples were served cold (4 °C) in transparent 2 oz plastic cups and water was offered as a palate cleanser. Information was collected anonymously with computers using SIMS Sensory Software® (Berkeley Heights, NJ, U.S.). Each participant was compensated with \$2 for completion of the sensory test.

2.3.5 pH, Titratable Acidity, Total Soluble Solids and Color Measurements

pH, titratable acidity (TA), and total soluble solids (°Brix) were evaluated following the methods reported by Mena *et al.* with minor modification [306]. Briefly, pH was measured using a Sartorius pH meter (Bohemia, NY, U.S.). TA was determined by titrating 5 g of kefir with 0.1 M NaOH solution. The results were expressed as % lactic acid [307] in kefir and % citric acid in juice. Total soluble solids were tested using a PAL-3 refractometer by Atago (Tokyo, Japan) and values were expressed as °Brix. The color was measured using a LabScan XE spectrophotometer manufactured by HunterLab (Reston, VA, US) and was recorded as L*

(lightness), a^* (redness/greenness), and b^* (yellowness/blueness). Hue angle and Chroma values were calculated by the following formulas [308, 309].

$$\text{Hue} = \text{Arc tan}(b^*/a^*) \text{ for the first quadrant (+a, +b)}$$

$$\text{Hue} = 360 + \text{Arc tan}(b^*/a^*) \text{ for the fourth quadrant (+a, -b)}$$

$$\text{Chroma} = \sqrt{(a^{*2} + b^{*2})}$$

pH and °Brix were measured in triplicate, TA was measured in duplicate due to limited sample availability. Color was measured in five independent tests to confirm the uniformity of the sample and obtain representative results.

2.3.6 Extraction

Phenolic compounds were extracted following the method reported by Scibisz *et al.* with modification [310]. After adding acidified 80% methanol (1% citric acid, w/v, 1:10, sample:solvent) to the kefir matrix, the mixture was vortexed then sonicated in a Branson 5510 sonicator (Danbury, CT, U.S.) for 1 hour. Samples were centrifuged at 16639×g (Eppendorf 5804R, Hamburg, Germany) for 30 minutes at 4°C. The supernatant was collected. This process was repeated three times. The combined supernatant was evaporated under a vacuum (Eppendorf Vacufuge plus, Hamburg, Germany) at room temperature. Dried samples were re-suspended using acidified 100% methanol (1%, citric acid), and kept at -20°C for one hour to precipitate the protein. The slurry was then centrifuged at 16639×g for 30 minutes at 0°C and the supernatant was collected. Supernatant was dried under a vacuum and re-suspended with 80% acidified methanol (1%, w/v). The extract samples were kept at -20°C until analyses.

2.3.7 Total Phenolic Content

TP content was determined using the Folin-Ciocalteu method as described by Velioglu *et al.*, with minor modifications [311]. Briefly, after mixing extract (20 μ l) and Folin-Ciocalteu reagent (90 μ l), the plate was left at room temperature for 5 minutes and then sodium bicarbonate (6 g/100 ml, 90 μ l) was added. The plate was covered and incubated at room temperature in the dark for 90 minutes. The absorbance was read at 750 nm with a Biotek plate reader (ELx 800, Winooski, VT, U.S.). All samples were measured in triplicate, and the results are expressed as gallic acid equivalents (GAE).

2.3.8 Total Monomeric Anthocyanin Content

TMA content was determined using the pH differential method developed by Lee *et al.* with modifications to fit a 96-well plate format [82]. Briefly, the extract (20 μ l) was diluted with 180 μ l of pH 1.0 buffer (0.025 M, potassium chloride) and 180 μ l pH 4.5 buffer (0.4 M, sodium acetate) separately. The mixture was incubated for 20 minutes at room temperature in the dark. The absorbance (abs) was read at 520 nm and 690 nm using the plate reader. The TMA content was calculated by the following formula and expressed as cyanidin-3-glucoside (C3G) equivalents.

$$\text{Anthocyanin (cyanidin - 3 - glucoside equivalents, mg/L)} = \frac{A \times MW \times DF \times 10^3}{\epsilon \times L}$$

Where: A = (abs520 nm – abs700 nm) pH 1.0 – (abs520 nm – abs700 nm) pH 4.5;

MW (molecular weight) = 449.2g/mol for cyanidin-3-glucoside;

DF = dilution factor;

L = pathlength in cm;

ϵ = 26900L/mol·cm, for cyanidin-3-glucoside;

10^3 = factor for conversion from g to mg.

2.3.9 Antioxidant Capacity

Antioxidant capacity was determined using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging method by Duymus *et al.* with minor modifications [312]. 150 μ l of DPPH solution (0.3 mM) were added to 150 μ l serially diluted extracts, the mixture was incubated in the dark at room temperature for 30 minutes and read at 515 nm. The inhibition rate was calculated by the following formula:

$$\% \text{inhibition} = \frac{\text{Abs}_{\text{Control}} - \text{Abs}_{\text{Sample}}}{\text{Abs}_{\text{Control}}} \times 100$$

Inhibition (%) was plotted against extract concentration, and the IC₅₀ (the concentration to scavenge fifty percent of DPPH free radical) was calculated. Gallic acid was used as a positive control.

2.3.10 Statistical Analysis

Data are shown as mean \pm standard deviation. Sensory data were analyzed with one-way analysis of variance using JMP 12 software by SAS Institute Inc. (Cary, NC, U.S.). Tukey's Honest Significant Difference (HSD) test was used for mean comparisons. Pearson test was used to determine correlations. A significance level was set at $\alpha = 0.05$.

2.4 Results and Discussion

2.4.1 Aronia Kefir Evaluations

2.4.1.1 Aronia Kefir Sensory Test

Three aronia kefir products containing different sweeteners were evaluated in this test. The only difference among aronia kefir products was the type of the sweetener. Participants' perception about different sweeteners could be assessed. Demographic results demonstrated that participants in this test were balanced in gender (54 female and 46 male), and the general age range was 18-34 years old. A question about the importance of purchasing food with potential health benefits was addressed and 94% of participants responded positively. Thus, functional food products, like aronia kefir, comply with consumer purchasing trends. Sensory attributes and overall acceptability results are shown in Figure 2.1. The results show that the participants accepted the color of all the products equally; however, significant differences in acceptability were detected for other attributes. Based on the ratings for sweetness, the participants liked the sucrose-sweetened sample best followed by stevia and monk fruit extract was the least favored. Similar results were obtained by another study where chocolate milk sweetened with sucrose received better acceptability compared to stevia or monk fruit extracts [313]. A study by Cardello *et al.* demonstrate that many non-nutritive sweeteners, such as stevia and aspartame have a residual bitter taste which is not well accepted by the consumers [314]. In our test, consumers noted that kefir products sweetened with non-nutritive sweeteners (stevia and monk fruit extract) had a "bad", "longer", or "unpleasant aftertaste" which lowered consumer liking of the products. Flavor was best received in the sucrose-sweetened aronia product. Kefir has a unique flavor due to the lactic acid and carbon dioxide

[273]. In this study, aronia juice and sweeteners were added to the kefir, and contributed to the overall flavor profile. Aftertaste of non-nutritive sweeteners may be a reason for the low hedonic scores of the products in the flavor attribute. The best texture was received in the sucrose-sweetened product where a thicker consistency was noticed. Cardoso and Bolini indicated that sucrose contributed a more viscous texture to a beverage product compared to non-nutritive sweeteners [315]. The unique foamy texture of kefir generated by carbonation during fermentation [267] may be novel to the American palate and may have an impact on the acceptability. Consumer preference of the texture may be influenced by the viscosity and the carbonation of the product. The best overall acceptability (6.3) was received in aronia kefir made with sucrose. Aronia kefir made with monk fruit extract, by contrast, received the lowest hedonic score (4.9). In addition to the influence by sweeteners, consumers' unfamiliarity with the kefir and/or aronia berry may be another reason which had an impact on the overall acceptability of the products. Over half of the participants (57%) were naive to kefir (never consumed kefir previously) and 78% were not familiar with aronia. Orjuela-Palacio *et al.* proved that repeated exposure to a high-polyphenol beverage increased the consumer acceptance [316]. This indicates that the acceptability of the kefir products in this study could be higher if our participants were more familiar with either kefir or aronia. Generally, overall acceptability of each aronia kefir product was better received by participants who were previously familiar with kefir compared to the kefir-naive participants (data not shown).

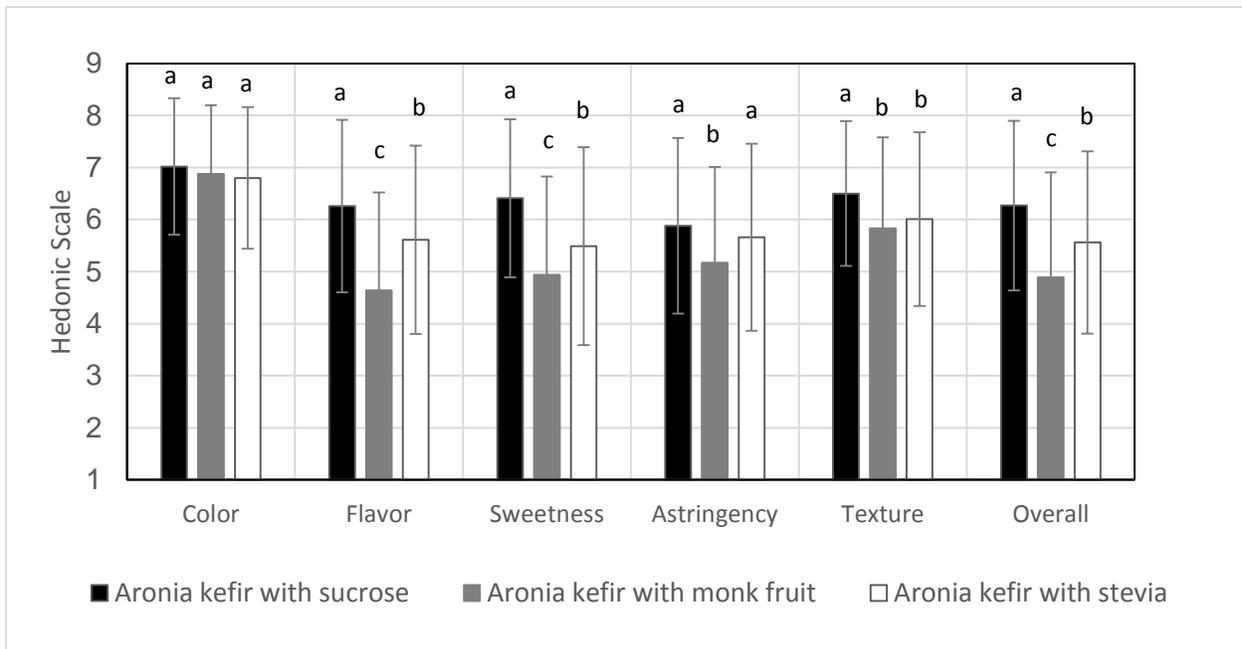


Figure 2.1. Consumer acceptability of aronia kefir made with different sweeteners

Note: Data are shown as means \pm standard deviation. n=100.

Different letters indicate significant differences among means within each attribute, $p < 0.05$.

2.4.1.2 Aronia Kefir Quality Parameters

The results of pH, TA, °Brix and color of aronia kefir are shown in Table 2.2. The pH values of aronia kefir were in the acidic range as expected and this acidic environment is necessary to maintain the integrity of the phenolic compounds [305]. TA is representative of the sour taste in the product. Lactic acid produced by the kefir culture and organic acids in aronia juice contributed to TA values. No correlation was observed between TA and pH ($r = -0.13$, $p = 0.73$). The highest °Brix value was observed in the sucrose-sweetened product as expected. Several factors contribute to the °Brix of the product. They include the added sucrose, fructose in aronia berries and the breakdown products of the milk disaccharides by the

living culture. Concentrations of viable lactic acid bacteria and yeasts in the commercial kefir starter were 9.83×10^8 CFU/g and 5.3×10^4 CFU/g, respectively.

Table 2.2 Quality evaluation of juices, aronia kefir, elderberry kefir made with commercial juice, and elderberry kefir made with fresh juice

Product		pH (n=3)	TSS (°Brix n=3)	%TA (w/w n=2)	Color (n=5)				
					L*	a*	b*	Hue angle	Chroma
Aronia kefir	Sucrose	4.4± 0.01	13.2 ± 0.10	0.64	53.1 ± 0.26	16.9 ± 0.29	-4.9 ± 0.14	359.7 ± 0.01	17.6 ± 0.24
	Stevia	4.4± 0.01	8.9 ± 0.06	0.67	53.2 ± 0.04	16.7 ± 0.11	-5.1 ± 0.04	359.7 ± 0.00	17.5 ± 0.09
	Monk fruit	4.4± 0.01	9.5 ± 0.12	0.66	54.0 ± 0.12	16.9 ± 0.17	-4.6 ± 0.09	359.7 ± 0.01	17.5 ± 0.14
Elderberry kefir (commercial juice)	4.3% sucrose	4.5± 0.01	13.1 ± 0.06	0.73	60.5 ± 0.11	10.9 ± 0.02	1.5 ± 0.04	360.1 ± 0.00	11.0 ± 0.03
	5.7% sucrose	4.6± 0.01	14.6 ± 0.06	0.70	60.4 ± 0.11	10.9 ± 0.03	1.6 ± 0.05	360.1 ± 0.00	11.1 ± 0.04
	0.4% stevia	4.5± 0.01	9.2 ± 0.00	0.77	60.8 ± 0.48	10.8 ± 0.07	1.7 ± 0.08	360.2 ± 0.01	10.9 ± 0.08
	0.6% stevia	4.5± 0.01	9.2 ± 0.06	0.75	61.6 ± 0.27	10.6 ± 0.16	1.6 ± 0.05	360.2 ± 0.00	10.7 ± 0.17
Elderberry kefir (fresh juice)	4.3% sucrose	4.5± 0.01	13.5 ± 0.01	0.74	60.7 ± 0.07	12.0 ± 0.09	0.1 ± 0.06	360.0 ± 0.01	12.0 ± 0.09
	5.7% sucrose	4.5± 0.01	14.9 ± 0.06	0.74	60.6 ± 0.02	12.1 ± 0.03	-0.3 ± 0.04	360.0 ± 0.00	12.1 ± 0.03
	0.4% stevia	4.6± 0.01	9.1 ± 0.06	0.76	59.9 ± 0.04	12.2 ± 0.03	0.6 ± 0.03	360.1 ± 0.00	12.2 ± 0.03
	0.6% stevia	4.6± 0.01	9.5 ± 0.01	0.74	59.6 ± 0.09	12.7 ± 0.05	0.3 ± 0.07	360.0 ± 0.01	12.7 ± 0.05
Juice	Aronia	3.4± 0.00	17.1 ± 0.06	1.15	0.4 ± 0.01	0.1 ± 0.02	-0.3 ± 0.03	358.7 ± 0.07	0.3 ± 0.02
	Commercial elderberry	4.0± 0.01	20.7 ± 0.06	1.90	0.3 ± 0.00	-0.1 ± 0.01	-0.8 ± 0.01	361.4 ± 0.01	0.8 ± 0.01
	Fresh elderberry	4.3± 0.01	12.5 ± 0.00	0.55	2.40 ± 0.12	2.7 ± 0.22	3.5 ± 0.22	360.9 ± 0.05	4.4 ± 0.24

Note: Data are shown as means ± standard deviation except for %TA.

Since hue values were close to 0°/360° on hue angle scale, hue angle values were transformed by adding 360° for comparison [308].

Chroma values indicate the saturation or the intensity of the color. High Chroma values of the aronia kefir products indicated the color was saturated. Based on the similar Chroma values among the aronia kefir products, there was no difference in the color intensity. Hue angle values close to 0° (360°) indicated reddish color. The hue angle values of aronia kefir indicate that the products displayed a reddish color. High L* values of the aronia kefir show the products had a bright color. Positive a* values and negative b* values of the products indicate the products presented a bluish-red color. Anthocyanins present a red to a blue color when the environment changes from acidic to alkaline. The bluish-red color was expected in the products due to the acidic environment of kefir.

2.4.1.3 Aronia Kefir Phytochemical Analyses

The results of the phytochemical analyses of aronia kefir samples are shown in Table 2.3. Aronia kefir products had high TMA contents (16.57-17.22 mg C3G/100 g kefir). A typical serving size of kefir is 8 oz. One serving of the aronia kefir product would provide more than 39 mg TMA. Average TMA intake in the United States is 12.5 mg/day/person [201]. Our products provided three times more than the average intake for TMA. An epidemiologic study shows that in a population with an intake of 22.3-24.3 mg/day of TMA there was a lower incidence of type 2 diabetes compared to a population with only 2.0-2.3 mg/day [3]. In addition, Jennings *et al.* indicated that a TMA intake of 39.9 mg/day was associated with lower inflammation levels and improved insulin resistance compared to a TMA consumption of only 3.54 mg/day [317]. According to Seymour *et al.*, consumption of cherries containing 25.83 mg TMA increased the plasma antioxidant capacity for 12 hours in healthy humans [149]. This evidence confirms that

the consumption of aronia kefir could increase anthocyanin intake in a normal diet and contribute to decreasing in inflammation and preventing type 2 diabetes.

Table 2.3 Phytochemical evaluation results of juices, aronia kefir, elderberry kefir made with commercial juice and elderberry kefir made with fresh juice

Product		Anthocyanin Content (mg C3G/100 g sample)	Total Phenolic Compounds (mg GAE/100 g sample)	DPPH IC ₅₀ (mg sample/mL)
Aronia kefir	Sucrose	17.22 ± 0.17	43.04 ± 1.05	28.84 ± 0.26
	Stevia	16.57 ± 0.44	40.32 ± 1.02	27.59 ± 0.26
	Monk fruit	16.94 ± 0.38	40.37 ± 0.51	27.84 ± 0.26
Elderberry kefir (commercial juice)	4.3% sucrose	1.02 ± 0.22	19.61 ± 0.25	59.19 ± 0.37
	5.7% sucrose	0.95 ± 0.01	18.76 ± 0.19	65.52 ± 0.13
	0.4% stevia	1.13 ± 0.02	19.86 ± 0.32	60.36 ± 0.56
	0.6% stevia	1.06 ± 0.02	20.13 ± 0.58	61.65 ± 0.76
Elderberry kefir (fresh juice)	4.3% sucrose	18.67 ± 0.08	42.31 ± 0.96	20.86 ± 0.43
	5.7% sucrose	20.10 ± 0.14	43.66 ± 1.90	20.36 ± 0.86
	0.4% stevia	17.05 ± 0.06	39.00 ± 0.46	24.11 ± 0.67
	0.6% stevia	18.90 ± 0.07	43.50 ± 0.82	20.68 ± 0.55
Juice	Aronia	275.64 ± 3.18	604.49 ± 14.90	1.54 ± 0.04
	Commercial elderberry	25.59 ± 1.58	337.28 ± 4.75	4.39 ± 0.03
	Fresh elderberry	416.92 ± 3.18	369.47.59 ± 1.38	2.14 ± 0.01

Note: Data are shown as means ± standard deviation, n=3.

TP content in aronia products were 40.32 – 43.04 mg GAE/100 g kefir. An 8 oz serving provides more than 95 mg GAE of phenolics to the consumers. This equals about one-fifth of the average daily consumption of TP (450 mg GAE/person) in the American diet [318]. Dall’Asta *et al.* proved that dietary polyphenols could stimulate insulin secretion and protect β-cells from

the damage induced by oxidative stress [106]. Aronia kefir products could add to the overall consumption of total phenolics in the diet and contribute to the prevention of diabetes. DPPH IC₅₀ values of aronia kefir products were between 27.59 to 28.84 mg kefir/mL. These results showed that the products had the ability to sequester free radicals. The antioxidant capacity of aronia kefir products is associated with the amount of TMA and TP. Kefir is a live culture and the environment is dynamic. TMA and TP are being actively metabolized which helps explain the low recovery rates. Recovery rates of TMA and TP were about 45% and 55% respectively. TMA ingested from fruit and vegetables are usually conjugated and have low bioavailability [319]. Using a food matrix like kefir that contains live cultures may enhance the bioaccessibility of the aronia bioactive compounds by increasing TMA due to the de-conjugation of the anthocyanins.

2.4.2 Elderberry Kefir Evaluations (Commercial Juice)

2.4.2.1 Elderberry Kefir Sensory Test

Four elderberry kefir products sweetened with either sucrose or stevia were evaluated in this test. In the previous sensory test of aronia kefir, monk fruit extract was not well accepted and it was eliminated from the test of elderberry kefir. The elderberry kefir products were sweetened to two levels to test the impact of sweetness. Commercial products, such as blueberry flavored Lifeway® kefir, usually have about 20% sugars including 8 g added sugars. The °Brix values of the products in this study without added sweetener were approximately 9% (data not shown). Thus 4.3% or 5.7% sucrose was used to sweeten elderberry kefir to keep the total °Brix at least 5% lower than commercial products. 0.4% and 0.6% stevia extracts were used to create equal sweetness to the sucrose products respectively. Demographic results

showed that participants in this test were balanced for gender (55 female and 45 male) and the major age range was 18-34 years old. All participants in this test responded positively that purchasing health-beneficial food was important.

