THESIS

CHARACTERIZATION OF PROTEIN-POLYPHENOL INTERACTIONS BETWEEN NOVEL PLANT PROTEINS (PEA AND HEMP) AND BLUEBERRY POLYPHENOLS WITH RESPECT TO POLYPHENOL BINDING AND DELIVERY

Submitted by

Bianca Chima

Department of Food Science and Human Nutrition

In partial fulfillment for the requirements

For the Degree of Master of Science

Colorado State University

Fort Collins, Colorado

Spring 2022

Master's Committee:

Advisor: Charlene Van Buiten

Sarah Johnson
Jessica Prenni

ABSTRACT

CHARACTERIZATION OF PROTEIN-POLYPHENOL INTERACTIONS BETWEEN NOVEL PLANT PROTEINS (PEA AND HEMP) AND BLUEBERRY POLYPHENOLS WITH RESPECT TO POLYPHENOL BINDING AND DELIVERY

Despite the numerous health benefits associated with polyphenols, dietary intake of this class of compounds is low in the United States due to low intake of fruits and vegetables. It has been shown that dairy foods (i.e. milk, yogurt) increase polyphenol bioavailability due to polyphenols interacting with whey protein, enhancing polyphenol stability and uptake throughout digestion. However, increasing concerns for sustainability and health have introduced a variety of novel plant-based proteins as dairy alternatives. This study aimed to investigate the abilities of edible pea and hemp protein isolates to form complexes with blueberry polyphenol extract (BPE) and characterize the physical and biological functionalities of these complexes compared to whey proteins. Protein/polyphenol solutions were analyzed using UV-Vis spectroscopy to determine if complexation occurred. Secondary structures and binding affinities were analyzed by far-UV CD Spectroscopy and fluorimetry, respectively. In vitro digestion was performed to determine whether the protein profile changed in the presence of BPE via SDS-PAGE and determination of free amino acids using the ninhydrin method. Protein isolates from pea and hemp successfully formed complexes with BPE with binding affinities for the compound similar to whey protein. Relative helicity of the hemp protein was higher than the other protein sources and increased upon complexation with BPE. Furthermore, the SDS-PAGE profiles of all the proteins were the same whether BPE was present or not and the free amino acid content

increased after digestion for the protein and protein/polyphenol solutions. Overall, complexation of BPE with plant proteins was successful. Fluorescence quenching and changes to the secondary structure of the proteins in the presence of BPE indicate that polyphenols were bound but the mechanisms and structures responsible for complexation seem to vary between proteins. More research is needed to determine the interactions that cause binding between the polyphenols and the proteins and whether the bioavailability of the compounds will increase when bound to the proteins in cell model and/or clinical study. This study provides a foundation for exploring the effects of plant-based proteins on phytochemical functionality in complex, “whole food” matrices.

ACKNOWLEDGEMENTS

Many people have helped me throughout this process which I will be forever grateful for. Firstly, I would like to thank my advisor Dr. Charlene Van Buiten for all of her insight and support and for giving me the opportunity to work on a project that so closely aligns with my future goals. I would also like to thank my committee members Dr. Sarah Johnson and Dr. Jessica Prenni for their assistance with materials, instrumentation and encouragement.

Special thanks to Scott Morgan, Emily Nock and Paul Matthews for their work on the turbidity, purification and circular dichroism experiments which I appreciate greatly considering it saved me many extra hours of research.

Thank you to my family, specifically my mother Augustina Chima, who has worked extremely hard to allow me to have the opportunities I currently have and has supported me throughout everything reminding me that I am capable of achieving any goals that I set for myself. Truly, I do not believe I would be where I am without her.