The consumer acceptability test (Figure 2.2) demonstrated that the products were accepted equally for color. The best acceptability in sweetness was observed in elderberry kefir sweetened with higher sucrose content (5.7%). Significantly lower ratings were observed in the two products sweetened with stevia extract. This is potentially driven by the unpleasant aftertaste of stevia extract based on the consumer comments. Sweeter products (5.7% sucrose and 0.6% stevia) were accepted better compared to less sweet products (4.3% sucrose and 0.4% stevia), but there was no significant difference between two stevia-sweetened products in this study. A similar trend was observed by Johansen *et al.* [320]. Their study indicated that yogurt sweetened with 13% sucrose significantly increased consumer preference compared to a product sweetened with 9% sucrose. Flavor was rated best in elderberry kefir sweetened with higher sucrose content (5.7%) which was not significantly different from elderberry kefir sweetened with 4.3% sucrose. The acid in kefir matrix, added sweetener and elderberry juice contributed to the complex flavor of the elderberry products. Fermented dairy products have a distinctive sour taste which may be a negative influencing factor on the consumers' acceptability [321]. The addition of sucrose to the products resulted in an increased sweet to sour ratio ($^{\circ}\text{Brix:TA}$) and may present a better-balanced flavor. The two products sweetened with stevia extract were less-accepted for flavor. The reason may be the unpleasant aftertaste of stevia extract. The best texture was received in the elderberry product with 5.7% sucrose. Sucrose contributes to a more viscous texture which was observed in this product.

Effervescence in dairy products induced by fermentation may be unfamiliar to consumers and might be an influencing factor. Best overall acceptability (6.6) was rated in elderberry kefir sweetened with high sucrose content (5.7%), and the product made with low stevia content (0.4%) received the lowest rating (5.8). Similar to what was observed in the previous aronia kefir consumer testing, participants' unfamiliarity with either kefir or elderberry may undermine the overall acceptability of the products. In this test, 40% of the consumers had never consumed kefir and 58% of the participants were unfamiliar or had never heard of elderberry. Elderberry kefir products were better accepted by previous kefir consumers compared to the kefir-naive group (data not shown).

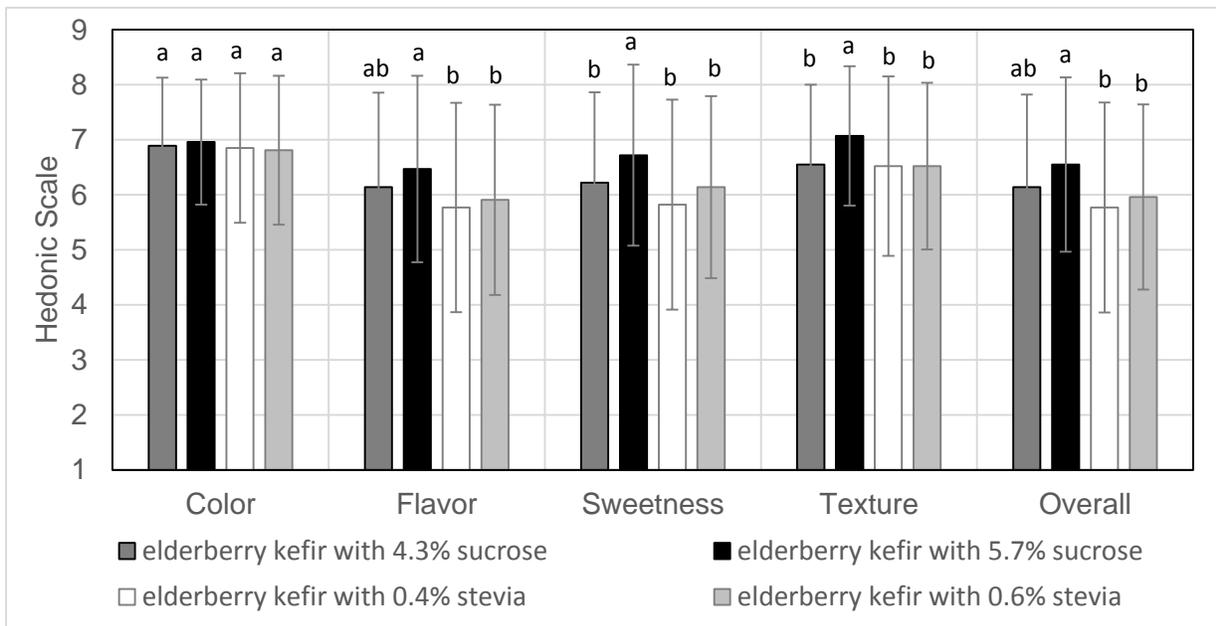


Figure 2.2 Consumer acceptability of elderberry kefir made with different sweeteners

Note: Data are shown as means \pm standard deviation. $n=100$. Different letters indicate significant differences among means within each attribute, $p<0.05$.

2.4.2.2 Elderberry Kefir Quality Parameters (Commercial Juice)

pH, TA, °Brix and color results of elderberry kefir made with commercial juice are shown in Table 2.2. The pH values demonstrated that the elderberry kefir products were acidic, which is necessary for the stability of bioactive compounds [305]. TA of products sweetened with 5.7% sucrose was the lowest while stevia-sweetened (0.4%) elderberry kefir was the highest. TA represents the sour taste. The sour taste in elderberry kefir was associated with the lactic acid generated by fermentation and the organic acids from the elderberry juice. There was no significant correlation between TA and pH ($r = 0.49$, $p = 0.25$). The highest °Brix value was observed in 5.7% sucrose-sweetened products as expected. Product sweetened with 4.3% sucrose was second highest. Stevia products both had low °Brix values. Color was analyzed for elderberry kefir products. All elderberry kefir products had similar color intensity based on the Chroma values. Hue angle values of all elderberry products were close to 0 (360) indicating that the products presented as a reddish color. High L^* values indicated that the products had a light color. a^* and b^* values of all products were positive. This indicated that elderberry kefir products present light red color. Since anthocyanins present a red color in an acidic environment, this color was reasonable.

2.4.2.3 Elderberry Kefir Phytochemical Analyses (Commercial Juice)

Phytochemical results of elderberry kefir made with commercial juice are shown in Table 2.3. Kefir products made with commercial elderberry juice contained a low amount of TMA (0.95 – 1.13 mg C3G/100 g kefir) and a moderate amount of TP (18.76 – 20.13 mg GAE/100 g kefir). The TMA and TP contents of commercial elderberry juice were 25.59 mg C3G/100 g and 337.28 mg GAE/100 g respectively. These amounts were much lower than the

reported values in the literature [322]. The recovery rates of TMA and TP were about 40% and 60% respectively (data not shown). Literature suggests several factors may impact TMA content in elderberry or elderberry products. For instance, plant variety and growing conditions can alter anthocyanin content [322, 323]. Processing, such as thermal treatments or filtration, could result in anthocyanin loss [324-326]. The processing parameters and elderberry varieties of commercial juice (a shelf stable product) are unknown, an additional set of kefir made with fresh juice was made to better understand the difference between the commercial and fresh elderberry juice. The same product formulas were used. Phytochemical evaluation of the additional elderberry kefir products is discussed in the next section.

2.4.3 Elderberry Kefir Evaluation (Fresh Juice)

The results for the biochemical measurements of the fresh elderberry juice are shown in Table 2.2. °Brix and TA values of the fresh elderberry juice were lower than the commercial juice. Color parameter values of commercial and fresh elderberry juice are different. Based on the Chroma values, fresh elderberry juice had a more saturated color than commercial juice. Phytochemical properties of the fresh and the commercial elderberry juices were measured to better understand the difference (Table 2.3). The results demonstrated that the TMA content in fresh elderberry juice was 16 times higher than the commercial juice. Higher TP content was observed in fresh elderberry juice. Gonzalez-Molina *et al.* revealed that anthocyanin content in elderberry juice decreased more than 50% during 56 days storage at room temperature [327]. Elderberry juice should be utilized fresh to ensure the highest levels of anthocyanins in products like elderberry kefir.

A sensory test was not conducted on the products made with fresh juice due to insufficient fresh elderberries. pH, TA, °Brix and color results of elderberry kefir made with fresh juice are shown in Table 2.2. The elderberry kefir products made with fresh juice exhibited similar pH, TA and °Brix values. No correlation was found between pH and TA ($r = 0.22$, $p = 0.61$). Based on the Chroma values, fresh-elderberry-juice-added kefir product with 0.6% stevia had a more saturated color.

Phytochemical analyses results of elderberry kefir made with fresh juice are shown in Table 2.3. TMA content of kefir made with fresh elderberry juice was 14 times higher than that in the product made with commercial juice. TP content in fresh-juice elderberry kefir was two times higher compared to the products made with commercial juice. Smaller IC_{50} values in the products made with fresh elderberry juice were observed. This indicates that kefir made with fresh elderberry juice exhibits stronger antioxidant capacity than the commercial-elderberry-juice kefir. The higher TMA and TP contents in fresh-elderberry-juice kefir due to the larger amount of TMA and TP in the fresh juice. One serving (8 oz) of the elderberry kefir products made with fresh juice contains more than 40 mg TMA and 93 mg TP. Elderberry kefir products made with fresh juice could contribute to the enhancement of consumers' dietary intake for both TMA and TP. Increased consumption of TMA and TP may contribute to a decrease in the chronic inflammation and in the risk of type 2 diabetes [3, 106]. Therefore, the freshness of juice and a shorter shelf life are important to maximize the delivery of bioactive compounds.

The recovery rate of TMA in products made with fresh elderberry juice was around 45%. For TP, the recovery rate exceeded 100%. Similarly, increased total phenolics were observed in myrtle berry homogenate after fermentation with *Lactobacillus plantarum* C2 and the main increase was in phenolic acids [176]. This study suggested that the increase was due to the esterase activity in the lactic bacteria. Esterase cleaves the ester bond which liberates the phenolic acids from their glycosylated form. During fermentation, the constituents of elderberry juice are metabolized by the living kefir culture. This enhancement may lead to a functional food with an increased amount of bioavailable phenolic compounds.

2.5 Conclusion

Kefir products made with aronia or elderberry were evaluated for their sensory attributes, quality parameters and phytochemical properties. Both sensory tests indicated that consumers preferred sucrose over non-nutritive sweeteners. Results of phytochemical analyses revealed that the freshness of juice was critical for maximum bioactive compounds in the products. The fermentation process may contribute to the liberation of phenolic compounds in part by esterase activity and may be important to enhance the bioavailability of bioactive compounds. Further research is needed to better understand the impact of fermentation on bioavailability and liberating the phenolic compounds to potentially increase their absorption. Developing value-added functional food products could be a good way to utilize aronia or elderberries. Kefir products with berries may increase dietary intake of anthocyanins and total phenolics, which may contribute to the prevention of type 2 diabetes and other inflammatory chronic diseases.

CHAPTER 3

FERMENTATION ALTERS THE BIOACCESSIBLE PHENOLIC COMPOUNDS AND INCREASES THE ALPHA-GLUCOSIDASE INHIBITORY EFFECTS OF ARONIA JUICE IN A DAIRY MATRIX FOLLOWING IN-VITRO DIGESTION

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3.1 Chapter Abstract

The prevalence of diabetes reached 415 million worldwide in 2015. Polyphenol-rich food intake can benefit the glycemic control for individuals with diabetes. Fermentation may increase the bioavailability of polyphenols, which is generally low. Aronia (*Aronia melanocarpa*) is a polyphenol-rich berry that is native to North America. Proanthocyanins and anthocyanins are the major phenolic compounds in aronia. In this study, aronia kefir was made by fermenting cow's milk with added aronia juice. The changes in bioaccessible polyphenols of aronia kefir during digestion were assessed using an *in-vitro* model. The impact of fermentation on the potential bioactivity of aronia polyphenols was evaluated. Results showed that the bioaccessible polyphenols in aronia kefir were elevated during digestion and the antioxidant capacity increased (IC₅₀ of DPPH scavenging decreased from 24.07 mg kefir/mL to 8.97 mg kefir/mL). Digested aronia kefir had stronger inhibitory activity on α -glucosidase (IC₅₀: 152.53 mg kefir/mL) compared to the non-fermented control (IC₅₀: 484.93 mg kefir/mL). Both aronia kefir and the non-fermented control had weak inhibition on pancreatic α -amylase. Specific inhibitors of α -glucosidase, such as aronia polyphenols, have the potential to delay carbohydrate digestion and reduce the absorption of glucose without side effects. Utilizing

aronia kefir in the diet is a good strategy to help control blood glucose levels. Fermentation may be an effective method to increase the bioavailability of dietary polyphenols in other food. More studies about the effects of fermentation on polyphenol-rich food are needed to optimize the potential health-promoting properties.

3.2 Introduction

Diabetes has been a global issue for the past few decades. In 2015, 30.3 million people (9.4%) in the United States and 415 million people in the world had diabetes [328]. Diabetes is associated with a decline in life expectancy and a reduction in life quality [32]. Tight blood glucose control is crucial for diabetic patients to delay the progression of complications, such as diabetic retinopathy, neuropathy and nephropathy [329]. The modulation of postprandial glucose absorption is one important method for management of hyperglycemia. Synthetic pharmaceutical agents are used to inhibit carbohydrate-hydrolyzing enzymes for delaying and reducing the absorption of glucose. Despite the effectiveness of the drugs, most of them have side effects that impact the gastrointestinal tract and hepatic system [329, 330]. Studies suggest that dietary polyphenols derived from plant-based food exhibit similar activity in inhibiting carbohydrate-hydrolyzing enzymes *in-vitro* and have the potential to aid in blood glucose control without side effects [329].

Dietary polyphenols are commonly ingested as a part of daily diet. They are found in high levels in plant-based foods, especially in berries. Generally, polyphenols have low bioavailability due to their instability in the small intestine and their large molecular size [331]. The food matrix may limit or improve the absorption of polyphenols and influence the

bioavailability [92]. Several methods have been suggested to increase the bioavailability of polyphenols, such as using encapsulation to increase the stability in the gastrointestinal tract [332, 333]. Additionally, some microorganisms are capable of breaking down the complex phenolic compounds and the metabolites may be more bioactive [177].

Aronia (*Aronia melanocarpa*) fruit is a berry native to eastern North America [334]. *Aronia* contains more total polyphenols (10-20 mg gallic acid equivalent/g fresh weight) than many other plant-based food, such as blueberry (1 to 4 mg gallic acid equivalent/g fresh weight) [202, 335, 336]. The astringent mouth-feel of *aronia* is caused by the high procyanidin content. *Aronia* is rarely consumed raw due to the astringency and the lack of sweetness. *Aronia* was traditionally used by Native Americans as medicine to treat the common cold [179]. The phenolic compounds are the major bioactives that are responsible for the therapeutic effects of *aronia*. In Russia, *aronia* has been used as a natural remedy to treat hypertension and atherosclerosis [179]. A recent study conducted by Loo *et al.* showed that consumption of *aronia* juice decreased low-grade inflammation in hypertensive patients [219]. Broncel *et al.* observed that consuming *aronia* extract reduced the oxidative stress in patients with metabolic syndrome [337].

Kefir is a fermented dairy product consisting of up to 30 species of microorganisms including lactic bacteria, yeast and sometimes acetic acid bacteria [338]. Functional properties of kefir are well documented, including anti-bacterial, anti-carcinogenic and anti-inflammatory effects [261]. Kefir is naturally lactose-free, making it a good calcium and protein source for lactose-intolerance individuals. In this study, kefir was selected as the matrix to incorporate

aronia polyphenols for the following reasons: 1) kefir is rich in protein, which can minimize the astringent mouth-feel of aronia [339] and may protect the polyphenols from degradation in the small intestine [340]; 2) the diverse microorganism community in kefir starter has the potential to metabolize phenolic compounds and increase the bioavailability [341]. Incorporating aronia into kefir may be a good way to optimize the potential health-promoting properties of aronia; 3) the acidic pH of kefir helps to protect the anthocyanins from degradation.

Digestion is a key process influencing the bioavailability of a dietary component [134] because factors such as pH and enzymes in the digestive tract can modify the components and alter their liberation behavior [333]. Understanding the changes of the aronia polyphenols during digestion is important to assess their potential bioactivity. The functional properties of aronia before ingestion is well studied, but knowledge about the changes in a fermented matrix and the potential anti-diabetic properties after digestion remain unknown. To our knowledge, this is the first study to examine the bioaccessibility of aronia polyphenols in a fermented dairy matrix. The objectives of this study were: 1) to investigate the effects of kefir-fermentation on the potential bioactivity of aronia polyphenols in a dairy matrix; 2) to evaluate the changes in the bioaccessibility and antioxidant capacity of aronia kefir in the digestive tract using an *in vitro* model.

3.3 Materials and Methods

3.3.1 Chemicals

Acetonitrile (HPLC grade), ammonium chloride, sodium phosphate dibasic, hydrochloric acid, methanol, potassium chloride, potassium thiocyanate, sodium hydroxide, sodium bicarbonate, soluble starch and urea were purchased from Fisher Scientific (Waltham, MA, U.S.). 2,2-diphenyl-1-picrylhydrazyl (DPPH), dinitrosalicylic acid, formic acid, ox-bile, p-nitrophenyl β -D-glucopyranoside (pnp-G), potassium sodium tartrate tetrahydrate, porcine α -amylase, rat intestinal powder and HPLC standards (quercetin, chlorogenic acid, neochlorogenic acid and cyanidin-3-galactoside) were obtained from Sigma-Aldrich (St. Louis, MO, U.S.). Bovine serum albumin, glucose, glucosamine hydrochloride, glucuronic acid, lipase, magnesium chloride, mucin, pepsin, sodium phosphate monobasic and uric acid were purchased from MP Biomedicals (Santa Ana, CA, U.S.). Calcium chloride was purchased from Ward's Science (Rochester, NY, U.S.), potassium dihydrogen phosphate was purchased from Alfa Aesar (Haverhill, MA, U.S.). All water used was obtained from a Millipore water system (EMD Millipore, Billerica, MA, U.S.).

3.3.2 Food Material

Aronia (*Aronia melanocarpa*, variety 'Viking') were harvest based on the apparent ripeness of uniform deep purple color from the University of Connecticut (Storrs, CT, U.S.) from the 2014 growing season. Berries were de-stemmed, washed and stored at -20 °C. Frozen berries were thermal-treated (100 °C, 5 minutes) and juiced with a domestic juicer (Hamilton Beach, Southern Pines, NC, U.S.). Juice was used immediately. Commercial kefir starter

(Yogourmet®, Lachute, QC, Canada) and 2% milk (Oakhurst®, Portland, ME, U.S.) were purchased from a local supermarket.

3.3.3 Sample Preparation

Aronia kefir was prepared by the following method: 2% milk was heated to 82 °C in an aluminum saucepan, and cooled to 26 °C in an ice bath. The cooled milk and the commercial starter (5 g per quart of milk) were combined in a glass bowl. The mixture was stirred for 5 minutes to ensure that the starter was fully dissolved. Freshly made aronia juice was added to the milk-starter matrix (15%, w/w) and mixed well. The mixture was covered with a breathable cloth and kept at room temperature (23 °C) to ferment overnight. After 24 hours of fermentation, the aronia kefir was homogenized with an immersion blender (Hamilton Beach®, Southern Pines, NC, U.S.). The homogenized aronia kefir was transferred into a sealed glass jar and stored at 4 °C for 24 hours before carrying out the *in-vitro* digestion. Three batches of aronia kefir were made and *in-vitro* digestion was performed individually.

Milk and aronia juice without the addition of kefir starter was used as a non-fermented control. Non-fermented control was made by mixing 2% milk with 15% (w/w) freshly made aronia juice in a glass jar and sealed with a lid. The mixture was kept at 4 °C for 24 hours (the fermentation time of making kefir) and then acidified to pH 4.5 (the pH of kefir). The acidified non-fermented control was stored in the refrigerator (4 °C) for another 24 hours before the *in-vitro* digestion process was carried out. Batches were made in triplicate.