Lastly, I would like to thank all the friends that I have made inside and outside of the department that have made this journey much easier and were always willing to support me through trials and tribulations of graduate school.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

LITERATURE REVIEW

1.1 Introduction

Polyphenols are a class of biologically active compounds found in plant foods such fruits, vegetables, coffee and chocolate. The main constituents of polyphenols are phenolic rings which are attached in various ways to create the different classes. Some of the major classes of polyphenols are phenolic acids, stilbenes and flavonoids (M. Abbas et al., 2016). Polyphenols are not essential to human life like vitamins and minerals but are believed to contribute many benefits to health. The quantity and type of polyphenols found in these foods contribute greatly to their beneficial effects. Berries such as blueberries have been found to have high numbers of polyphenols and are popular among many consumers making them valuable to study for their benefits (Kang et al., 2015). One major complication with polyphenols is that they are not highly bioavailable which limits the ability of the compounds to improve health. The aim of this review is to discuss the health benefits of blueberry polyphenols and their bioavailability. Also, it addresses the current solutions being studied to improve polyphenol bioavailability and why food proteins specifically plant proteins, may be adequate to solve the issue naturally based on how they are customarily consumed as a part of a food matrix.

1.2 Health Benefits of Consuming Blueberry Polyphenols

Polyphenols, which are a class of secondary plant metabolites, have gained interest in recent years for providing additional health benefits from consuming fruits and vegetables. Some of the most notable benefits have been the ability of these compounds to acts as antioxidants, reduce

the risk for metabolic syndrome, obesity, cardiovascular disease and inflammation. In general, polyphenols consumed as a part of a Mediterranean diet were found to reduce the risk of mortality in Spanish individuals by 37% as a part of a parallel-group, randomized, controlled feeding trial that occurred over the course of about five years (Tresserra-Rimbau et al., 2014). Moreover, various types of foods and drinks such as tea and berries have been shown to provide these effects. Blueberries have been studied for their potential health effects because of their popularity with consumers and high nutritional value (Kang et al., 2015).

Obesity is currently a major health concern in America. In 2015, approximately 75% of Americans were overweight or obese, 41% being obese and these values are projected to grow (Wang et al., 2020). Research continues to expand in this area to find solutions to this epidemic. Blueberry polyphenols have been found to potentially reduce the risk for obesity and negative outcomes associated with the disease. One study conducted on rats that consumed a high fat diet in addition to a blueberry polyphenol extract for four weeks found that weight gain was reduced by 6.7% in comparison to the high fat diet only group. Additionally, LDL cholesterol levels were significantly decreased by the addition of polyphenols to the diet while HDL cholesterol increased. Furthermore, factors of lipid metabolism such as PPARγ, FAS and SREBP-1 were downregulated as a result of consumption of the polyphenols (Jiao et al., 2019). Similarly, a study conducted in rats, found that blueberry proanthocyanidins reduced visceral obesity and weight gain (Morissette et al., 2020).

Researchers have investigated the effects of cranberries which have similar polyphenolic constituents to blueberries, on conditions related to obesity *in vivo*. Cardiovascular disease (CVD) and type II diabetes mellitus are diseases typically related to obesity that are also being targeted for treatment. The study showed that consumption of cranberry polyphenols in humans

was able to increase insulin sensitivity which would reduce the risk for type II diabetes. Also, HDL cholesterol increased, and the presence of C-reactive protein decreased which can dimmish the potential for developing cardiovascular disease (Chew et al., 2019). Vascular function was also found to improve *in vivo* after the consumption of a blueberry drink in healthy men. Flow mediation dilation increased after intake, which could lead to decreased blood pressure and reduced risk for CVD (Rodriguez-Mateos et al., 2013). Nonalcoholic fatty liver disease is another condition typically associated with metabolic diseases such as obesity. Blueberry polyphenols were found to reduce the production of triglycerides which could prevent or prolong the first stage of the disease (Liu et al., 2011).