3.3.4 *in-vitro* Digestion Procedure

The digestion process was simulated using a modified method from Oomen *et al.* to assess the changes of polyphenols in the digestive tract after ingestion [342]. For each digestion, three compartments in the digestive tract were simulated: mouth, stomach and small intestine. Artificial digestive juices (saliva, gastric juice, intestinal juice and bile) were prepared fresh before the *in-vitro* digestion was performed. The composition of digestive juices are listed in Table 3.1. pH values of the digestive juices were adjusted with concentrated HCl or 2M NaOH to the appropriate range. The digestion process was carried out as follows: the process was initiated by adding saliva to 27 mL aronia kefir (2:3, v/v). The mixture was stirred gently for 5 minutes at 37°C in an Isotemp™ water bath (Fisher's Scientific, Waltham, MA, U.S.). One-third of the oral-digested sample was removed and collected as the oral-digested fraction. Gastric digestion was initiated by adding in gastric juice to the remaining oral-digested sample (4:5, v/v) and incubating the mixture at 37 °C in a shaking water bath (Edvotek®, Washington D.C., U.S.) for 2 hours. This process consisted of two steps because the pH environment in the stomach is not stable at the beginning of gastric digestion due to food influx: for the first hour, one portion of gastric juice was added to the remaining oral digesta and the pH of the mixture was not adjusted; for the second hour, three portions of gastric juice were added to the mixture, the pH was adjusted to 2.0 with concentrated hydrochloric acid. At the end of gastric digestion, half of the gastric digesta was removed and collected as the gastric-digested fraction. Digestion in the small intestine was initiated by adding NaHCO₃ (1 M) to the remaining gastric-digested sample, resulting in a pH of 5.7. Intestinal juice and bile were added to the mixture (4:2:9, v/v). pH of the mixture was adjusted to 7.5 with 2 M sodium hydroxide and the mixture was incubated at

37 °C in the shaking water bath for two hours. All of the intestinal digesta were collected as the small intestine-digested fraction. Though the collected volume of the individual fractions was different, each fraction contained an equal amount of aronia kefir (9 mL). All incubations were conducted in the dark and the mixtures were sealed with parafilm to reduce oxygen exposure. The aronia kefir controls for each stage of digestion were processed by the same procedure in the absence of enzymes and bile. Non-fermented control was treated with the same *in-vitro* digestion procedure.

All collected samples were centrifuged at 16639×g (Eppendorf 5804R, Hamburg, Germany) for 10 minutes at 0 °C. The collected supernatant was acidified to pH 2.0 with concentrated hydrochloric acid to inactivate the digestive enzymes and to stabilize the phenolic compounds [331]. Methanol was added to the supernatant (2:1, v/v) and it was chilled at -20 °C to precipitate proteins. After 30 minutes, proteins in the mixture were removed by centrifugation at 0 °C for 30 minutes (16639×g, Eppendorf 5804R). Samples were filtered through a 0.20 µm syringe filter (Corning Inc., Corning, NY, U.S.) where an aliquot of the filtered supernatant was stored at -80 °C for phenolic compound quantification and antioxidant capacity evaluation. The remaining filtered supernatant was evaporated under a vacuum (Eppendorf Vacufuge plus, Hamburg, Germany) to remove methanol. The aqueous supernatant was purified with a C18 cartridge (Sigma-Aldrich, St. Louis, MO, U.S.) and washed with water to remove reducing sugars. The phenolic compounds in the supernatant were eluted with methanol. The purified sample was dried under a vacuum and resuspended in ultrapure water. The re-suspended samples were stored at -80 °C for enzyme inhibition activity analyses. Digestion was carried out in triplicate.

Table 3.1 The composition of digestive juices

Artificial saliva	Gastric juice	Intestinal juice	Bile
Inorganic compounds			
10 mL KCl 89.6 g/L	15.7 ml NaCl 175.3g/L	40 ml NaCl 175.3 g/L	30 mL NaCl 175.3 g/L
10 mL KSCN 20 g/L	18 mL CaCl ₂ ·2H ₂ O 22.2 g/L	40 mL NaHCO ₃ 84.7 g/L	68.3 mL NaHCO ₃ 84.7 g/L
10 mL NaH ₂ PO ₄ 88.8 g/L	9.2 mL KCl 89.6 g/L	10 mL KH ₂ PO ₄ 8.0 g/L	4.2 mL KCl 89.6 g/L
10 mL Na ₂ PO ₄ 57.0 g/L	3 mL NaH ₂ PO ₄ 88.8 g/L	6.3 mL KCl 89.6 g/L	200 µl HCL 37% g/g
1.7 mL NaCl 175.3 g/L	10 mL NH ₄ Cl 30.6 g/L	10 mL MgCl ₂ 5 g/L	
1.8 mL NaOH 40.0 g/L	8.3 mL HCl 37% g/g	180 µl HCl 37% g/g	
Organic compounds			
8 mL urea 25.0 g/L	10 mL glucose 65.0 g/L 10 mL glucuronic acid 2.0 g/L 3.4 mL urea 25.0 g/L 10 mL glucosamine hydrochloride 33.0 g/L	4 mL urea 25.0 g/L	10 mL urea 25.0 g/L
Others			
145 mg α-amylase	1.0 g BSA	9 mL CaCl ₂ ·2H ₂ O 22.2 g/L	10 mL CaCl ₂ ·2H ₂ O 22.2 g/L
15 mg uric acid	1.0 g pepsin	1.0 g BSA	1.8 g BSA
50 mg mucin	3.0 g musin	3.0 g pancreatin 0.5 g lipase	6.0 g bile
pH			
6.5 ± 0.2	1.07 ± 0.07	7.8 ± 0.2	8.0 ± 0.2

Note: The organic and inorganic solutions were adjusted into 500mL with distilled water separately. Other constituents were added to the mixture of organic and inorganic solutions and the pH was adjusted to the appropriate intervals with 2 M NaOH or concentrated HCl

3.3.5 UPLC Analysis of Phenolic Compounds

The profile of polyphenols would be altered during digestion, monitoring these changes is important to understand the possible metabolism of polyphenols in the digestive tract and the impacts of digestion on the potential bioactivity of polyphenols. In this study, the quantification of the anthocyanins and phenolic acids (chlorogenic acid and neo-chlorogenic acid) in the collected digesta was performed on an Ultra Performance Liquid Chromatography (UPLC) (Agilent Technologies 1290 Infinity, Santa Clara, CA, U.S.) with a Photodiode Array (PDA) detector. The method used was modified from Teleszko *et al.* [343]. Separation was carried out using a C18 column (3 μ m, 150 \times 4.6 mm, Thermo Scientific, Waltham, MA, U.S.) at 25 $^{\circ}$ C. Samples were injected at a flow rate of 1.3 mL/min. Phenolic compounds were eluted with a gradient mobile phase consisting 4.5% formic acid in water (phase A) and 4.5% formic acid in acetonitrile (phase B). The gradient was as follows: 0 min: 1% phase B; 4.5 min: 10% phase B; 7 min: 20% phase B; 10 min: 24% phase B; 14 min: 36% phase B; 15 min: 60% phase B; 16 min: 1% phase B. The post run time was 5 min. Samples were spiked with quercetin (25 μ g/mL) as an internal standard. An external calibration curve was drawn using cyanidin-3-galactoside (3.9, 7.8, 15.6, 31.2, 62.5, 125 and 250 μ g/mL, $r^2 = 0.9987$), chlorogenic acid (3.9, 7.8, 15.6, 31.2, 62.5, 125 and 250 μ g/mL, $r^2 = 0.9999$) and neo-chlorogenic acid (3.9, 7.8, 15.6, 31.2, 62.5, 125 and 250 μ g/mL, $r^2 = 0.9998$) standards. Anthocyanins were detected at 520 nm and expressed as cyanidin-3-galactoside equivalents. The individual anthocyanins were identified by the elution order reported by Jakobek *et al.* [202]. Chlorogenic acid and neo-chlorogenic acid were detected at 320 nm. Peak areas were used for quantification and the results were expressed as

mg polyphenols per part (one part contains 9 mL aronia kefir). Measurements were conducted in triplicate.

3.3.6 DPPH Free Radical Scavenging Assay

Antioxidant capacity of polyphenols is a crucial parameter to evaluate their potential health benefits. In the presented study, the antioxidant capacity of each digested fraction was evaluated by 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity. The method was slightly modified from Duymus *et al.* [312]. Briefly, equal amounts of 0.3 mM DPPH solution (150 μ L) and the diluted samples (150 μ L) were loaded to a 96-well plate. The mixture was incubated in the dark at room temperature for 30 minutes and the absorbance was read at 515 nm with a Biotek plate reader (ELx800, Winooski, VT, U.S.). A mixture of DPPH solution and water was used as the negative control for this assay. Scavenging percentage was calculated with the following formula:

$$\% \text{Scavenging} = \frac{\text{Abs}_{\text{Control}} - \text{Abs}_{\text{Sample}}}{\text{Abs}_{\text{Control}}} \times 100$$

Scavenging activity of each fraction was measured at five different concentrations to calculate IC₅₀ values, which is the concentration of the sample to scavenge 50% of the DPPH free radicals. Measurements were conducted in triplicate.

3.3.7 Rat Intestinal α -glucosidase Inhibitory Activities

Alpha-glucosidase is a vital carbohydrate-hydrolyzing enzyme catalyzes the breakdown of disaccharides and oligosaccharides to release glucose in the small intestine [344]. In this study, α -glucosidase inhibitory activity was evaluated using a method reported by Oki *et al.*

with modification [345]. Alpha-glucosidase was extracted from rat intestine powder by using 0.1 M sodium phosphate buffer at pH 6.9 (1:30, w/v) in an ice bath with sonication. Sonication was performed 12 times (30 seconds for each round) and the mixture was vortexed after each sonication. The mixture was centrifuged at 0 °C for 10 minutes at 16639×g. The supernatant was filtered through a 0.45 µm syringe filter (Phenomenex, Torrance, CA, U.S.) and kept on ice until use in the assay. Samples (50 µL) and α-glucosidase extract (100 µL) were mixed and incubated at 37 °C for 10 minutes in the dark. Water was used to prepare controls. The reaction was initiated by the addition of 50 µL 4-nitrophenyl α-glucopyranoside (pnp-G, 5 mM). The mixture was incubated at 37 °C for 30 minutes in the dark and read at 405 nm. Phosphate buffer (0.1 M) was used to prepare sample blank to correct for the background color. The inhibitory activity of the sample on intestinal α-glucosidase was calculated as follows:

$$\%inhibition = \frac{Abs_{control} - (Abs_{sample} - Abs_{sample\ blank})}{Abs_{control}} \times 100$$

Five dilutions of each sample were measured to calculate IC₅₀ values. Measurements were carried out in triplicate.

3.3.8 Porcine Pancreatic α-amylase Inhibitory Activities

Pancreatic α-amylase is a key enzyme that starts the digestion of complex carbohydrates by hydrolyzing the glycosidic linkages in the small intestine. Inhibitory effects of samples on porcine pancreatic α-amylase were conducted with the method reported by Nampoothiri *et al.* with modification [346]. Briefly, sodium phosphate buffer (0.02 M, pH 6.9) with 0.006 M sodium chloride was used to dissolve α-amylase and the starch. 100 µL sample and 100 µL α-amylase solution (100 unit/mL) were mixed and incubated at 25 °C for 10 min.

The reaction was initiated by adding 100 μL starch solution (1 g/mL). The mixture was incubated at 25 $^{\circ}\text{C}$ for an additional 10 min. The reaction was stopped by adding 200 μL dinitrosalicylic acid reagent and incubating the mixture in a water bath for 5 minutes at 100 $^{\circ}\text{C}$. The dinitrosalicylic acid reagent was made of 1 g/mL dinitrosalicylic acid in water containing 2% NaOH (2 M, v/v) and 30% (w/v) potassium sodium tartrate tetrahydrate. When the mixture temperature reached the room temperature (23 $^{\circ}\text{C}$), 50 μL of the mixture was loaded to a 96-well microplate, diluted with 200 μL water and read at 540 nm. Sample blank was prepared using sodium phosphate buffer to correct for the background color. The control was prepared with sodium phosphate buffer instead of samples. The α -amylase inhibitory activity of the samples was calculated as follows:

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

The inhibitory activities of individual samples were tested at five different dilutions. IC_{20} values, the concentration of the sample required to inhibit 20% porcine pancreatic α -amylase, were calculated. The measurements were conducted in triplicate.

3.3.9 Statistical Analysis

Data are shown as means \pm standard deviations. Statistical analyses were conducted using SAS Studio (Cary, NC, USA). Analysis of Variance and Tukey's HSD post hoc were carried out to evaluate the differences. A significance level was set at $\alpha = 0.05$.

3.4 Results

3.4.1 Quantification of Bioaccessible Phenolic Compounds

The contents of the bioaccessible phenolic compounds in aronia kefir and non-fermented control during *in-vitro* gastrointestinal digestion are presented in Table 3.2. Four major monomeric anthocyanins (cyanidin-3-galactoside, cyanidin-3-glucoside, cyanidin-3-arabinoside and cyanidin-3-xyloside) and two dominant phenolic acids (chlorogenic acid and neo-chlorogenic acid) were identified and quantified via UPLC analyses. Caffeic acid, a metabolite of chlorogenic acid, was not detected. Most phenolic compounds in aronia kefir increased after gastric digestion, with the exception of cyanidin-3-glucoside which showed no change compared to the oral-digested sample. During intestinal digestion, the chlorogenic acid content was increased (from 1.04 ± 0.02 mg/part to 1.29 ± 0.09 mg/part) and the other identified anthocyanins and phenolic acids remained the same.

The total anthocyanin content is shown in Figure 3.1. After the entire gastrointestinal digestion, total bioaccessible anthocyanins were increased by 96.9% compared to the undigested aronia kefir. Total anthocyanins in intestinal-digested kefir was 5.09 ± 0.40 mg/part (1 part = 9 mL aronia kefir). One serving of commercial kefir is 240 mL thus one serving aronia kefir would provide 135.73 mg bioaccessible anthocyanins.

Table 3.2 Quantification of individual phenolic compounds (mg/part)

Fractions		Cyanidin-3-galactoside	Cyanidin3-glucoside	Cyanidin-3-arabinoside	Cyanidin-3-xyloside	Chlorogenic acid	Neo-chlorogenic acid	
Aronia Kefir	Undigested	1.87 ±0.11 d	0.09 ±0.01 cd	0.57 ±0.03 e	0.05 ±0.01 e	0.61 ±0.03 g	0.49 ±0.02 e	
	Oral	C*	1.87 ±0.15 d	0.07 ±0.02 d	0.55 ±0.07 e	0.05 ±0.01 e	0.76 ±0.02 efg	0.59 ±0.02 cde
		D*	1.96 ±0.13 d	0.07 ±0.02 d	0.58 ±0.06 e	0.06 ±0.01 e	0.78 ±0.02 efg	0.60 ±0.02 cde
	Gastric	C*	2.60 ±0.34 cd	0.10 ±0.03 bcd	0.85 ±0.14 de	0.08 ±0.01 de	0.92 ±0.04 cde	0.69 ±0.02 abc
		D*	3.14 ±0.42 c	0.12 ±0.04 abcd	1.13 ±0.19 cd	0.11 ±0.02 cd	1.04 ±0.02 bc	0.75 ±0.02 ab
	Intestinal	C*	3.10 ±0.33 c	0.11 ±0.02 abcd	1.08 ±0.12 cd	0.10 ±0.01 cd	1.17 ±0.11 ab	0.74 ±0.03 ab
		D*	3.50 ±0.29 bc	0.13 ±0.01 abcd	1.33 ±0.10 bc	0.13 ±0.01 bc	1.29 ±0.09 a	0.70 ±0.09 abc
	Non-fermented control	Undigested	2.60 ±0.13 cd	0.13 ±0.01 abcd	0.87 ±0.06 de	0.08 ±0.01 de	0.70 ±0.04 fg	0.54 ±0.03 de
Oral		C*	3.10 ±0.45 c	0.12 ±0.03 abcd	1.06 ±0.21 cd	0.11 ±0.02 cd	0.84 ±0.06 def	0.65 ±0.04 bcd
		D*	3.29 ±0.16 bc	0.13 ±0.02 abcd	1.15 ±0.07 cd	0.11 ±0.01 cd	0.85 ±0.05 def	0.64 ±0.04 bcd
Gastric		C*	4.13 ±0.32 ab	0.16 ±0.04 abc	1.56 ±0.17 ab	0.16 ±0.02 ab	0.97 ±0.02 cd	0.72 ±0.02 ab
		D*	4.59 ±0.41 a	0.18 ±0.04 a	1.79 ±0.21 a	0.18 ±0.02 a	1.08 ±0.05 bc	0.77 ±0.03 a
Intestinal		C*	4.67 ±0.29 a	0.17 ±0.02 ab	1.85 ±0.11 a	0.19 ±0.01 a	1.19 ±0.07 ab	0.78 ±0.01 a
		D*	4.69 ±0.36 a	0.17 ±0.03 ab	1.88 ±0.19 a	0.19 ±0.02 a	1.31 ±0.06 a	0.72 ±0.07 ab

Note: data are shown as means ± standard deviations (n=3), values in the same column with the same letter are not significantly different at p < 0.05; *: C stands for control and D stands for digesta; one part was equivalent to 9 mL sample; anthocyanins were expressed as mg cyanidin-3-galactoside/part; chlorogenic acid was expressed as mg chlorogenic/part; neo-chlorogenic acid was expressed as mg neo-chlorogenic/part.

No differences were observed among phenolic compounds in the individual stages of digestion between aronia kefir digesta and aronia kefir controls (Table 3.2 and Figure 3.1). After intestinal digestion, the non-fermented control contained larger amount of cyanidin-3-

galactoside, cyanidin-3-arabinoside and cyanidin-3-xyloside (4.69 ± 0.36 mg/part, 1.88 ± 0.19 mg/part and 0.19 ± 0.02 mg/part) compared to aronia kefir (3.50 ± 0.29 mg/part, 1.33 ± 0.10 mg/part and 0.13 ± 0.01 mg/part respectively). Differences in the other identified phenolic compounds between non-fermented control and aronia kefir were not observed. The total bioaccessible anthocyanins in intestinal-digested non-fermented control was 88.5% higher than in the undigested non-fermented control. After digestion, the increase of anthocyanins was lower in non-fermented control compared to the increase in aronia kefir.

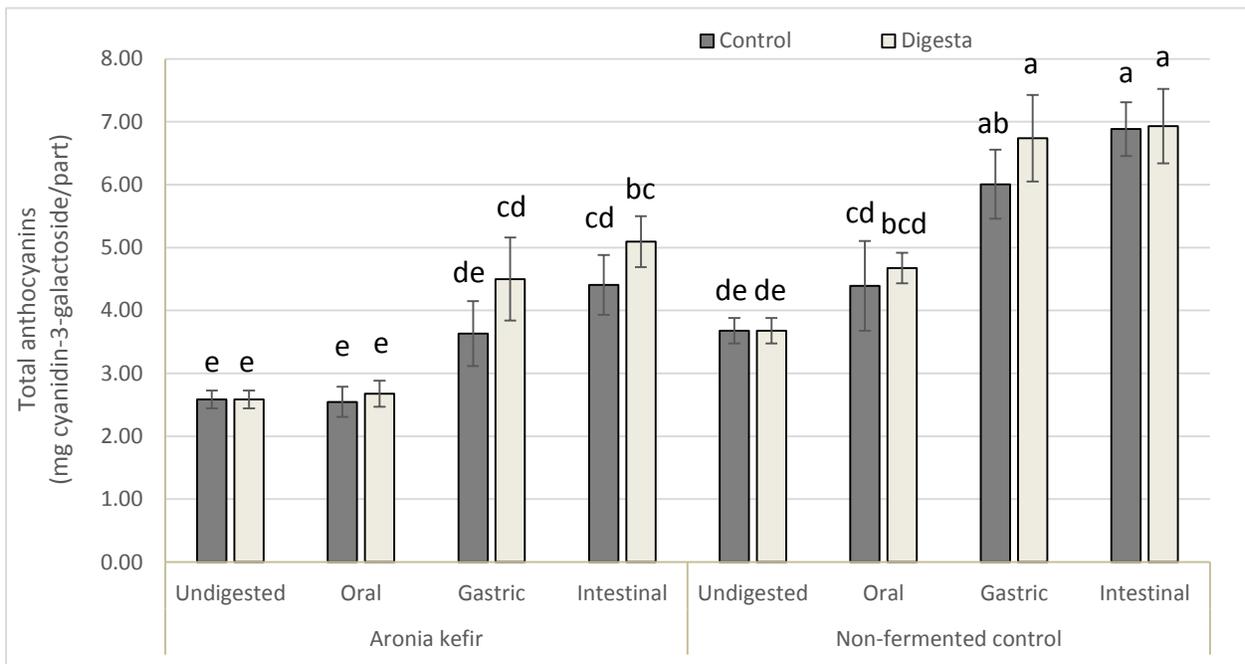


Figure 3.1 Quantification of total anthocyanins during *in-vitro* digestion

Note: Data are shown as means \pm standard deviations ($n=3$), one part was equivalent to 9 mL sample, bars with the same letter are not significantly different at $p < 0.05$.