Polyphenolic compounds have also been cited to reduce inflammation in a number of diseases including cancer and act as antioxidants to prevent some inflammatory responses from occurring in the first place. Several studies have discussed the ability for blueberry polyphenols to reduce the presence of inflammatory cytokines such as IL-6, TNF-α, IL-1β *in vitro* (ben Lagha et al., 2015; Kang et al., 2015). Also, nitric oxide synthase and cyclooxygenase, enzymes involved in lipopolysaccharide induced inflammation, have been found to be reduced *in vitro* after treatment with blueberry polyphenol extract (Lau et al., 2009).

Studies have shown that blueberry polyphenols have the potential to reduce oxidative stress in cardiomyocytes, erythrocytes and after exercise (Kang et al., 2015; Louis et al., 2014; McAnulty et al., 2004). Furthermore, inflammation and oxidative stress are conditions associated with the development of all cancers, therefore the disease may be mediated by the consumption of blueberry polyphenols. One study found that crude fractions of blueberry polyphenols reduced tumor growth in rats. Also, breast cancer cell metastasis was reduced as well as the severity of lung tissues damage showing that blueberry polyphenols may be able to slow down the

progression of the disease (Yang et al., 2021). Despite the numerous benefits of blueberry polyphenols discussed, many of these outcomes may not actually be observed in humans due to the low intake and low bioavailability of these compounds.

1.3 Scope of Polyphenol Consumption in the United States (US)

Fruit and vegetables contain numerous health promoting compounds such as fiber, vitamins, minerals and polyphenols. Although these benefits are well known, consumption of fruits and vegetables remains low in the United States. The United States Department of Agriculture recommends consuming 5 to 9 servings of fruit and vegetables a day, which many Americans fall below (Tkacz et al., 2021). The National Health and Nutrition Examination Survey (NHANES) from 2013-2016 found that about 70% of US consumers take in 4 servings or less of fruit and vegetables on any given day and 37% consume 2 servings or less (Katherine Hoy et al., 2020). As a result of low intake of fruits and vegetables, polyphenol consumption also remains low in the US.

Polyphenol intake has been estimated to be around 800 to 1600 mg per day (Burkholder-Cooley et al., 2016; Huang et al., 2020). This would be equivalent to around two to four servings of fruit or vegetables per day, but this is not how many Americans obtain their daily polyphenol intake (Zhong et al., 2017). Most polyphenol consumption comes from non-alcoholic beverages, the main source being coffee. Coffee was found to make up around 40 to 65 percent of the consumption of polyphenols, while fruits and vegetables made up only 15 to 20 percent (Burkholder-Cooley et al., 2016; Huang et al., 2020). Furthermore, older, educated, non-Hispanic white females with a normal BMI between 18.5-24.9 typically had higher intakes of polyphenols than other groups (Huang et al., 2020). This parallels with groups that traditionally

have greater access to these foods and more education on nutrition (Gundersen et al., 2011). Access to fruits and vegetables is limited for many individuals, which would hinder their ability to be able to consume polyphenols despite their many health benefits.

1.4 Bioavailability of Polyphenols

The bioavailability of all polyphenols has been found to be between 5 to 10 percent in the gastrointestinal tract, which indicates that most of the polyphenols consumed are not being fully absorbed (Fang & Bhandari, 2010). In a clinical trial where participants consumed a blueberry drink containing polyphenols equivalent to two servings of blueberries, only 1% of anthocyanins were present in the plasma (Zhong et al., 2017). Likewise, another study in which human subjects consumed a blueberry juice with similar phenolic composition, found that anthocyanin content was also low in the plasma after intake. The absorption rate for two types of anthocyanins, malvidin-3-glucoside and petunidin-3-glucoside, was measured in a cell model and found to be 0.5% and 0.6%, respectively (Kuntz et al., 2015).