3.4.2 Antioxidant Capacity

Antioxidant activity of aronia kefir and the non-fermented control was measured using the capacity to scavenge DPPH free radicals. IC₅₀ values were calculated and the results are shown in Figure 3.2. Aronia kefir exhibited antioxidant capacity during the entire gastrointestinal digestion. Antioxidant capacity of aronia kefir digesta was improved during gastric digestion (DPPH IC₅₀ values from 24.07 ± 0.78 mg/part to 12.01 ± 0.57 mg/part) and held consistent after intestinal digestion (DPPH IC₅₀: 8.97 ± 0.93 mg/part). Aronia kefir digesta exhibited similar antioxidant capacity compared to the corresponding aronia kefir controls at each digestive stage. Aronia kefir digesta and non-fermented control digesta exhibited similar antioxidant capacity after gastric- and intestinal-digestion. A strong correlation between IC₅₀ values of DPPH and total anthocyanins was observed ($r = -0.89$) as well as between IC₅₀ values and the sum of chlorogenic and neo-chlorogenic acid contents ($r = -0.90$).

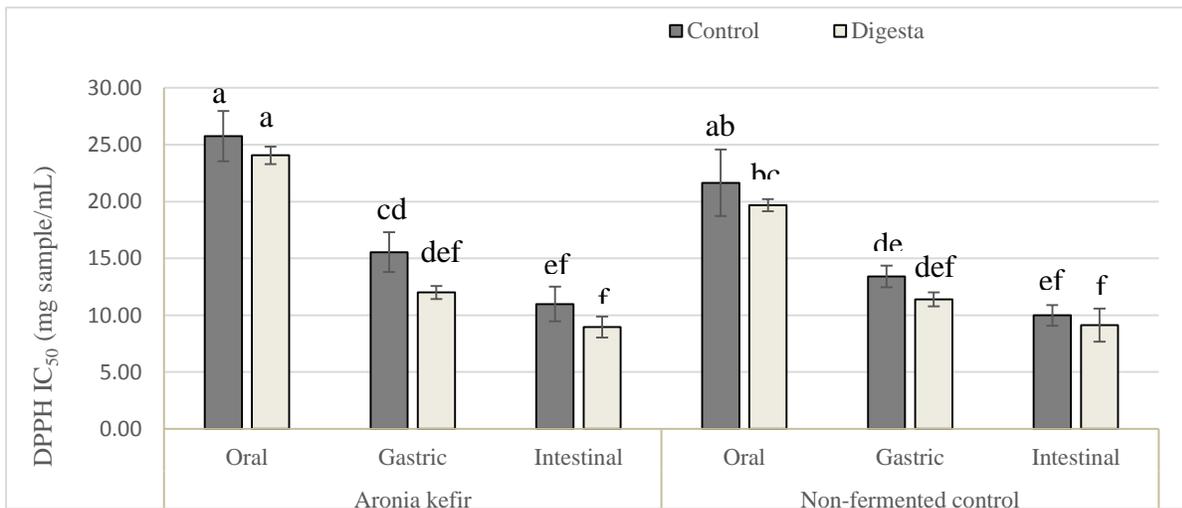


Figure 3.2 Antioxidant capacity of kefir during *in-vitro* digestion

Note: Data shown as means ± standard deviations (n=3), bars with the same letter are not significantly different at $p < 0.05$.

3.4.3 Inhibitory Activity of Carbohydrate-hydrolyzing Enzymes

The inhibitory effects of intestinal digested aronia kefir and non-fermented control on α -glucosidase and pancreatic α -amylase were tested. The results are shown in Figure 3.3 and Figure 3.4. Digested aronia kefir exhibited strong inhibitory activity toward α -glucosidase and weak inhibitory activity on α -amylase. Compared to the digested non-fermented control, digested aronia kefir had a stronger inhibitory effect on α -glucosidase. The IC_{50} values for α -glucosidase inhibition of aronia kefir and non-fermented controls were 152.53 ± 15.24 mg kefir/mL and 365.16 ± 48.84 mg non-fermented control/mL respectively. Digested aronia kefir as well as the digested non-fermented control exhibited similar inhibitory activity against pancreatic α -amylase. IC_{20} values of α -amylase for the aronia kefir and the non-fermented control were 146.52 ± 5.37 mg kefir/mL and 196.21 ± 5.50 mg non-fermented control/mL. Plain kefir was processed using the *in-vitro* digestion system with the same method as the samples. Inhibitory activity of plain kefir on enzymes was not observed.

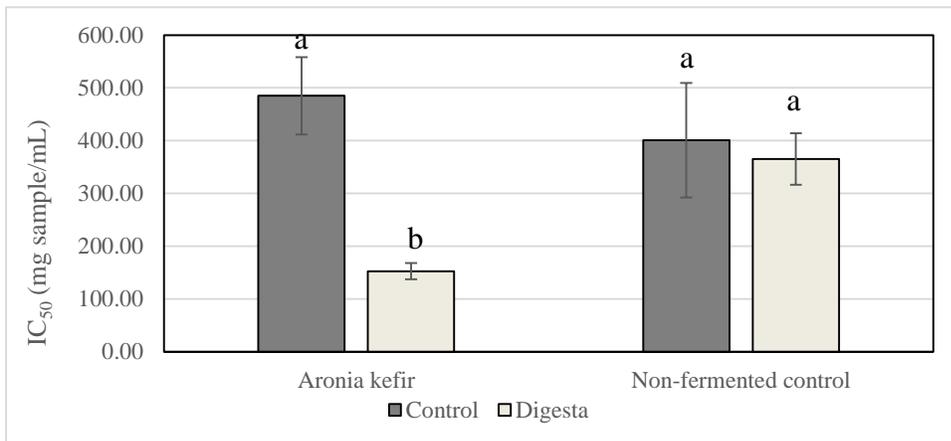


Figure 3.3 α -glucosidase inhibitory activity

Note: Data shown as means \pm standard deviations ($n=3$), bars with the same letter are not significantly different at $p < 0.05$.

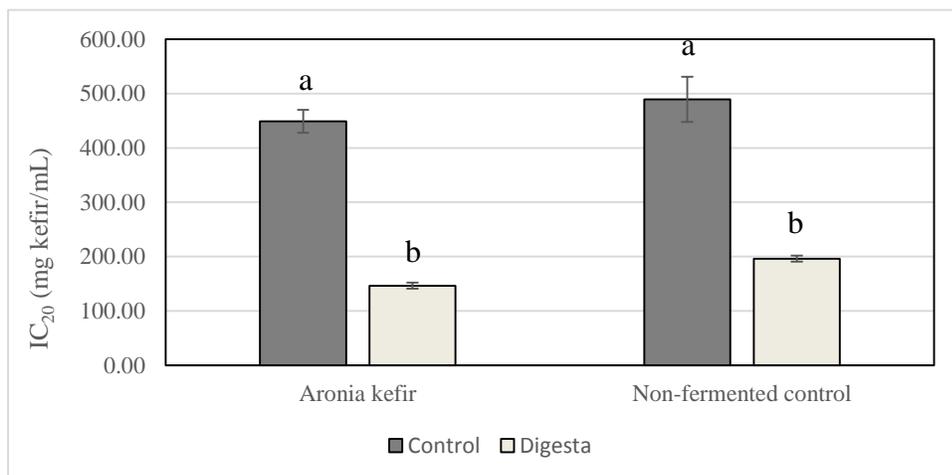


Figure 3.4 Pancreatic α -amylase inhibitory activity

Note: Data shown as means \pm standard deviations (n=3), bars with the same letter are not significantly different at $p < 0.05$.

3.5 Discussion

This study examined the bioaccessibility and the antioxidant capacity of phenolic compounds in aronia kefir during a simulated gastrointestinal digestion. The impacts of fermentation on aronia polyphenols and on their carbohydrate-hydrolyzing enzyme inhibitory activities were evaluated.

The *in-vitro* digestion model used in this study simulated three compartments of the digestive tract: mouth, stomach and small intestine. Digestive juices (saliva, gastric juice, duodenal juice and bile) used in this model contained not only corresponding enzymes but also other compounds that exist in human digestive juices, such as calcium chloride which may chelate phenolic compounds in the digestive tract and alter their bioaccessibility [347].

In this study, salivary α -amylase, which is the main digestive enzyme in the mouth, had negligible effects on the release of bioaccessible phenolic compounds in aronia kefir as expected, because the aronia kefir is a protein-rich beverage and the duration for the simulated oral digestion is short.

During gastric digestion, the acidic environment helps to stabilize the free anthocyanins and phenolic acids in aronia kefir. The low pH environment in the stomach contributes to the liberation of the phenolic compounds from the phenolic-protein complex and lead to the increase in bioaccessible anthocyanins and phenolic acids [143, 305, 333]. In addition, proanthocyanins, the oligomeric and/or polymeric flavan-3-ols, are the most abundant bioactive constituents in aronia. The depolymerization of proanthocyanins due to the acidic environment may contribute to the enhancement of the monomeric anthocyanin levels and potentially increase the bioavailability of aronia polyphenols [22, 135]. Bermudezsoto *et al.* reported that digestive enzymes did not affect the aronia polyphenol content in the absence of food matrix [331]. In this study, a similar trend was observed. Though the amount of bioaccessible polyphenols in the gastric-digested aronia kefir (4.50 ± 0.66 mg/part for total anthocyanins, 1.04 ± 0.02 mg/part for chlorogenic acid and 0.75 ± 0.02 mg/part for neo-chlorogenic acid) was slightly higher than that in the gastric control (3.63 ± 0.52 mg/part for total anthocyanins, 0.92 ± 0.04 mg/part for chlorogenic acid and 0.69 ± 0.02 mg/part for neo-chlorogenic acid), the difference was not significant.

The small intestine is the major absorption site for most phenolic compounds so the quantity of bioaccessible polyphenols is important [141]. Many studies demonstrated that phenolic compounds are labile in the small intestine due to the mild alkaline environment. Bermudezsoto *et al.* conducted a study demonstrating that more than 35% of anthocyanins and 20% phenolic acids were lost after *in-vitro* intestinal digestion of aronia juice [331]. Similar results were reported by Correa-Betanzo *et al.* where anthocyanins in blueberry decreased to 10% – 15% during *in-vitro* intestinal digestion [348]. Bouayed *et al.* reported a complete loss of anthocyanins but an increase in phenolic acids after *in-vitro* intestinal digestion of apples [349]. However, depending on the type of polyphenols and the food matrix, the changes of bioaccessible polyphenols in the small intestine may be different. In the present study, the bioaccessible chlorogenic acid in aronia kefir increased and the anthocyanins content remained the same during intestinal digestion. The increases in chlorogenic acid may be attributed to the degradation of anthocyanins in addition to the liberation from the kefir matrix. Similar results were observed in other studies that utilized a protein-rich food matrix to protect the polyphenols from degradation in the small intestine. A study conducted by Lamothe *et al.* showed that the stability of tea polyphenol in the small intestine was improved by dairy matrices (milk, yogurt and cheese) [340]. The protective effects of food matrices (dairy and egg) on the stability of grape anthocyanins during the intestinal digestion were observed by Pineda-Vadillo *et al.* [350]. Stanisavljevic *et al.* reported that after *in-vitro* digestion of aronia juice in a food matrix, bioaccessible anthocyanins and total phenolic compounds increased [351]. It is important to note that the referenced study only tested the anthocyanin and the total phenolic contents before and after the entire gastrointestinal digestion process (not at the

individual digestive stage). The changes of the soluble anthocyanins in the small intestine remains unknown. Digestive enzymes and bile did not contribute to the liberation of phenolic compounds in aronia kefir since the differences in anthocyanins, chlorogenic acid and neochlorogenic acid between the digested aronia kefir and aronia kefir controls were not significant.

The antioxidant capacity of polyphenols is associated with their health-promoting properties. The consumption of polyphenols may help to decrease oxidative stress, attenuate the production of pro-inflammatory biomarkers and lower the risk of chronic diseases, such as type 2 diabetes [352]. Foods that have strong antioxidant capacity before consumption may lose their antioxidant activity during the digestion process. This is caused by the structural alterations that occur due to the harsh conditions in the digestive tract and/or the interaction with other food ingredients. A loss of antioxidant capacity of polyphenol-rich food after *in-vitro* gastrointestinal digestion was documented in many studies and this loss was associated with the degradation of phenolic compounds [348, 353]. In this study, the antioxidant capacity of the intestinal-digested aronia kefir was higher than the oral-digested aronia kefir. The progressive release of phenolic compounds during digestion may contribute to the increase [350]. It is important for food to exhibit antioxidant capacity in the gut lumen, where dietary polyphenols could inhibit the proliferation of abnormal cells and slow the progression of cancer [331]. In addition, dietary polyphenols in the lumen may have protective effects on other food components during digestion, such as protecting unsaturated fatty acids from oxidation [350, 354]. The protective activity of polyphenols on unsaturated fatty acids may contribute to a healthier cardiovascular status.

Alpha-glucosidase and pancreatic α -amylase are carbohydrate-hydrolyzing enzymes that play a vital role in catalyzing the breakdown of complex carbohydrates. Inhibition of these enzymes can delay the absorption of carbohydrates and aid in the management of hyperglycemia. In the present study, only intestinal-digested samples were tested for enzyme inhibitory activity because pancreatic α -amylase and α -glucosidase exist in the small intestine. It is important to note that yeast α -glucosidase was frequently used in other research, but this study used α -glucosidase extracted from rat small intestinal powder because mammalian α -glucosidase is more relevant to human α -glucosidase [355]. This study demonstrated that polyphenols in aronia were the major compounds affecting the enzyme inhibitory activity because plain kefir treated in the same method did not show any inhibitory activity (data not shown). The inhibitory effects of dietary polyphenols on pancreatic α -amylase and α -glucosidase are well documented [356]. In this study, intestinal-digested aronia kefir exhibited strong inhibitory activity on α -glucosidase and minor inhibitory effect on pancreatic α -amylase. Strong inhibition of pancreatic α -amylase may lead to undigested complex carbohydrates in the large intestine and cause abdominal pain, flatulence, and/or diarrhea [357]. Therapeutic agents, such as acarbose, can cause gastrointestinal side effects because of their non-specific inhibitory effects on both pancreatic α -amylase and α -glucosidase. Due to this effect, the specific inhibitory activity of aronia kefir on α -glucosidase over pancreatic α -amylase might be desirable for hyperglycemia management [358, 359]. Incorporating aronia kefir into a normal diet may be a good strategy to control postprandial plasma glucose level without causing side effects.

Fermentation altered the composition of bioaccessible aronia polyphenols in kefir and changed their potential bioactivity. The impacts of the fermentative microorganisms on polyphenols from various studies suggest that the phenolic metabolites produced by microorganisms might be more bioavailable. This is due to the smaller size of the metabolites and thus they are better absorbed compared to the parent compounds [173, 177]. A study conducted by Curiel *et al.* observed that fermentation by lactic acid bacteria increased the antioxidant capacity of Myrtle berry homogenate [176]. In addition, Hunaefi *et al.* stated that 24 hours lactic acid fermentation decreased the total phenolic compounds in red cabbage sprouts but increased the antioxidant activity [360]. Zhao *et al.* also reported that fermentation by lactic acid bacteria decreased the flavan-3-ols content and increased phenolic acid derivatives in tea extract [341]. There was also evidence that the antioxidant activity was elevated [341]. These results demonstrate that fermentation may be a feasible method to enhance the antioxidant capacity of dietary polyphenols in different food matrices. However, in this study, there was no difference in the antioxidant capacity between aronia kefir and the non-fermented control after gastric- and intestinal-digestion.

In this study, digested aronia kefir had stronger inhibitory activity on α -glucosidase than the digested non-fermented control (IC_{50} values are 152.53 mg kefir/mL and 365.16 mg non-fermented control/mL, respectively) though digested non-fermented control had higher cyanidin-3-galactoside, cyanidin-3-arabinoside and cyanidin-3-xyloside. The stronger enzyme inhibitory effects of digested aronia kefir may due to the metabolites of polyphenols generated by the fermentation. Frediansyah *et al.* observed similar results where fermentation by lactic acid bacteria increased the inhibitory activity of black grape juice for α -amylase and α -

glucosidase [174]. Fermentation may be a good strategy to increase the bioavailability of polyphenols in other foods. In addition, a fermented dairy matrix may be a suitable carrier for dietary polyphenols due to their protective effects on the stability of phenolics in the small intestine. More research is needed to better utilize the potential activity of fermentation on improving the bioavailability of dietary polyphenols.

3.6 Conclusion

In this study, the stability and bioaccessibility of the polyphenols in aronia kefir were evaluated using an *in-vitro* gastrointestinal digestion model, where the impacts of fermentation on aronia polyphenols were evaluated. After digestion, the bioaccessible polyphenols in aronia kefir and its antioxidant capacity increased. The digested aronia kefir exhibited strong inhibitory activity toward α -glucosidase but weak inhibition of pancreatic α -amylase. Intestinal-digested aronia kefir contained less cyanidin-3-galactoside, cyanidin-3-arabinoside and cyanidin-3-xyloside compared to the intestinal-digested non-fermented control but exhibited similar antioxidant capacity. Fermentation enhanced the inhibitory activity of aronia polyphenols on α -glucosidase. In conclusion, consuming aronia kefir may aid in controlling blood glucose level without side effects. Fermentation may be a good strategy to enhance the bioavailability of dietary polyphenols. In order to better understand the positive impacts of fermentation on the bioavailability of dietary polyphenols, the identification of the metabolites in aronia kefir is necessary.

CHAPTER 4

OVERALL CONCLUSIONS AND FUTURE DIRECTIONS

4.1 Study Conclusions

Aronia and elderberry are underutilized fruits with great health-promoting properties. They are rarely consumed raw due to the astringent sensation caused by a large amount of phenolic compounds. In this research, berries were incorporated into a fermented dairy matrix, kefir, and sweetened with different natural sweeteners (sucrose, stevia extract and monk fruit extract) to mask the astringency. Studies were conducted to evaluate the consumer acceptability and health-promoting properties of these products. The key findings are summarized in table 4.1.

The first objective of this study was to develop new palatable kefir products using aronia or elderberries. The levels of sucrose used in the aronia and elderberry kefir were lower than most flavored commercial kefir products in the market, such as blueberry, mango and raspberry flavored Lifeway® kefirs. The reason for minimizing the amounts of added sucrose is to ensure that these products are attractive to health-conscious consumers. The final level of sucrose in aronia and elderberry kefirs was at least 5% lower than flavored commercial kefirs. In order to test how berry kefirs were received by the potential consumers, two separate sensory tests on either aronia or elderberry kefir products were conducted to evaluate the consumer acceptability. In the first sensory test, aronia kefir products were sweetened with sucrose, stevia extract or monk fruit extract to the same level of sweetness. Both the sucrose- and stevia-sweetened aronia kefirs were slightly liked by the consumers where the overall acceptability of the sucrose-sweetened products were higher (6.3). Monk fruit-sweetened

aronia kefir was not well accepted by the consumers. In the second sensory test, elderberry kefir was sweetened with either sucrose or stevia extract to two levels of sweetness. The highest overall acceptability was observed in elderberry kefir sweetened with a higher amount of sucrose (5.7%). All elderberry kefir samples were accepted by the consumers where all ratings were higher than 5. In summary, berry kefir, which were less sweetened than most commercial products, were accepted by consumers. Sucrose appeared to be the best accepted sweetener than monk fruit or stevia extract based on the consumers' rating. Aronia and elderberry kefir have the potential to be successful commercial products. Additionally, the berry kefir made with stevia and monk fruit extracts are suitable for pre-diabetic and diabetic individuals.

The second objective of this study was to evaluate the health-related characteristics of the aronia and elderberry kefir, including the total phenolic levels, monomeric anthocyanins content and antioxidant capacity. The results showed that all the berry kefir contained high levels of phenolic compounds and exhibited moderate antioxidant capacity. Compared to elderberry kefir made with commercial juice, a pasteurized shelf-stable product, elderberry kefir made with fresh juice had approximately twenty times more anthocyanins, and two times more total phenolics. One serving of aronia kefir or elderberry kefir made with fresh juice can provide three times more than the average intake for anthocyanins and contribute to approximately one-fifth of the average intake of phenolic compounds in the United States. The consumption of phenolic compounds may help to decrease the risk of T2DM and slow the progression of its complications. Currently, the available food products of aronia and elderberries are limited in the United States. More commercial available products using aronia or elderberry may help to increase the consumption of phenolic compounds among consumers.

In addition, the development of aronia and elderberry products may enhance consumers demand for these berries, which may encourage farmers to grow them and gain profits.