The lack of bioavailability of polyphenols in general is believed to result from pH changes, enzymes and fluids throughout the gastrointestinal tract which impacts their solubility. Many polyphenols tend have low solubility or permeability through the cell membrane. Their intestinal impermeability may be due to lack of receptors to transport polyphenols which makes only low molecular weight polyphenols able to diffuse through the membrane. Another factor that affects the bioavailability of polyphenols is the transformation of these compounds by gut microorganisms. During phase I and II metabolism of polyphenols, gut microorganisms alter the structure of polyphenols which leads to them being excreted in the urine rather than being transported to the small intestine where they can be absorbed (Annunziata et al., 2020). All of

these factors contribute to the lack of bioavailability of these compounds, but various strategies are being investigated to improve their absorption.

1.5 Strategies for Improving the Bioavailability of Polyphenols

Nanodelivery systems have been the main strategy for trying to enhance the bioavailability of polyphenols. These systems include the use of nanoemulsions, micelles, microencapsulation, and lipid, polymeric, and silica nanoparticles (Z. Zhang et al., 2021). Fiber has been a material researched to be used to form these delivery systems. One type of fiber that has been studied is chitosan, which comes from the outer skeleton of shellfish, and has been found to enhance the bioavailability and bioactivity of polyphenols such as quercetin and curcumin in nanodelivery systems (Tang et al., 2020). Pectin, a fruit fiber, in a nanoencapsulation system has been found to increase the release of bilberry anthocyanins into the gastrointestinal tract (Oidtmann et al., 2012). Furthermore, chitin, a fiber found in the cell wall of fungi and arthropods, were used to create microspheres that enhanced the amount of anthocyanins delivered to the GI tract. When the polyphenols were also coated in ethyl cellulose, it increased their delivery to up to 85% (Wang et al., 2017). Synthetic polymers including poly(lactide-co-glycolide), polyethelene gycol, polycaprolactone and polylactide have also been used to encapsulate resveratrol and found to increase uptake of the compound 7-fold in a cell model. Another type of delivery system that has been used are liposomes which have also been shown to increase bioavailability of polyphenols (Annunziata et al., 2020).

Many of the strategies currently being studied to enhance the bioavailability of polyphenols are addressing the pharmaceutical and supplement industries, targeting methods such as

nanoencasuplation. Therefore, few approaches have been studied on whether the food matrix can be used improve the bioavailability of these compounds.

1.6 Whey Protein as a Strategy for Delivering Polyphenols

Various types of proteins have been found to interact with polyphenols to be used in nanodelivery systems. Some of these proteins include ferritin, gelatin, albumin, and collagen but studies focusing on the effects of these compounds on bioavailability as a part of the food matrix are limited (Z. Zhang et al., 2021). Whey protein is one of the most popular proteins currently studied for its potential to increase polyphenol bioavailability. Several studies have shown that whey proteins interact with many different types of polyphenols spontaneously (Meng & Li, 2021; Ni et al., 2020; Schneider et al., 2016). The complexes that are formed have been found to have the same antioxidant activity as the polyphenols alone (Ming et al., 2020; Schneider et al., 2016). One study even found that radical scavenging ability of chlorogenic acid was greater when bound to whey protein than just chlorogenic acid alone (Jiang et al., 2018). Another study that assessed the complexes as a part of a food matrix, found that cookies containing freeze dried whey protein and blackcurrant complexes had greater antioxidant activity than the control cookies. Furthermore, the cookies containing the polyphenol-protein complexes exhibited a lower glycemic response which could help with blood glucose control in those with poor regulation (Wu et al., 2021).

The complexation of whey protein and polyphenols has been found to improve bioavailability and bioaccessibility *in vitro*. In an intestinal cell model, the binding of apple polyphenols to whey protein increased the bioavailability of the polyphenols by approximately

15% (Li et al., 2022). Another study found that blueberry polyphenols and whey protein aggregates would be able to deliver approximately two servings worth of polyphenols (Diaz et al., 2020). Similarly, the bioaccessibility of sea buckthorn polyphenols complexed to whey protein increased by 20% which indicates that the polyphenols would be more available for cell processes (Ashwar & Gani, 2021).