The third objective of this research was to evaluate the bioaccessibility of phenolic compounds in aronia kefir and their potential to assist blood glucose control. The levels of free phenolic compounds and their antioxidant capacity during digestion were assessed using an *in-vitro* model of simulated digestion. The inhibitory activity of digested aronia kefir on the carbohydrate-hydrolyzing enzymes was measured. After digestion, the bioaccessible phenolic compounds and antioxidant capacity of the aronia kefir increased. The digested aronia kefir exhibited a strong inhibitory activity toward α -glucosidase and weak inhibitory activity for pancreatic α -amylase. The inhibition of α -glucosidase and pancreatic α -amylase can slow the digestion of carbohydrates and thus reduce their absorption. However, strong inhibition of pancreatic α -amylase can lead to un-digested complex carbohydrates in the large intestine, affect the bacterial fermentation and then cause side effects, such as abdominal pain and flatulence. Thus, the consumption of aronia kefir may aid in blood glucose control without side effects due to its specific inhibition of α -glucosidase over pancreatic α -amylase. In addition, the impacts of fermentation on the potential bioactivity of aronia kefir were evaluated in this part. Compared to the non-fermented control, aronia kefir exhibited stronger inhibitory activity of α -glucosidase after digestion. Both the digested aronia kefir and the non-fermented control had weak inhibition on pancreatic α -amylase. This is the first study to investigate the impact of kefir fermentation on berry polyphenols and their health-promoting properties. Kefir fermentation may be a good strategy to improve the bioavailability of dietary polyphenols.

In summary, this research provides evidence that both aronia and elderberries have the potential to be used as food ingredients in commercial food products. Also, the results of sensory tests show a possibility for the industry to lower the sucrose content of their products without sabotaging the consumers' acceptability. A reduction in sucrose content may lead to healthier products which may be favored by health-conscious consumers. The berry-containing kefirs developed in these studies may be beneficial to pre-diabetic and diabetic individuals for their potential to help control blood glucose. Additionally, the berry-containing kefirs are a good source of protein and calcium for individuals with lactose intolerance due to the lactose-free properties of kefir.

Table 4.1 Key findings of these studies

Chapter No.	Samples	Evaluations	Key findings
2	Elderberry and aronia kefirs sweetened with sucrose, stevia or monk fruit extract	Consumer acceptability test	1. Kefirs that were less sweetened than commercial products were accepted by consumers; 2. Sucrose-sweetened kefirs were best accepted compared to the products sweetened with stevia or monk fruit extract.
		Phytochemical analyses	1. Berry kefirs contained high levels of phenolic compounds and exhibited moderate antioxidant capacity; 2. Freshness of juice used in making kefirs affected their phenolic content
3	Aronia kefir	Bioaccessibility	Levels of bioaccessible phenolic compounds, including anthocyanins and phenolic acids, increased during digestion
		Benefits to diabetes	Consumption of aronia kefir may aid in blood glucose control
		Impact of fermentation	Fermentation increased the inhibitory activity of polyphenols toward α -glucosidase

4.2 Study Limitations

In the sensory test (chapter 2), we investigated the impacts of sweetener type and sweetness levels on consumers' acceptability toward aronia or elderberry kefir. The first limitation of this study is that we did not include a control sample in sensory tests. If we included an aronia or elderberry kefir without additional sweetener in each individual test, we could know if the addition of sweetener can increase the consumer acceptability. Alternatively, we could add a plain kefir sample with the same sweetness levels to each sensory tests, which would allow us to draw a conclusion if the addition of berry juice is favored by consumers. However, more than four samples are not recommended to be evaluated in one sensory session because increased sample numbers will lead to participant fatigue, which may negatively affected the accuracy of the evaluation. Thus, in order to obtain accurate results, we did not add a control sample. The second limitation was that the phenolic metabolites in digested aronia kefir could not be identified (chapter 3). We observed new peaks in the digested aronia kefir compared to the digested non-fermented control. However, due to lack of mass spectrometry, the identification could not be conducted.

4.3 Future Directions

4.3.1 Impacts of Kefir Culture on Proanthocyanins

In a previous study (presented in Appendix A), we made an assumption that the increases of monomeric anthocyanins in elderberry kefir during storage may be related to the microbial depolymerization of proanthocyanins. Proanthocyanins exist in many berry fruits and cereals. One major restriction of the bioavailability of proanthocyanins is the large molecular size. Kefir culture may contain microorganisms that can depolymerize proanthocyanins and

produce smaller, more bioavailable molecules, such as monomeric anthocyanins. To date, there is no study that investigates the impact of kefir culture on the bioavailability of proanthocyanins. It is worth investigating the impact of kefir fermentation on the bioavailability of proanthocyanins from various foods. Studies aiming at separation and identification of the microorganisms in kefir culture may be needed. The microorganisms which could depolymerize proanthocyanins may be good candidates for the bioprocessing of proanthocyanin-rich foods to increase their bioavailability.

4.3.2 Identification of the Metabolites in Aronia Kefir after Digestion

In chapter four of this dissertation, the *in-vitro* digested aronia kefir exhibited stronger α -glucosidase inhibitory activity than the non-fermented control. This result may be due to the phenolic metabolites generated by microorganisms in the kefir. However, due to limitations of our experiments, the metabolites in aronia kefir were not identified. The identification of these metabolites needs to be conducted in order to better understand how kefir microorganisms interact with phenolic compounds. In addition, the metabolites may be isolated and used as nutraceutical agents to aid in blood glucose control of diabetic individuals. Currently, there is no available literature investigating the phenolic metabolites from kefir fermentation.

4.3.3 Evaluation of Berry-incorporated Kefir with *in-vivo* Studies

For this research study, the strong inhibitory activity of aronia kefir on α -glucosidase was observed *in-vitro*. However, the question if aronia kefir can alter the activity of α -glucosidase *in-vivo* and decrease the postprandial blood glucose level has not been answered. Though *in-vitro* studies showed positive results, the results of *in-vivo* studies may be different.

In order to confirm the potential benefits of berry kefir in an integrated metabolic system, *in-vivo* studies need to be conducted. Currently, no research in regards to the *in-vivo* bioactivity of polyphenol-enriched kefir has been done. In addition, no publication is available on the bioavailability of kefir-fermented phenolic metabolites using animal models. Studies utilizing animal models are needed to investigate if kefir fermentation improves the bioavailability of dietary polyphenols *in-vivo*.

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APPENDIX A: EFFECTS OF SWEETENERS ON THE LEVELS OF PHENOLIC COMPOUNDS AND ANTIOXIDANT CAPACITY OF ARONIA AND ELDERBERRY KEFIRS DURING STORAGE

1. Chapter Abstract

Aronia berries and elderberries are rich in phenolic compounds including anthocyanins. Some phenolic compounds, specifically anthocyanins, are very labile. The instability of phenolic compounds can restrict their applications in the food industry. The addition of sweeteners may help the stability of phenolic compounds. In this study, aronia and elderberry juice were individually incorporated into kefir, a fermented dairy beverage. Products were sweetened with natural sweeteners, sucrose, stevia extract or monk fruit extract, at varying concentrations. The total phenolic (TP) content, total monomeric anthocyanin (TMA) content and DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical scavenging capacity of each product was tested every seven days during twenty-eight days of storage at 4°C. The results demonstrated that, compared to aronia kefir without added sucrose, the addition of sucrose at 4.8% and 6.3% negatively affected the levels of TP and TMA. Compared to sucrose, monk fruit extract appeared to be a better sweetener for aronia kefir and elderberry kefir with a low level of sweetness to produce kefirs with a higher amount of TP and TMA. Stevia-sweetened berry kefirs contained more TMA but exhibited lower antioxidant capacity than sucrose-sweetened products. After twenty-eight days of cold storage (4°C), TP content decreased in most berry kefirs. The levels of TMA decreased in aronia kefirs but increased in elderberry kefirs during storage. DPPH scavenging capacity of all kefir products declined after twenty-eight days of storage. The changes in the levels of TP, TMA and antioxidant capacity over time may be associated with both natural degradation and microbial metabolism of polyphenols.

2. Introduction

Aronia (*Aronia melanocarpa*) and elderberry (*Sambucus nigra* L. spp. *canadensis*) fruits are underutilized berries that are rich in polyphenols, especially anthocyanins. Development of new products using these berries could increase the diversity of food products on the market and potentially help to increase the consumer's daily intake of anthocyanins. The utilization of anthocyanins in food products is restricted because anthocyanins are labile, which means the levels of anthocyanins in food products will decrease during processing and storage. Reque *et al.* reported that after 10 days of storage at 4°C, the anthocyanins in blueberry juice degraded by 83% [83]. Queiros *et al.* demonstrated that the level of anthocyanins in sweet cherry juice was reduced by 42% after 28 days of refrigeration [361]. Anthocyanins are stable at pH levels ranging from 1 to 4. The degradation of anthocyanins is accelerated in an alkaline environment [83]. An acidic food matrix such as a fermented dairy beverage, may help to decrease the degradation of anthocyanins compared to a neutral food matrix. The presence of sweeteners may alter the stability of anthocyanins during storage. Kopjar *et al.* reported that blackberry juice with sucrose added contained a higher amount of anthocyanins than juice without added-sucrose after ten days of storage stored at 4°C [362]. In order to ensure the quality of an anthocyanin-rich product during its shelf life, methods to increase the stability of anthocyanins are important.

Kefir is a fermented dairy product that originated in the Caucasus Mountain region. Kefir is an effervescent beverage with a sour taste and creamy texture [261]. Kefir was chosen to be the food matrix for incorporating aronia and elderberries for the following reasons: 1) kefir is acidic, which may help to stabilize the phenolic compounds during storage; 2) studies have

showed that fermentation with lactic acid bacteria or yeast increased the antioxidant capacity of polyphenol-rich foods [176, 177, 360]. Kefir is a symbiotic community of various lactic acid bacteria and yeast. Some lactic acid bacteria and yeast in kefir may have the ability to increase the antioxidant capacity of phenolic compounds in berries. 3) kefir is a suitable dairy beverage for lactose-intolerant individuals due to the consumption of lactose by the microorganisms. In this study, aronia or elderberry juice was incorporated into kefir, and different sweeteners were added. The objective of this study was to investigate the influence of sweeteners on the phenolic compounds and the antioxidant capacity of berry kefirs during storage. In the first part of this study, the impacts of sucrose on phenolic compounds in berry-added kefirs were tested at five concentrations. The effects of sucrose and two non-nutritive sweeteners (stevia and monk fruit extracts) were evaluated in the second part.

3. Material and Methods

3.1 Chemicals and Dietary Ingredients

Citric acid anhydrous, hydrochloric acid, methanol, potassium chloride, and sodium bicarbonate were purchased from Fisher's Scientific (Waltham, MA, U.S.). 2, 2-diphenyl-1-picrylhydrazyl (DPPH), Folin - Ciocalteu's phenol reagent and gallic acid monohydrate were obtained from Sigma-Aldrich (St. Louis, MO, U.S.). Sodium acetate was purchased from Chem-Impex international, Inc. (Wood Dale, IL, U.S.). Ultra pure water used in this study was obtained from a Millipore water system (EMD Millipore, Billerica, MA, U.S.).

Aronia berries (*Aronia melanocarpa*, variety 'Viking') used in the first part of this experiment were obtained from the University of Connecticut (Storrs, CT, U.S.) in 2013. Aronia

berries used in the second part of this study to test the effects of different sweeteners were harvested from the University of Connecticut (Storrs, CT, U.S.) in 2014. Elderberries (*Sambucus nigra* L. spp. *canadensis*) were harvested from the Littlefield experimental garden at the University of Maine from the 2014 growing season (Dr. Myracle's Hatch Project, Hatch # ME021924). The berries were harvested at their full ripeness based on their deep purple color. The berries were de-stemmed, washed and stored at -20°C until use. A commercial kefir culture (Yogourmet®, Lachute, QC, Canada) containing lactic bacteria (*Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris*, *Lactococcus lactis* subsp. *bv. diacetylactis*, *Lactobacillus acidophilus*) and lactic yeasts was used to make kefir products. Dietary sweeteners and low-fat milk (2%, Oakhurst®, Portland, ME, U.S.) were purchased from a local supermarket. The dietary sweeteners used in this study include sucrose (Great Value®, Bentonville, AR, U.S.), stevia extract (Stevia in the Raw®, New York City, NY, U.S.) and monk fruit extract (Monk Fruit in the Raw®, New York City, NY, U.S.).

3.2 Kefir Preparation and Formulas

The kefir formulas products are shown in Table A.1, Table A.2 and Table A.3. All kefir products were prepared using the following method: low-fat milk was heated to 82°C on the gas stove and then cooled to 26°C using an ice bath within 30 min. The temperature was measured using a digital thermometer. During the cooling process, frozen aronia berries or elderberries were heated (100°C, 5 min) and then juiced using a domestic juicer (Hamilton Beach®, Southern Pines, NC, U.S.). The cooled milk was transferred to a glass bowl and the commercial starter was added (5 g/quart). The mixture was stirred for 5 minutes to fully dissolve the starter. Aronia or elderberry juice was added to the mixture, and then the

sweeteners were added. The amounts of non-nutritive sweeteners (stevia and monk fruit extracts) used in the individual samples were adjusted according to the instructions on the package to standardize the sweetness to equal sucrose. The sweetness levels of the aronia kefir presented in Table A.2 was the same. Elderberry kefir (Table A.3) were grouped according to the sweetness levels. The products with 3 g sucrose, 0.3 g stevia or 0.6 g monk fruit extract were grouped as products with a low level of sweetness (Group L). The other elderberry kefir were grouped as products with a high level of sweetness (Group H). Once prepared, kefir mixture samples were covered with a cloth and incubated at room temperature (23°C) for fermentation. After 24 hours, pH values of the kefir products were 4.5. Then the kefir products were homogenized with a blender (Hamilton Beach®, Southern Pines, NC, U.S.) and transferred to sealable glass containers, and each sample batch was divided into five aliquots, sealed and stored at 4°C. The phenolic analyses were performed after 2 hours of storage (day 0), and subsequent analyses were conducted on days 7, 14, 21 and 28 of storage. Samples are duplicated.

Table A.1 Aronia kefir formulas sweetened with sucrose

Product Code	Milk (g)	Aronia Juice (g)	Sucrose (g)
Aronia-S0.0	100	20	0
Aronia-S1.6	100	20	2
Aronia-S3.2	100	20	4
Aronia-S4.8	100	20	6
Aronia-S6.3	100	20	8

Note: Aronia-S0.0: aronia kefir without added sucrose; Aronia-S1.6: aronia kefir with 1.6% added sucrose; Aronia-S3.2: aronia kefir with 3.2% added sucrose; Aronia-S4.8: aronia kefir with 4.8% added sucrose; Aronia-S6.3: aronia kefir with 6.3% added sucrose.

Table A.2 Aronia kefir formulas sweetened with different sweeteners

Aronia kefir	Milk (g)	Aronia Juice (g)	Sweetener (g)		
			Sucrose	Stevia	Monk fruit
AroniaSucrose	100	20	4	-----	-----
AroniaStevia	100	20	-----	0.4	-----
AroniaMonk	100	20	-----	-----	0.8

Note: ----- indicates the ingredient was not used in the product.

AroniaSucrose: aronia kefir sweetened with sucrose; AroniaStevia: aronia kefir sweetened with stevia extract; AroniaMonk: aronia kefir sweetened with monk fruit extract.

Table A.3 Elderberry kefir formulas sweetened with different sweeteners

Group*	Elderberry kefir	Milk (g)	Elderberry juice (g)	Sweetener (g)		
				Sucrose	Stevia	Monk fruit
L	EberrySucrose-L	80	20	3	-----	-----
	EberryStevia-L	80	20	-----	0.3	-----
	EberryMonk-L	80	20	-----	-----	0.6
H	EberrySucrose-H	80	20	4.5	-----	-----
	EberryStevia-H	80	20	-----	0.5	-----
	EberryMonk-H	80	20	-----	-----	0.9

Note: ----- indicates the ingredient was not used in the product

*: Products in each group had a similar level of sweetness. Products in group L were less sweet than products in group H.

EberrySucrose-L: elderberry kefir sweetened with 3% sucrose; EberryStevia-L: elderberry kefir with 0.3% stevia extract; EberryMonk-L: elderberry kefir with 0.6% monk fruit extract; EberrySucrose-H: elderberry kefir sweetened with 4.5% sucrose; EberryStevia-H: elderberry kefir with 0.5% stevia extract; EberryMonk-H: elderberry kefir with 0.9% monk fruit extract.

3.3 Extraction

Phenolic compounds were extracted from the berry kefir following a modified method from Scibisz *et al.* [310]. Briefly, kefir products were extracted with 80% acidified methanol (1% citric acid, w/v). The kefir to solvent ratio was 1:10. The samples were sonicated for one hour in a Branson 5510 sonicator (Danbury, CT, USA) and centrifuged at 16639×g (Eppendorf 5804R, Hamburg, Germany) for 30 min at 4°C. The supernatant was collected and the residue was extracted two additional times. The supernatant was pooled and dried under a vacuum (Eppendorf Vacufuge plus, Hamburg, Germany). The dried extracts were re-suspended using 100% acidified methanol (citric acid, 1%, w/v) and kept at -20°C to precipitate proteins. The slurry was centrifuged at 0°C for 30 minutes at 16639×g. The supernatant was vacuum-dried at room temperature and re-suspended with 80% acidified methanol (citric acid, 1%, w/v). The samples were stored at -20° C until analysis.

3.4 Total Phenolic Content

The total phenolic (TP) content in kefir extracts was measured using a modified Folin-Ciocalteu method reported by Velioglu *et al.* [311]. The extract (20 µL) and Folin-Ciocalteu reagent (90 µL, 1:9 diluted with water) were loaded into a 96-well plate. After 5 min incubation at room temperature in the dark, sodium bicarbonate (6 g/100 mL, 90 µL) was added to samples. The mixture was incubated for 90 minutes at room temperature in the dark. Gallic acid was used to create the standard curve. Absorbance was read at 750 nm using a Biotek plate reader (Elx 800, Winooski, VT, USA). The data are presented in gallic acid equivalents (GAE), as the average of triplicate measurements of duplicated samples.

3.5 Total Monomeric Anthocyanin Content

The total monomeric anthocyanin (TMA) levels in the kefir products was measured using the pH differential method established by a modified method from Lee *et al.* [82]. Generally, the extract (20 µL) was mixed with either pH 1.0 buffer (0.025 M potassium chloride, 180 µL) or pH 4.5 buffer (0.4 M sodium acetate, 180 µL) in 96-well plates. The mixture was incubated at room temperature for 20 minutes in the dark. The absorbance (Abs) was read at 515 nm and 690 nm using a Biotek plate reader (Elx 800, Winooski, VT, USA). The total monomeric anthocyanin content was calculated using the following formula and expressed in cyanidin-3-glucoside (C3G) equivalents. Samples were measured in triplicate.

$$\text{Anthocyanin (mg C3G/L)} = \frac{A \times MW \times DF \times 10^3}{\epsilon \times L}$$

Where: A = (Abs₅₁₅-Abs₆₉₀) pH 1.0 – (Abs₅₁₅-Abs₆₉₀) pH 4.5;

MW (molecular weight) = 449.2/mol for cyanidin-3-glucoside;

DF = dilution factor;

L = pathlength in cm;

ε = 26900 L/mol·cm, for cyanidin-3-glucoside;

10³ = factor for conversion from g to mg.

3.6 Antioxidant capacity

The antioxidant capacity of kefir products was evaluated using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging capacity method. The method was modified from Duymus *et al.* [312]. Equal amounts (150 µL) of the sample and DPPH methanol solution (0.3 mM) were loaded into a 96-well plate. The plate was incubated at room temperature in the

dark for 30 min. A mixture of 80% acidified methanol (solvent of the extract) and DPPH solution was used as the control. The absorbance was read at 515nm. The inhibition rate of the sample was calculated using the following formula:

$$\%inhibition = \frac{Abs_{control} - Abs_{sample}}{Abs_{control}} \times 100$$

Five dilutions of each sample were measured for DPPH scavenging capacity. The IC₅₀ values, which is the concentration of the sample required to scavenge 50% of DPPH radicals, were calculated and expressed as mg/mL. Measurements were conducted in triplicate.

3.7 Statistical Analysis

Data are shown as mean ± standard deviations. Significant differences among sample means were evaluated using one way Analysis of Variance and Tukey's HSD post hoc tests ($p \leq 0.05$). Pearson correlation coefficient test was conducted to determine the correlation between the levels of phenolic compounds and DPPH scavenging capacity. Data were analyzed using SAS University Edition (Cary, NC, U.S.).

4. Results

4.1 The Impacts of Sucrose on the Levels of Phenolic Compounds, Monomeric Anthocyanins and Antioxidant Activity of Aronia Kefir

The impacts of sucrose concentration on the phenolic compounds including anthocyanins were evaluated by comparing the five samples (Aronia-S0.0, Aronia-S1.6, Aronia-S3.2, Aronia-S4.8 and Aronia-S6.3) that had been stored for the same duration. TP of aronia kefir sweetened with different concentrations of sucrose are shown in Figure A.1. Compared to Aronia-S0.0 (no added sucrose), the TP levels were higher in the sample with 1.6% added

sucrose on day 0 (95.23 ± 2.02 mg GAE/100 g) and 3.2% sucrose on day 14 (110.57 ± 0.52 mg GAE/100 g). On every individual testing day, TP content of Aronia-S4.8 and Aronia-S6.3 were lower than Aronia-S0.0.