Not only do whey protein-polyphenol complexes serve the potential to be beneficial to health but they can also improve the functional properties of the foods that they are used in. Several studies have shown that whey protein and polyphenol aggregates can act as food ingredients to improve emulsification properties, solubility, stability and foaming capacity of a product (Meng & Li, 2021; Thongkaew et al., 2014; Tian et al., 2021). Soy protein, one of the most well-known and well researched plant proteins, also has exhibited similar outcomes when complexed with polyphenols. Bioaccessibility of soy protein bound to polyphenols from a plant commonly known as Russian tarragon, increased by 15% and the polyphenols were found to be eight times more bioavailable (Ribnicky et al., 2014). Soy protein and polyphenols aggregates were also found to maintain their antioxidant activity and produced hypoglycemic affects in rats (Djuardi et al., 2020; Roopchand et al., 2013). All in all, whey protein is traditionally consumed as a part of a dairy and fruit food matrix, which makes it a good candidate to help improve the bioavailability of fruit polyphenols in its natural form.

1.7 Popularity and History of Consuming Fruit and Dairy

Yogurt is a popular way to consume dairy due to its convivence and nutritional value. The yogurt industry is estimated to reach 9.1 million dollars by the end of 2021 and is expected to grow another 2.5% (*US Yogurt and Yogurt Drinks Market Report 2021*, n.d.). Furthermore, one

of the most popular varieties of yogurt are fruit flavored and fruit containing yogurts which are produced by adding fruit puree to the bottom or stirring it throughout (Chandan et al., 2017). Since the introduction of yogurt, consumers have had a strong interest in consuming fruit flavored yogurt. Nearly 50% of consumers were found to have a preference for purchasing a fruit flavored yogurt beverage (Ryan et al., 1984). Fruit inclusions are preferred by US consumers in their yogurt to offset the tart flavors present from lactic acid (Das et al., 2019). Additionally, epidemiological studies have shown that consuming fruit and yogurt can reduced the risk for obesity, type II diabetes, and cardiovascular disease which is believed to be caused by the symbiotic affects from probiotics and fiber found in fruit but could potentially be due to other compounds as well (Fernandez & Marette, 2017).

Smoothies are another popular way to consume dairy and fruit. In 2020, the global smoothie market was worth 12.1 billion and is expected to reach just over 17 billion dollars by 2025 (Cano-Lamadrid et al., 2020). Smoothies have gained popularity due to consumer preferences for ready to eat and on the go options (Tkacz et al., 2021). Also, smoothies are a convenient way to increase the intake of fruits and vegetables considering one smoothie can have more than one fruit or vegetables. Both of these food options have been customarily prepared with dairy, but in recent years there has been an increase in consumption of plant-based alternatives for a multitude of reasons.

1.8 History, Popularity and Consumer Reasons for Consuming Plant-Based Dairy Alternatives

The plant-based food industry has grown drastically in the last decade. From 2017 to 2019, the market for these food products grew 29% and is estimated to reach a market value of nearly 5

billion dollars (McClements & Grossmann, 2021). Plant-based dairy is one of the largest sections of the plant-based foods market, making up approximately 40%. The market consists of milk, yogurt, cheese, creamers and ice cream alternatives produced using a variety of different plant sources such as almond, soy, and coconut (Alcorta et al., 2021). The consumption of plant-based dairy dates back thousands of years. Soymilk has been estimated to be produced in China for the past 2000 years, but commercial soymilk production began within the last 100 years (Mendly-Zambo et al., 2021). Since its introduction, plant-based dairy has become a staple in many households all over the world. In a 2020 survey of nearly a thousand households, it was determined that 40% of households consume plant-based dairy on a regular basis. On the other hand, dairy milk sales have decreased nearly 3 billion from 2013 to 2018 (Wolf et al., 2020).

People consume plant-based dairy for numerous reasons including health, ethical, and environmental considerations. A global survey in 2019 found that 40% of consumers are trying to reduce their consumption of animal protein. In the US, the number of vegans increased from nearly 4 million in 2014 to 19.6 million in 2017. (Alcorta et al., 2021). Lactose intolerance and milk allergy prevalence have also increased over the years. It is estimated worldwide that 75% of people are lactose intolerant. Lactose intolerance results in gastrointestinal symptoms such as bloating, flatulence, and abdominal pain after consumption of dairy products. Asian, Black, and Hispanic populations are affected at a greater rate and the only solution is to avoid consumption of lactose containing dairy products (Mäkinen et al., 2015).

Consumers have a perception of plant-based milk being more sustainable than cow's milk. Almond-based products, which are preferred by many consumers, contradict this assumption due to irrigation issues caused by the use of large amounts of water in the production of almond milk. However, legume production has been shown to produce less greenhouse gas

emissions than other agricultural sectors. Likewise, emerging plant protein sources, such as microalgae, require less land for protein production in comparison to pork, chicken, and beef. Even the Academy of Nutrition and Dietetics considers plant-based diets more environmentally sustainable than other diets due to its use of fewer natural resources and decreased environmental damage. In general, plant-based industries offer more environmentally friendly practices than animal-based ones (Alcorta et al., 2021).

Lastly, one of the major reasons why people consume plant-based alternatives is for their health. Reducing cholesterol is one the benefits most desired from the consumption of non-dairy milk, and studies have shown that consumption of cow's milk has been associated with increased risk of certain cancers (Vanga & Raghavan, 2017). A review of plant-based diets has also found that they were associated with decreased risk of cardiovascular disease (Satija & Hu, 2018). Furthermore, a review of the nutritional content of four types of plant-based milks: almond, soy, rice, and coconut found that overall, the milks were lower in calories, lack cholesterol, and have a comparable mineral content to normal milk. The reduction of LDL cholesterol levels from almond and soy milk is attributed to monounsaturated fatty acids which have a host of other health benefits as well (Vanga & Raghavan, 2017). Overall, a healthful plant-based diet has been associated with decreased risk of disease, which is why many consumers are moving towards these products increasing the need for more research in this area.

CHAPTER II

STATEMENT OF PURPOSE

Polyphenols have been shown to have positive effects on human health. One of the major pitfalls associated with their consumption, is that despite these benefits, many polyphenols are not highly bioavailable (Fang & Bhandari, 2010). Numerous techniques have been studied to help improve the bioavailability of these compounds, but few have taken into account how these foods are consumed as a part of a food matrix. Polyphenols can interact and form complexes with different types of dietary proteins which has led to improved bioavailability and bioaccessibility (Z. Zhang et al., 2021). There is evidence to support that fruit and vegetables are often consumed in mixed meals rather than as a snack or side on their own (Katherine Hoy et al., 2020). Specifically, dairy and fruit have been consumed together in many food products such as yogurt and smoothies. Therefore, assessing the interactions between dairy proteins and polyphenols would be a viable option to try to investigate the potential for these proteins to aid in the delivery of polyphenols when consumed as a part of a whole food. Consumers are moving towards plant based dairy alternatives for health and ethical reasons (Alcorta et al., 2021)which is why it is important to establish these relationships between plant proteins in addition to dairy proteins.

The objective of this study was to determine whether commercially available protein powders from whey and novel plant protein sources (pea and hemp) would form complexes with blueberry polyphenol extract. Moreover, if complexation occurred, we sought to investigate the affinities of each protein for polyphenols, and how complexation would affect the digestibility and structural characteristics of the proteins. We hypothesized that all three protein types would

form complexes with blueberry polyphenols and that the proteins would remain digestible in the presence of the compounds. Additionally, we expected the protein structure to change in the presence of the phenolics and that the different protein sources would exhibit similar binding affinities. The characterization of these interactions will act as a basis for future studies to determine whether the bioavailability of blueberry polyphenols is improved by its complexation with these proteins in a transepithelial cell model.