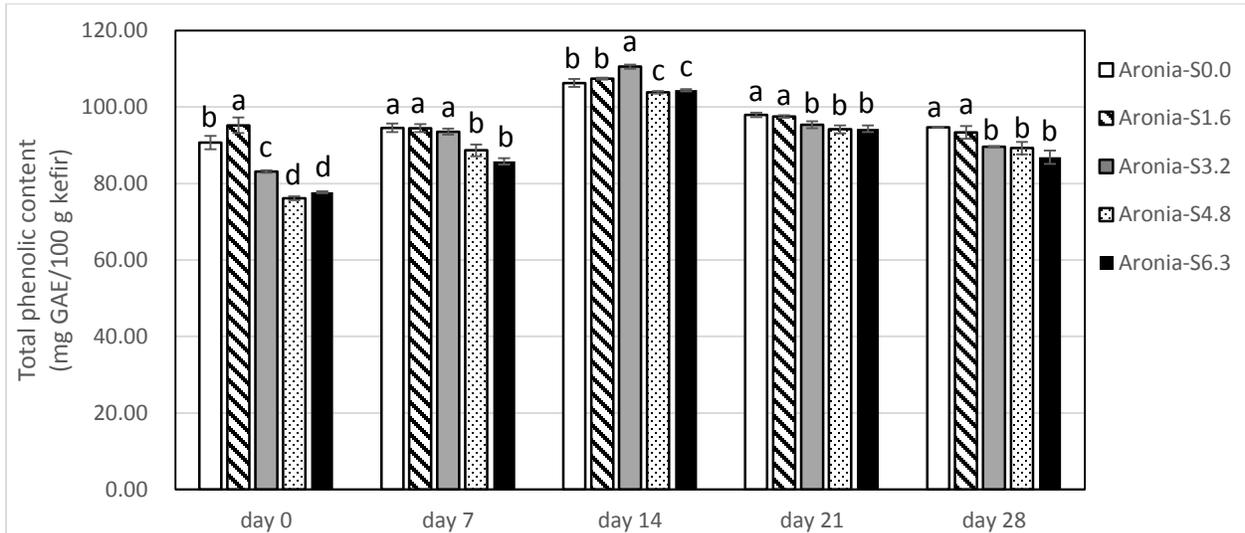


Figure A.1 Total phenolic compound levels of aronia kefir sweetened with different concentrations of sucrose

Note: Data are shown as mean \pm standard deviation, $n=2$ (batches) \times 3 (measurements). Among the samples that were tested on the same day, different letters indicate significant differences at $p \leq 0.05$.

TMA in different concentrations of sucrose-sweetened aronia kefir are shown in Figure A.2. All samples contained the highest TMA on day 0. After fermentation (on day 0), only aronia kefir sweetened with 6.3% sucrose (Aronia-S6.3) contained less monomeric anthocyanins (41.14 ± 0.85 mg C3G/100 g) than Aronia-S0.0 (44.50 ± 1.95 mg C3G/100 g). No significant differences of TMA among all samples were detected on day 7. After day 14, 21 or 28

of storage, Aronia-S4.8 and Aronia-S6.3 contained less monomeric anthocyanins compared to Aronia-S0.0 that had been stored for the same period.

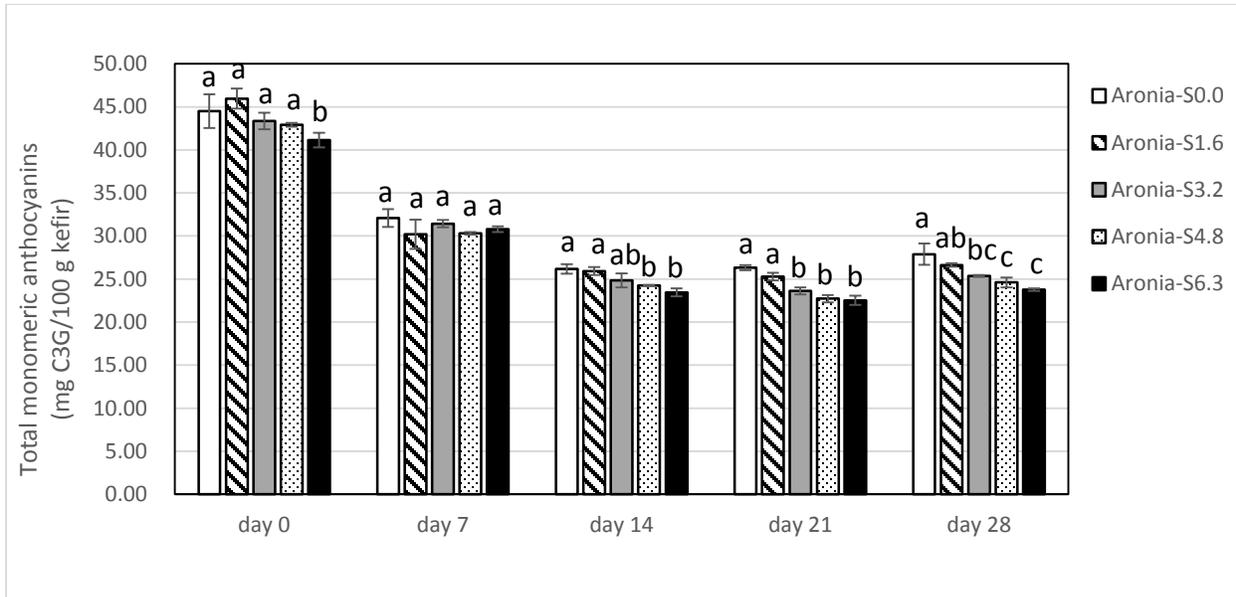


Figure A.2 Total monomeric anthocyanin levels in aronia kefir sweetened with different concentrations of sucrose

Note: Data are shown as mean \pm standard deviation, $n=2$ (batches) \times 3 (measurements). Among the samples that were tested on the same day, different letters indicate significant differences at $p \leq 0.05$.

The antioxidant capacity of the aronia kefir samples were evaluated and the results are shown in Figure A.3. Aronia-S0.0 exhibited either higher or similar antioxidant capacity compared to the other samples on every testing day during storage, with the exception of Day 21. On day 21, Aronia-S1.6 exhibited the strongest scavenging capacity (IC_{50} was 13.25 ± 0.27 mg kefir/mL). Overall, the antioxidant capacity of aronia kefir samples sweetened with different concentrations of sucrose were slightly decreased during 28 days of storage.

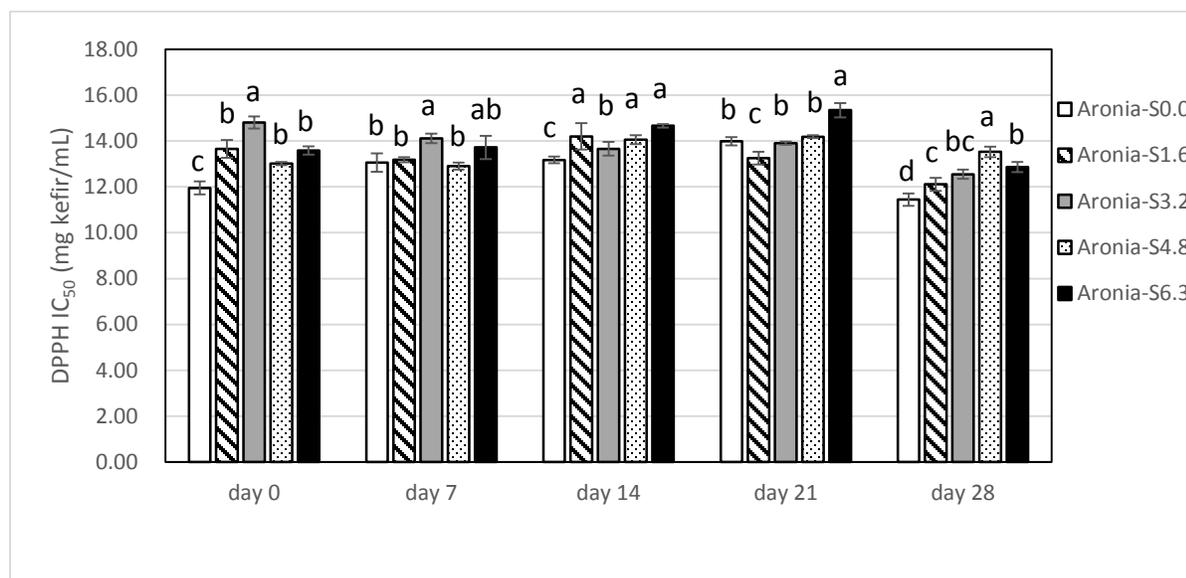


Figure A.3 DPPH IC₅₀ values of aronia kefir sweetened with different concentrations of sucrose

Note: Data are shown as mean \pm standard deviation, n=2 (batches) x 3 (measurements). Among the samples that were tested on the same day, different letters indicate significant differences at $p \leq 0.05$.

4.2 The Impacts of Different Sweeteners on the Levels of Total Phenolic Compounds, Monomeric Anthocyanins and Antioxidant Capacity in Aronia Kefir

TP content of aronia kefir samples are shown in Figure A.4. The impact of sweetener type (sucrose, stevia and monk fruit) on TP levels were evaluated by comparing the three aronia kefir samples stored for the same time period (day). Compared to AroniaSucrose, AroniaMonk contained more TP on each testing day except for day 0. On day 0, the TP content of AroniaSucrose and AroniaMonk was the same. Compared to AroniaSucrose, AroniaStevia had an equal amount of TP on day 0, higher levels on day 7, 14 and 21, and then a lower level on day 28.

The effects of storage time on the TP content of samples assessed. TP levels of AroniaSucrose decreased on day 14, and then increased on day 28. TP levels of AroniaMonk decreased on day 14 and gradually increased on days 21 and 28. A decreases of the TP in the AroniaStevia sample was observed after 14 days of storage, and the TP content of AroniaStevia increased on day 21. After twenty-eight days of storage, the TP levels of AroniaSucrose and AroniaMonk were almost the same as their corresponding values on day 0. The AroniaStevia sample had a lower amount of TP on day 28 compared to day 0.

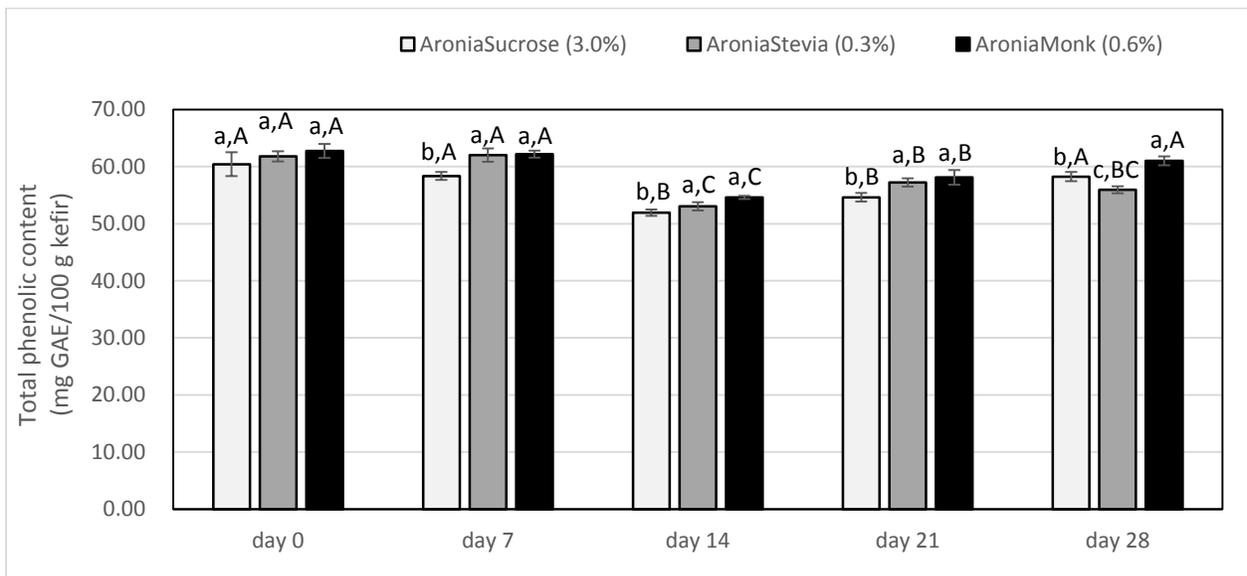


Figure A.4 Total phenolic compound levels of aronia kefir sweetened with sucrose, stevia extract and monk fruit extracts

Note: 1. Data are shown as mean \pm standard deviation, $n=2$ (batches) \times 3 (measurements).

2. Lowercase letters indicate significant differences ($p \leq 0.05$) among the three samples that were tested on the same day;

3. Capital letters indicate significant differences ($p \leq 0.05$) of the same sample over storage time.

The TMA content of aronia kefir is shown in Figure A.5. The impacts of sweetener types were evaluated by comparing the three products tested on the same day. On each testing day, AroniaMonk contained higher levels of TMA than AroniaSucrose. AroniaStevia also had slightly higher levels TMA than AroniaSucrose on day 0 (31.53 ± 0.18 and 29.94 ± 0.76 mg C3G/100 g, respectively) and day 28 (13.67 ± 0.36 and 11.50 ± 0.11 mg C3G/100 g, respectively). The TMA levels of AroniaStevia and AroniaSucrose were similar on all other testing days.

The TMA content of all aronia kefirs dramatically decreased after seven days of storage. TMA levels of AroniaMonk declined until day 21 and slightly increased during the last week of storage. The amount of TMA in AroniaStevia decreased until Day 14, remained the same on day 21 and slightly increased on day 28. The level of TMA in AroniaSucrose maintained the same from day 14 to the end of the storage study.

The antioxidant capacity levels of the aronia kefirs are shown in Figure A.6. Comparisons among products stored for the same time period were conducted to evaluate the impacts of the sweeteners. On days 0, 7 and 14, the antioxidant activity of AroniaMonk (IC_{50} s were 16.31 ± 0.47 , 15.59 ± 0.16 , 16.54 ± 0.34 mg kefir/mL) was higher than AroniaSucrose (IC_{50} s were 17.86 ± 0.47 , 16.97 ± 0.10 , 16.95 ± 0.57 mg kefir/mL). However, the antioxidant capacity of AroniaMonk (IC_{50} s were 19.87 ± 0.33 and 20.07 ± 0.32 mg kefir/mL) was lower than AroniaSucrose (IC_{50} s were 18.09 ± 0.36 and 19.42 ± 0.21 mg kefir/mL) on Day 21 and 28. AroniaStevia exhibited similar antioxidant capacity as AroniaSucrose on most testing days except for day 21 where the antioxidant capacity of AroniaStevia (IC_{50} was 19.02 ± 0.28 mg kefir/mL) was lower than AroniaSucrose (IC_{50} was 18.09 ± 0.36 mg kefir/mL). The antioxidant capacity of all aronia kefir

samples were maintained over 14 days of storage but slightly decreased on either day 21 or day 28. Decreases in antioxidant capacity of AroniaMonk and AroniaStevia were observed on day 21. The antioxidant activity of AroniaSucrose decreased on day 28.

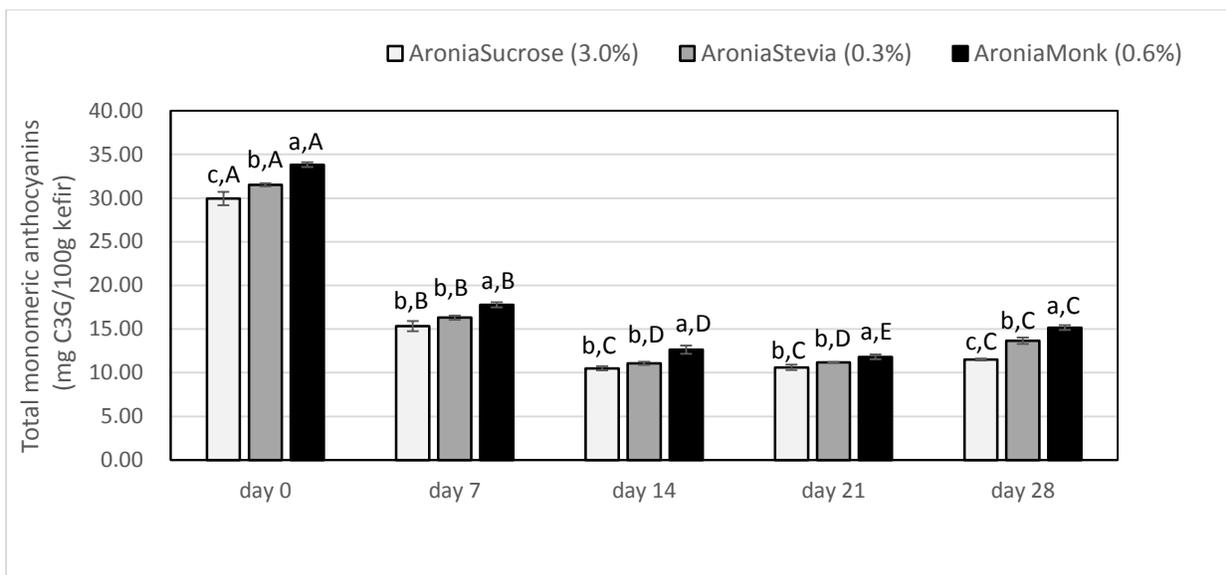


Figure A.5 Total monomeric anthocyanin levels of aronia kefir sweetened with sucrose, stevia extract and monk fruit extracts

Note: 1. Data are shown as mean \pm standard deviation, n=2 (batches) x 3 (measurements).

2. Lowercase letters indicate significant differences ($p \leq 0.05$) among the three samples that were tested on the same day;

3. Capital letters indicate significant differences ($p \leq 0.05$) of the same sample over storage time.

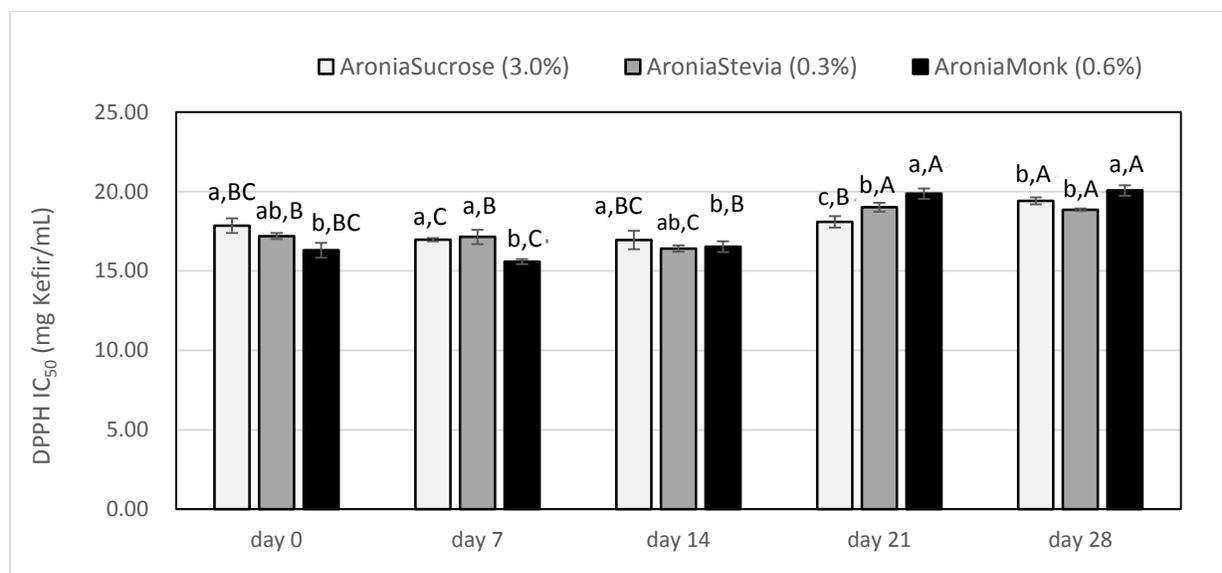


Figure A.6 DPPH IC₅₀ means of aronia kefir sweetened with sucrose, stevia extract and monk fruit extracts

Note: 1. Data are shown as mean \pm standard deviation, n=2 (batches) x 3 (measurements).

2. Lowercase letters indicate significant differences ($p \leq 0.05$) among the three samples that were tested on the same day;

3. Capital letters indicate significant differences ($p \leq 0.05$) of the same sample over storage time.

4.3 The Impacts of Different Sweeteners on the Levels of Total Phenolic Compounds,

Anthocyanins and Antioxidant Capacity of Elderberry Kefir

The TP contents of elderberry kefir samples are shown in Figure A.7. Comparisons were conducted among the products with a comparable level of sweetness. The impacts of sweetener type were evaluated by comparing the different products over storage time. In group L (products with a low level of sweetness), EberryMonk-L higher TP levels than EberrySucrose-L on days 0, 14 and 28. Compared to EberrySucrose-L, EberryStevia-L had a

higher TP content on day 14 but a less amount on day 21. No significant differences of TP levels were noted between EberryStevia-L and EberrySucrose-L on other testing days. In group H (products with a high level of sweetness), the EberryMonk-H and EberryStevia-H samples contained higher TP content than EberrySucrose-H on day 0. There was no significant difference in TP content among EberrySucrose-H, EberryStevia-H and EberryMonk-H on day 7, 14 and 21.

The TP levels of elderberry kefir appeared to decrease over storage time for samples with both high and low sweetness levels. In group L, TP content of EberrySucrose-L decreased after one week of storage and maintained the same level until day 21 and then decreased again. The TP levels of EberryStevia-L and EberryMonk-L fluctuated during the first 21 days of storage. The lowest amount of TP for all elderberry kefir samples was observed on day 28. In group H, TP levels of EberryStevia-H decreased over storage time. The TP content of EberrySucrose-H and EberryMonk-H slightly fluctuated during storage time. After 28 days, all elderberry kefir samples in group H contained less TP levels than day 0.

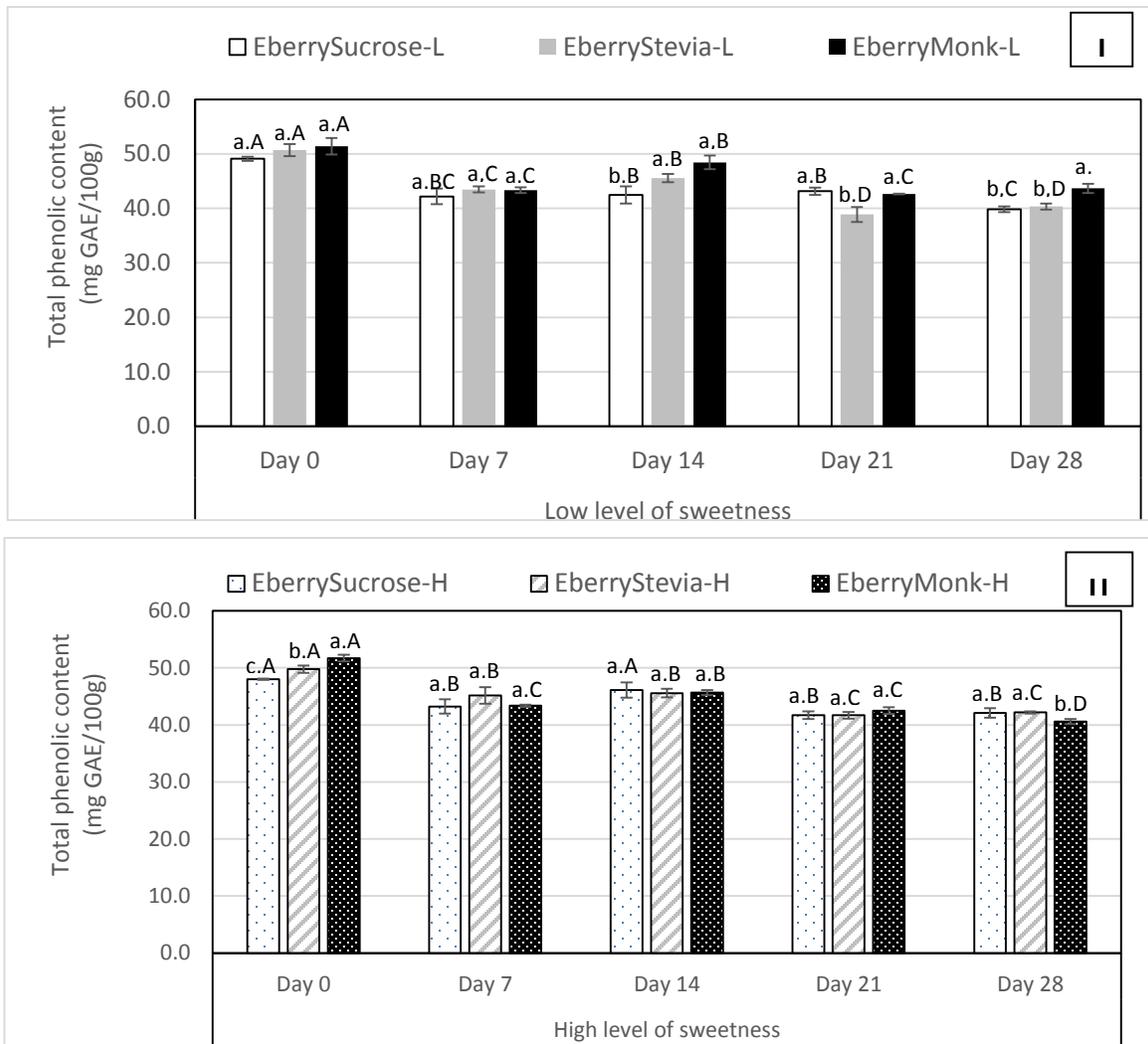


Figure A.7 Total phenolic compound levels of elderberry kefir sweetened with sucrose, stevia extract and monk fruit extracts

Note: 1. Data are shown as mean \pm standard deviation, $n=2$ (batches) \times 3 (measurements).

2. Graph I: Results of elderberry kefir with a low level of sweetness;

Graph II: Results of elderberry kefir with a high level of sweetness.

3. Lowercase letters indicate significant differences ($p \leq 0.05$) among the three samples that were tested on the same day;

Capital letters indicate significant differences ($p \leq 0.05$) of the same sample over storage time.

The TMA content of elderberry kefir samples is shown in Figure A.8. In group L, EberrryMonk-L sample contained higher TMA content than EberrrySucrose-L samples on days 7, 14, 21 and 28. EberrryStevia-L levels were also higher than EberrrySucrose-L on day 7, 14 and 28. In Group H, EberrryMonk-H sample contained higher TMA content than EberrrySucrose-H on days 0, 21 and 28. No significant differences among all samples were observed on day 7. EberrryStevia-H and EberrrySucrose-H contained similar amounts of TAM content on days 0, 7, 14 and 21. On Day 28, EberrryStevia-H had higher TAM content than EberrrySucrose-H.

The TMA content of elderberry kefir samples for both sweetness level groups increased over time. Over storage time, the highest TMA amount for each elderberry kefir treatment was observed on day 28. After twenty-eight days of storage, the amount of TMA in EberrrySucrose-L, EberrryStevia-L, EberrryMonk-L, EberrrySucrose-H, EberrryStevia-H, EberrryMonk-H increased by 32.6%, 36.2%, 46.4%, 32.3%, 43.8% and 35.6% compared to their corresponding values on day 0, respectively.

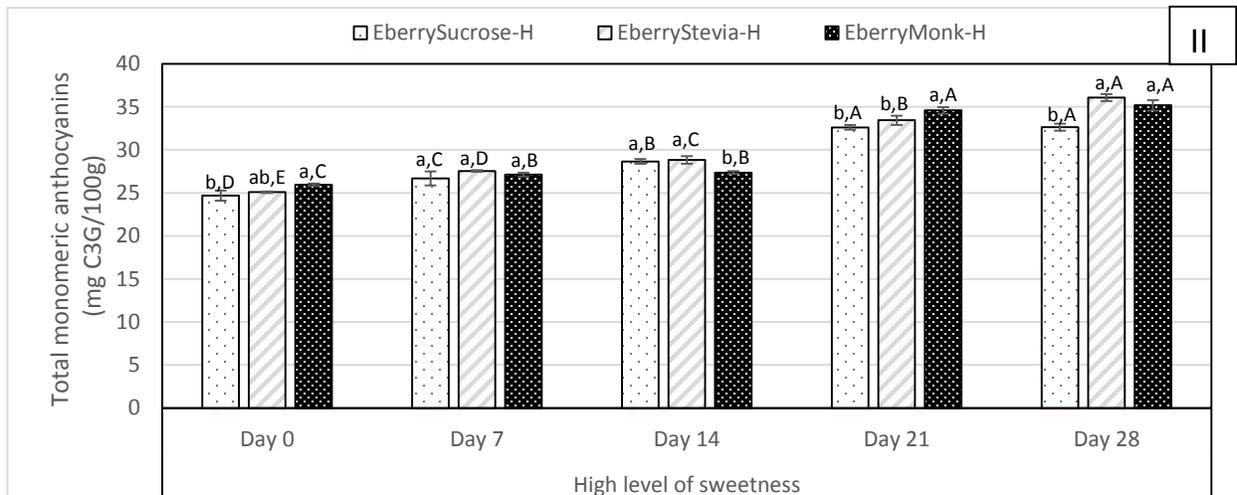
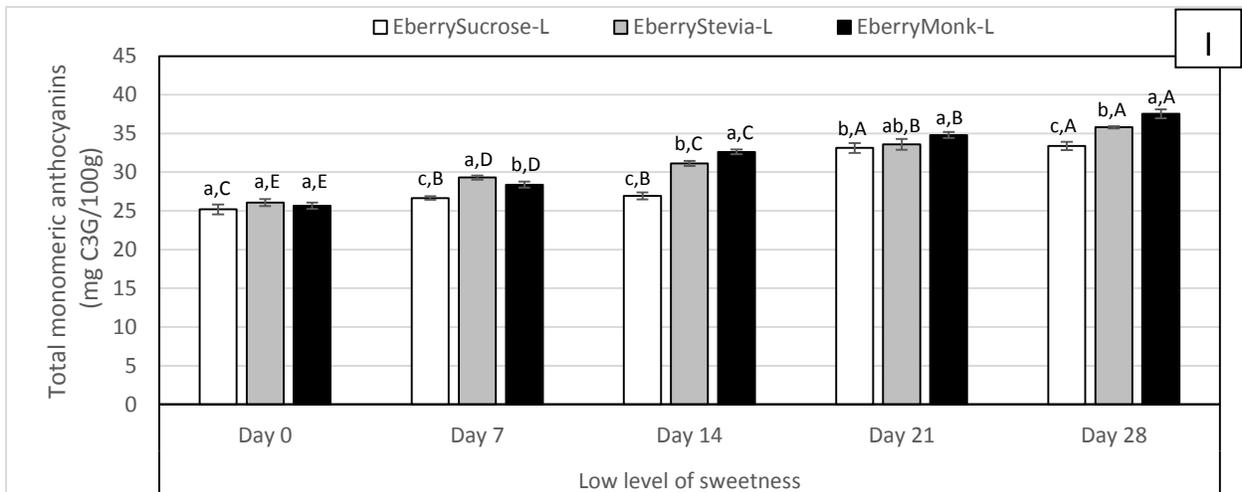


Figure A.8 Total monomeric anthocyanin content of elderberry kefir sweetened with sucrose, stevia extract and monk fruit extracts

Note: 1. Data are shown as mean \pm standard deviation, n=2 (batches) x 3 (measurements).

2. Graph I: Results of elderberry kefir with a low level of sweetness;

Graph II: Results of elderberry kefir with a high level of sweetness.

3. Lowercase letters indicate significant differences ($p \leq 0.05$) among the three samples that were tested on the same day;

Capital letters indicate significant differences ($p \leq 0.05$) of the same sample over storage time.

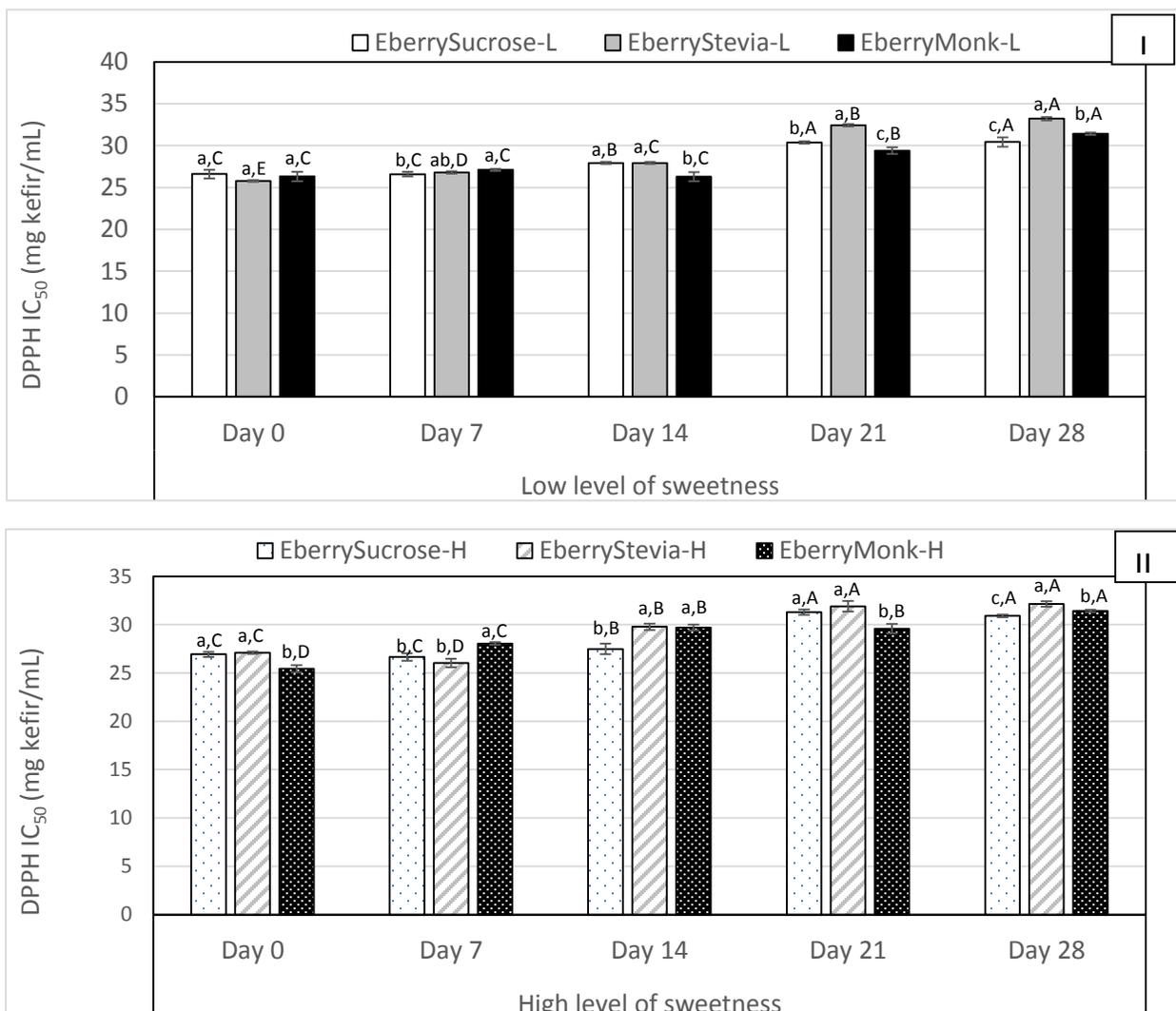


Figure A.9 DPPH IC₅₀ means of elderberry kefir sweetened with sucrose, stevia extract and monk fruit extracts

Note: 1. Data are shown as mean ± standard deviation, n=2 (batches) x 3 (measurements).

2. Graph I: Results of elderberry kefir with a low level of sweetness;

Graph II: Results of elderberry kefir with a high level of sweetness.

3. Lowercase letters indicate significant differences ($p \leq 0.05$) among the three samples that were tested on the same day;

Capital letters indicate significant differences ($p \leq 0.05$) of the same sample over storage time.

The antioxidant activity levels of elderberry kefir samples are shown in Figure A.9. In group L, no significant differences in antioxidant capacity were detected among kefir samples on day 0. EberryMonk-L exhibited a higher antioxidant capacity than EberrySucrose-L on days 14 and 21 but a lower antioxidant capacity on days 7 and 28. The antioxidant capacity of EberryStevia-L was similar with EberrySucrose-L samples on day 0, 7 and 14 but lower than EberrySucrose-L on day 21 and 28. In group H, the antioxidant capacity of EberryMonk-H sample was higher than EberrySucrose-H on days 0 and 21 but lower on all other testing days. Compared to EberrySucrose-H, EberryStevia-H exhibited a lower antioxidant capacity on days 14 and 28 but no significant difference on days 0, 7 and 21.

After twenty-eight days of storage, the antioxidant capacity of elderberry kefir samples decreased over storage time. The decreases in antioxidant capacity of EberrySucrose-L, EberryStevia-L, EberryMonk-L occurred on day 14, day 7 and day 21, respectively. The antioxidant capacity of EberrySucrose-H, EberryStevia-H and EberryMonk-H reduced on day 14, day 14 and day 7, respectively.

5. Discussion

5.1 Impacts of Sucrose

In our study, aronia kefir samples with 4.8% and 6.3% added-sucrose contained less TP and TMA levels than aronia kefir without added-sucrose on each testing day. The addition of sucrose did not appear to protect the TP or TMA levels during storage. To our knowledge, this is the first study to investigate the impacts of sucrose concentration on polyphenols in a fermented dairy matrix. The aronia phenolic compounds in our study were exposed to various

fermentative microorganisms, such as lactic bacteria and yeasts in kefir. Some fermentative microorganisms may have the ability to alter the amount of phenolic compounds by releasing the bound phenolic compounds and/or metabolizing them [173, 363]. The ability of yeast (*Kluyveromyces marxianus*) isolated from kefir to increase the antioxidant capacity of grape polyphenols synergistically has been observed in other research studies [364]. In addition, researchers noted that the presence of added-sucrose (1%) altered the growth pattern of both lactic acid bacteria and yeast in soymilk kefir compared to samples without sucrose [365]. Thus, the concentration of sucrose may influence TP and TMA levels by altering the capacity of microorganisms to metabolize phenolic compounds. The protective impacts of sucrose on phenolic compounds in fruit juice have been documented by several studies [366-368]. These protective impacts may be related to a decrease in water activity [367, 369]. In our study, the addition of 6.3% sucrose may not be sufficient to substantially affect water activity levels of the kefir sample to a critical point that would be beneficial to the stabilize polyphenols over storage time.

The antioxidant capacity of aronia kefir is contributed by the phenolic compounds. However, in this study, the correlation analyses did not show a significant relationship between TP and the DPPH radical scavenging activity. These results indicate that in addition to changes of TP levels, there were other alterations that may have affected the antioxidant capacity of aronia kefir samples, such as changes in polyphenol composition. The changes in phenolic composition may lead to altered antioxidant capacity levels because the antioxidant capacity of individual phenolic compound differs [370]. Fermentation may have affected the composition of polyphenols in our study. Aronia kefir was stored at 4°C, at which temperature the

fermentation process might be slowed but not stopped because the °brix values of sucrose-added aronia kefir decreased over storage (data not shown). The addition of sucrose at different concentrations may lead to varied activities of microorganisms during storage and influence the profile of the fermentative metabolites. Wang *et al.* reported that fermentation with yeasts (*Saccharomyces cerevisiae bayanus* EC 118) decreased the amount of total polyphenols but increased the level of gallic acid and the antioxidant capacity of maqui berry juice [177]. Jimenez-Lopez *et al.* demonstrated that fermentation decreased the concentration of epicatechin, increased the amount of total phenolic compounds and resulted in slightly enhanced antioxidant capacity in caper berries [371]. In summary, the impacts of sucrose on the antioxidant capacity of aronia kefir during storage may be achieved by altering the metabolism activity of microorganisms in addition to the changes of phenolic levels. More research is needed to verify this assumption.

5.2 Effects of Non-nutritive Sweeteners

In our study, monk fruit-sweetened aronia kefir contained more TP and TMA compared to sucrose-sweetened kefir samples at the lower sweetness levels. However, among the elderberry kefir samples in group H, the levels of TP and TMA between monk fruit- and sucrose-sweetened samples fluctuated at different testing points. Based on our results, no clear apparent trend occurred between antioxidant capacities of monk fruit and sucrose-sweetened berry kefir samples. These results indicate that monk fruit extract may be a preferred sweetener to use than sucrose for aronia kefir and elderberry kefir samples with a low level of sweetness to produce products with higher levels of TP and TMA. Our results are similar to a study conducted by Nowicka and Wojdylo [372]. They demonstrated that after six months storage at 4°C, sour

cherry puree with the addition of 1% monk fruit (referred as “Luo Han Kuo”), had higher TP and TMA levels compared to the sample with 7% added sucrose [372]. To our knowledge, this research is the only study involving monk fruit extract on the stability of phenolic compounds to date. However, the food matrix and the amount of sweeteners used in our study was different from the referenced study. In addition to the direct interactions between sweeteners and polyphenols, the impacts of sweeteners on the microorganisms may also contribute to our results. There has been no published study about the impacts of monk fruit on fermentation.

Compared to sucrose, the addition of stevia appeared to be beneficial to maintain TMA levels but decreased the antioxidant capacity of berry kefir over time. However, the results did not show a clear trend of the TP content among stevia- and sucrose-sweetened aronia kefir and elderberry kefir in group L. Some of our findings are in agreement with a former study conducted by Skapska *et al.*, where the replacement of sucrose with stevia extract reduced the antioxidant capacity of an aronia-herbal beverage [373]. However, Wozniak *et al.* demonstrated that in the absence of microorganisms, sucrose exhibited protective effects on anthocyanins but steviol glycosides (the sweet compounds in stevia extract) did not, which was different than our findings [374]. One factor that may influence the impact of sweeteners on TMA levels in our study is the presence of fermentative microorganisms. Both sucrose and stevia are carbon sources for lactic acid bacteria [375]. The addition of sucrose or stevia may alter the fermentation activity of microorganisms in kefir and then influence their pattern to interact with polyphenols. One other factor that may result in this difference is the type of anthocyanins. In the referenced study, purified cyanidin-3-glucoside and pelargonidin-3-

glucoside were used. In our study, both aronia and elderberries contain various types of anthocyanins.

Twenty-eight days of storage at 4°C decreased the TP levels in most berry kefir and reduced the antioxidant capacity of all berry kefir. The decreases may be caused by the natural and microbial degradation of phenolic compounds. In a study conducted by Muniandy *et al.*, decreases of TP levels in tea extract-enriched yogurt was observed during 21 days of storage. However, the DPPH scavenging capacity of tea extract-added yogurt was not altered [376]. The different results may be due to the different fermentation culture and polyphenol sources used in our study versus the referenced study.

During storage, the TMA content of the aronia kefir decreased but the TMA levels in the elderberry kefir increased over time. The decreases in aronia kefir are associated with the degradation of monomeric anthocyanins over time. Reque *et al.* also observed that 10 days of storage at 4°C decreased the anthocyanin content in blueberry juice by 83% [74]. The increases of TMA content in elderberry kefir may be related to the microbial activity in the kefir. Some microorganisms can produce enzymes that can catalyze the hydrolysis of proanthocyanins, such as tannase [377, 378]. The hydrolysis of proanthocyanins can yield monomeric anthocyanins. The proanthocyanin compositions of aronia and elderberries are different [198], and this may be a reason for the different trends of TMA changes in aronia versus elderberry kefir. The majority of the proanthocyanins in aronia are polymers with more than ten degrees of polymerization. In elderberries, the polymerization degrees of the predominant proanthocyanins are lower than seven. More studies are needed to test this assumption.

6. Conclusion

The impacts of sweeteners (sucrose, stevia and monk fruit) on the levels of TP, TMA and antioxidant capacity of berry-added kefir were tested during 28 days of storage at 4°C. Results showed that compared to Aronia kefir without sucrose, the addition of sucrose at 4.8% and 6.3% decreased the contents of TP and TMA. Compared to sucrose, monk fruit extract may be a preferred sweetener for aronia kefirs and elderberry kefirs with a low level of sweetness for protecting the levels of TP and TMA. Berry kefirs sweetened with stevia contained more TMA than the samples sweetened with sucrose, but the free radical scavenging capacity showed an opposite trend.

Twenty-eight days of storage at 4°C decreased the antioxidant capacity of all berry kefirs. Decreases of TP were observed during storage, but with fluctuations. The TMA content in aronia kefir decreased over time, but the content in elderberry kefir increased. The different trends may be related to the distinct polyphenol compositions of aronia and elderberries. In addition, it may be associated with the activity of microorganisms in kefir. More studies are needed to understand the influences of microorganisms on polyphenols during storage.

APPENDIX B: APPLICATION FOR APPROVAL OF RESEARCH WITH HUMAN SUBJECTS

1. Summary of the proposal

Chronic diseases have become a global issue causing many health issues with increased morbidity and mortality. A healthy diet is critical to the prevention of many chronic diseases such as type 2 diabetes and cancer. The functional properties of food and the bioactive compounds they contain are current research interests to increase health benefit. Fermented products such as kefir and berries that contain phytonutrients are two attractive topics in this area.

Fermented milk products have a good reputation in maintaining gut health due to their microorganisms that influence the fermentation process and make them a rich source of probiotics. Probiotics are also linked to healthy metabolic effects such as reducing blood cholesterol, boosting the immune system, preventing cancer, and attenuating lactose intolerance symptoms [1]. Kefir, a fermented dairy product, has been proven to have similar impacts on human health similar to other fermented dairy products such as yogurt. In addition, due to the composition of several different microorganisms in kefir, it is naturally free of lactose, which is very important. As reported, lactose intolerance is a popular disorder that influences approximately 75% of the world's adults [2]. Considering there is no lactose in kefir, it would be a good source of protein and calcium for the lactose intolerance population.

Berries are rich in phenolic compounds, especially anthocyanins which make them an attractive resource for health related research. Anthocyanins are secondary plant metabolites that are proven to have high antioxidant capacity [3]. The health benefits of

anthocyanins, especially their antioxidant effects which are related to their role in lowering the risk of several chronic diseases, have been reported by many articles [4].

Aronia and elderberries are under-utilized berries, reported to have high anthocyanin content [5, 6]. Due to the astringent mouth feel caused by the high phenolic content, they are considered unpleasant to consumers. The use of these berries in a dairy matrix may minimize this flavor, and the acidic environment of fermented dairy could stabilize the phenolic compounds in berries. The aim of this study is to develop new desirable products that incorporate elderberry and aronia into kefir. The consumption of this berry product might help to decrease the prevalence of chronic diseases by boosting the public's intake of antioxidant compounds, especially anthocyanins.

New products will be made with low fat milk (2%) and a commercial-available kefir starter mix (Yogourmet™), either aronia or elderberry will be added to the product. Each product will be sweetened by natural sweeteners (monk fruit or stevia extract) or table sugar. One concentration of each sweetener will be applied to aronia products, while two different concentrations will be used in elderberry products. Overall, three products will be developed using aronia berry and six p tests will be carried out in the Consumer Testing Center in University of Maine in four days, two days for aronia products and another two days for elderberry products. During each test, three or six samples (about one ounce each) marked with three randomized digit numbers will be served to every participant. Flyers, Consent form, and Questionnaires are presented in Appendices A to F.

2. Personnel

Principal Investigator: Xue Du, MS.

Xue Du is a PhD student in the School of Food and Agriculture in University of Maine, majoring in Food Science and Nutrition. She will be in charge of participant recruitment, sample preparation, conduction of sensory evaluation, and data analysis. CITI training is current.

Mentor (Faculty Sponsor): Angela Myracle, MPH, PhD.

Dr. Myracle is an Assistant Professor in the School of Food and Agriculture in University of Maine. She obtained her PhD in Human Nutrition, Purdue University. She has 25 years of experience in biochemical analysis, epidemiological studies, and sensory evaluation. She is the major supervisor of this project and will oversee the entire project. CITI training is current.

Research Associates:

Mary Ellen Camire, PhD.

Dr. Camire is a Professor of the School of Food and Agriculture, University of Maine. She has more than 20 years' sensory evaluation experience, and presently teaches the graduate course: Sensory Evaluation. CITI training is current.

Katherine Davis-Dentici.

Ms Davis-Dentici is an employee in University of Maine since 1993, and she has rich experience with sensory evaluation and product development. She will help through the entire project. CITI training is current.

Students: Some students in the School of Food and Agriculture might help with this project, and all of them will complete CITI training before assisting the project.

3. Participant recruitment

100 participants who aged 18 or older will be recruited for each sensory evaluation test (elderberry or aronia products tasting) via flyers, Facebook, and FirstClass notices (attached as Appendix C and D); both male and female will be recruited. Consumers who are allergic to any ingredient of the products, such as milk and elderberry / aronia, will be excluded. The number of subjects for the study is chosen according to Gacula and Rutenbeck [7], to allow enough power to obtain statistically significant results.

4. Informed consent

Subjects will be asked to read the printed informed consent form (as presented in Appendices E and F), which will be written at an 8th reading level, before participation. Participation in the study will be assumed to indicate consent.

5. Confidentiality

Subjects will login on the SIMS program in the Consumer Testing Center of University of Maine; data will be collected anonymously and only available to the study investigators. Password protected data file will be saved in the computers that are kept in a locked room.

Data will be deleted once the analysis is completed and the resulting article has been accepted; data will not be kept for more than one year after the completion of the study, which is December 20th, 2016.

6. Risks to participants

Potential food allergens will be announced to the subjects in the recruitment notices and informed consent forms.

Subjects will risk losing personal time. Even though each test should take no more than 30 minutes, unfamiliarity of the SIMS computer program may lead to longer testing time. Assistance will be provided during the test to minimize the time.

In general, the risks involved are minimal, and should be no greater than daily eating.

7. Benefits

Participants will have the opportunity to taste new products and may like them.

Developing new products in this project may help the elderberry and aronia farmers to utilize the berries. In addition, new products will contribute to the varieties of dairy food products, and may also boost the consumption of polyphenols, which is a health-beneficial phytochemical that has been related to lower the risk of chronic disease.

Risk of this project will be no more than associated with normal eating, so the benefits are expected to outweigh the risk.

8. Compensation

Participants who complete the whole evaluation process will receive \$2 as compensation.

References

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APPENDIX C: ELDERBERRY KEFIR TASTING RECRUITMENT NOTICE

Would you like to taste kefir, a fermented milk beverage? New kefir products are developed and volunteers are needed to provide their opinions; if you are aged 18 or older, and have about 20 minutes spare time, please come and help the researchers!

Testing session will be held in the Consumer Testing Center, Hitchner Hall 158. The date will be announced. Scheduling a time by calling (207)889-7117 or e-mailing xue.du@maine.edu will be preferred; however, volunteers are welcome to show up at any time.

Volunteers will receive \$2 for participating in the study; however, if you are allergic to milk, elderberry, sugar, monk fruit or stevia, please do not participate.

Contacts: Xue Du (Magic): (207)889-7117 or e-mail xue.du@maine.edu;



Dr. Angela Myracle at (207)581-1617 or angela.myracle@maine.edu.

APPENDIX D: ARONIA KEFIR TASTING RECRUITMENT NOTICE

Would you like to eat kefir, a fermented milk beverage? New kefir products are developed and volunteers are needed to provide their opinions; if you are aged 18 or older, and have about 20 minutes spare time, please come and help the researchers!

Testing session will be held in the Consumer Testing Center, Hitchner Hall 158. The date will be announced. Scheduling a time by calling (207)889-7117 or e-mailing xue.du@maine.edu will be preferred; however, volunteers are welcome to show up at any time.

Volunteers will receive \$2 for participating in the study; however, if you are allergic to milk, aronia berry (chockberry), sugar, monk fruit or stevia, please do not participate.

Contacts: Xue Du (Magic): (207)889-7117 or e-mail xue.du@maine.edu;

Dr. Angela Myracle at (207)581-1617 or angela.myracle@maine.edu.



APPENDIX E: ELDERBERRY KEFIR INFORMED CONSENT FORM

Dear Consumer,

You are invited to participate in a research study being carried out by Xue Du, a PhD student under the supervision of Dr. Angela Myracle, Assistant Professor of Human Nutrition from the School of Food and Agriculture at the University of Maine. The purpose of this study is to determine whether new kefir products with underutilized berries (elderberries) and different sweeteners are well accepted by consumers.

If you know that you are allergic or sensitive to milk, elderberry, sugar or stevia, please do not participate; and you must be at least 18 years of age to participate.

What Will You Be Asked to Do?

If you decide to participate in this study, you will be asked to answer a list of questions about yourself. The questions will include your age, gender, and your familiarity of elderberry and kefir. You will be given 6 different samples to taste and evaluate. The test might take 20 minutes.

Risks

The risks of participating in this study should be minimal and no greater than normal daily eating.

Benefits

You will taste new products and may like them. Also, this research may help the elderberry farmers and industries to develop new products and increase food varieties. In addition, this research may increase the fruit intake of consumers.

Compensation

After completing all the questions listed, you will receive \$2. No compensation will be offered if the questions are not completed.

Confidentiality

The data will be collected anonymously, and your name will not be shown in any document. Data will be kept on pass-word protected computers in a locked room. All data will be destroyed no later than 12 months after this study is completed (December 20th, 2016).

Voluntary

Participation is voluntary. You may choose to not participate at the beginning or stop at any time during the study. Compensation will be provided only if you complete the tasting and the questions.

Contact Information

If you have any questions about this study, please contact Xue Du at (207)889-7117 or xue.du@maine.edu; or Dr. Angela Myracle at (207)581-1617 or angela.myracle@maine.edu. If you have any questions about your rights as a research participant, please contact Gayle Jones, Assistant to the University of Maine's Protection of Human Subjects Review Board, at 581-1498 (or e-mail gayle.jones@umit.maine.edu).

Your participation in the study indicates that you have read and understand the above information and agree to participate in the study.

APPENDIX F: ARONIA KEFIR INFORMED CONSENT FORM

Dear Consumer,

You are invited to participate in a research study being carried out by Xue Du, a PhD student under the supervision of Dr. Angela Myracle, Assistant Professor of Human Nutrition from the School of Food and Agriculture at the University of Maine. The purpose of this study is to determine whether new kefir products with underutilized berries (aronia berries) and different sweeteners are well accepted by consumers.

If you know that you are allergic or sensitive to milk, aronia berry, sugar, monk fruit or stevia, please do not participate; and you must be at least 18 years of age to participate.

What Will You Be Asked to Do?

If you decide to participate in this study, you will be asked to answer a list of questions about yourself. The questions will include your age, gender, and your familiarity of aronia (chockberry) and kefir. You will be given 3 different samples to taste and evaluate. The test might take 20 minutes.

Risks

The risks of participating in this study should be minimal and no greater than normal daily eating.

Benefits

You will taste new products and may like them. Also, this research may help the aronia farmers and industries to develop new products and increase food varieties. In addition, this research may increase the fruit intake of consumers.

Compensation

After completing all the questions listed, you will receive \$2. No compensation will be offered if the questions are not completed.

Confidentiality

The data will be collected anonymously, and your name will not be shown in any document. Data will be kept on a pass-word protected computer in a locked room. All data will be destroyed no later than 12 months after this study is completed (December 20th, 2016).

Voluntary

Participation is voluntary. You may choose to not participate at the beginning or stop at any time during the study. Compensation will be provided only if you complete the tasting and the questions.

Contact Information

If you have any questions about this study, please contact Xue Du at (207)889-7117 or xue.du@maine.edu; or Dr. Angela Myracle at (207)581-1617 or angela.myracle@maine.edu. If you have any questions about your rights as a research participant, please contact Gayle Jones, Assistant to the University of Maine's Protection of Human Subjects Review Board, at 581-1498 (or e-mail gayle.jones@umit.maine.edu).

Your participation in the study indicates that you have read and understand the above information and agree to participate in the study.

APPENDIX G: SENSORY EVALUATION QUESTIONNAIRE OF ELDERBERRY KEFIR

Attribute 1: Instruction

Thank you for agreeing to participate in our research. Please click the bottom button on the screen to start the evaluation, and evaluate the samples following the order shown on the screen and verify that each 3-digit code matches the sample that you taste. Please choose the answers that best describe your attitude towards the product. Please take a sip of water between tasting samples.

Attribute 2: check all that apply

Please indicate your gender:

- ◆ Male
- ◆ Female
- ◆ Would prefer not to say

Attribute 3: check one

Please indicate your age range:

- ◆ 18-24
- ◆ 25-34
- ◆ 35-44
- ◆ 45-54
- ◆ 55-65
- ◆ Over 65

Attribute 4: yes or no

Have you ever consumed kefir?

- ◆ Yes
- ◆ No
- ◆ I don't know what kefir is

Attribute 5: check one

How familiar are you with elderberries?

- ◆ Never heard of
- ◆ Somewhat unfamiliar
- ◆ Somewhat familiar
- ◆ Very familiar

Attribute 6: check one

How important is it to you to consider the health benefits of food before purchasing?

- ◆ Not important
- ◆ Slightly important
- ◆ Neutral
- ◆ Moderately important
- ◆ Extremely important

Attribute 7: hedonic scale

How much do you like the color of this sample?

1	2	3	4	5	6	7	8	9
Dislike extremely	Dislike very much	Dislike moderately	Dislike slightly	Neither like nor dislike	Like slightly	Like moderately	Like very much	Like extremely

Attribute 8: hedonic scale

How much do you like the flavor of this sample?

1	2	3	4	5	6	7	8	9
Dislike extremely	Dislike very much	Dislike moderately	Dislike slightly	Neither like nor dislike	Like slightly	Like moderately	Like very much	Like extremely

Attribute 9: hedonic scale

How much do you like the sweetness of this sample?

1	2	3	4	5	6	7	8	9
Dislike extremely	Dislike very much	Dislike moderately	Dislike slightly	Neither like nor dislike	Like slightly	Like moderately	Like very much	Like extremely

Attribute 10: hedonic scale

How much do you like the texture of this sample?

1	2	3	4	5	6	7	8	9
Dislike extremely	Dislike very much	Dislike moderately	Dislike slightly	Neither like nor dislike	Like slightly	Like moderately	Like very much	Like extremely

Attribute 11: hedonic scale

How do you rate the overall acceptability of this product?

1	2	3	4	5	6	7	8	9
Dislike extremely	Dislike very much	Dislike moderately	Dislike slightly	Neither like nor dislike	Like slightly	Like moderately	Like very much	Like extremely

Attribute 12: comment

If you want to refer to any product you have tasted, please use the three-digit code of the product.

Thank you for your time.

Please lift the window up to let the staff know that you are finished.

APPENDIX H: SENSORY EVALUATION QUESTIONNAIRE OF ARONIA KEFIR

Attribute 1: Instruction

Thank you for agreeing to participate in our research. Please click the bottom button on the screen to start the evaluation, and evaluate the samples following the order shown on the screen and verify that each 3-digit code matches the sample you taste. Please choose the answers that best describe your attitude towards the product. Please take a sip of water between tasting samples.

Attribute 2: check all that apply

Please indicate your gender:

- ◆ Male
- ◆ Female
- ◆ Would prefer not to say

Attribute 3: check one

Please indicate your age range:

- ◆ 18-24
- ◆ 25-34
- ◆ 35-44
- ◆ 45-54
- ◆ 55-65
- ◆ Over 65

Attribute 4: yes or no

Have you ever consumed kefir?

- ◆ Yes
- ◆ No
- ◆ I don't know what kefir is

Attribute 5: check one

How familiar are you with aronia berries (Chokeberries)?

- ◆ Never heard of
- ◆ Somewhat unfamiliar
- ◆ Somewhat familiar
- ◆ Very familiar

Attribute 6: check one

How important is it to you to consider the health benefits of food before purchasing?

- ◆ Not important
- ◆ Slightly important
- ◆ Neutral
- ◆ Moderately important
- ◆ Extremely important

Attribute 7: hedonic scale

How much do you like the color of this sample?

1	2	3	4	5	6	7	8	9
Dislike extremely	Dislike very much	Dislike moderately	Dislike slightly	Neither like nor dislike	Like slightly	Like moderately	Like very much	Like extremely

Attribute 8: hedonic scale

How much do you like the flavor of this sample?

1	2	3	4	5	6	7	8	9
Dislike extremely	Dislike very much	Dislike moderately	Dislike slightly	Neither like nor dislike	Like slightly	Like moderately	Like very much	Like extremely

Attribute 9: hedonic scale

How much do you like the sweetness of this sample?

1	2	3	4	5	6	7	8	9
Dislike extremely	Dislike very much	Dislike moderately	Dislike slightly	Neither like nor dislike	Like slightly	Like moderately	Like very much	Like extremely

Attribute 10: hedonic scale

How much do you like the astringency (dry, shrinking mouth-feel of dry wine, tea, or coffee) of this sample?

1	2	3	4	5	6	7	8	9
Dislike extremely	Dislike very much	Dislike moderately	Dislike slightly	Neither like nor dislike	Like slightly	Like moderately	Like very much	Like extremely

Attribute 11: hedonic scale

How much do you like the texture of this sample?

1	2	3	4	5	6	7	8	9
Dislike extremely	Dislike very much	Dislike moderately	Dislike slightly	Neither like nor dislike	Like slightly	Like moderately	Like very much	Like extremely

Attribute 12: hedonic scale

How do you rate the overall acceptability of this product?

1	2	3	4	5	6	7	8	9
Dislike extremely	Dislike very much	Dislike moderately	Dislike slightly	Neither like nor dislike	Like slightly	Like moderately	Like very much	Like extremely

Attribute 13: comment

If you want to refer to any product you have tasted, please use the three-digit code of the product.

Thank you for your time.

Please lift the window up to let the staff know that you are finished.

BIOGRAPHY OF THE AUTHOR

Xue Du was born in Jinzhou, Liaoning Province, China on August 25, 1986. She was raised in Jinzhou, Liaoning Province and graduated from Jinzhou High School in 2005. She attended the Sichuan University in China and graduated in 2009 with a Bachelor's degree in Food Science and Engineering. She went to the United Kingdom and entered the Food Science and Nutrition graduate program at the University of Leeds in the fall of 2009. After receiving her Master of Science degree, Xue went back to Jinzhou and worked in Shuangba Dairy Co. for two years. Xue is a candidate for the Doctorate of Philosophy degree in Food Science and Nutrition from the University of Maine in August 2018.