CHAPTER III

MANUSCRIPT

3.1 Introduction

Polyphenols are a class of phytochemicals that have been shown to have to confer beneficial effects on health. Berries such as blueberries (*Vaccinium cyanococcus*) contain high levels of polyphenols specifically anthocyanins. Polyphenols present in blueberries have been found to have protective effects against various diseases such as CVD, obesity, and cancer (Chew et al., 2019; Jiao et al., 2019; Yang et al., 2021). Blueberries are second-most consumed berry by Americans which makes them an important berry to study for their health effects (Kang et al., 2015). Even though blueberry consumption is high, studies have shown that overall consumption of polyphenols is low in Americans due to their low consumption of fruits and vegetables (Huang et al., 2020). Furthermore, the functional foods market is growing as a result of consumers being more interested in health-promoting foods, so research is also increasing in this area to find ways to maximize the health benefits of certain foods (Devcich et al., 2007).

Although polyphenols have many health benefits, the bioavailability of many of these compounds is typically low. The bioavailability for anthocyanins has been found to be between 0.7 and 1.1 %, which means most of the polyphenols consumed are not readily absorbed (Zhong et al., 2017). Strategies for increasing intake have been explored, including incorporation of polyphenol-rich foods into mixed meals. It has been shown that binding of these compounds to macromolecules such as proteins in dairy foods may be able to improve their absorption. Smoothies are a popular way to consume fruits and vegetables since consumers are able to reap the benefits of these foods without the hassle of preparation. In 2020, the global smoothie market was worth 12.1 billion and is expected to reach just over 17 billion dollars by 2025 (Cano-Lamadrid et al., 2020). Smoothies are typically made of fruit and some liquid (juice or dairy

milk) blended together and is a common way to consume dairy foods (Teleszko & Wojdylo, 2014). Additionally, yogurt, another dairy product typically flavored with fruit has gained popularity in recent years for its beneficial effects on gut health. The spoonable and drinkable yogurt industry is estimated to reach 9.1 million dollars by the end of 2021 and is expected to grow another 2.5% (*US Yogurt and Yogurt Drinks Market Report 2021*, n.d.).

As these dairy foods become more popular, non-dairy alternatives are also being created as consumers look for more health promoting and sustainable foods. A global survey in 2019, found that 40% of consumers are trying to reduce their consumption of animal protein (Alcorta et al., 2021). Moreover, some of the most commonly consumed non-dairy products are sourced from oat, soy, almond and coconut. Although, there are several plant protein options on the market, innovative plant sources are being studied considering the allergenicity and low functionality of the types of proteins available currently. Pea and hemp proteins are novel plant proteins being considered for use in these products due to their sustainability, lack of allergenicity and high nutritional value (Gao et al., 2020; Mamone et al., 2019).

It has been established on multiple accounts that dairy protein specifically whey protein can form complexes with polyphenols (Baba et al., 2021). Despite the fact that polyphenols have been found to have a low digestibility and can demonstrate anti-nutritional effects against proteins (Samiya et al., 2020), studies have shown that when bound to whey protein there is no effect on the protein digestibility. A study conducted by de Morais et al. demonstrated that when whey protein was digested with caffeic acid and epigallocatechin gallate (EGCG), there was no profound difference in protein digestibility (de Morais et al., 2020). However, this phenomenon has not been studied in the context of plant proteins with specifically blueberry phenolics which

can provide a new perspective on how to improve the bioavailability of these compounds maximizing their potential health benefits.

The goal of this study was to determine whether commercially available plant protein powders from novel plant protein sources (pea and hemp) would form complexes with blueberry polyphenol extract similarly to what has been observed for whey protein. Upon successful complexation of the proteins and polyphenols, we aim to assess the affinities for the polyphenols to bind the proteins and how binding would affect protein structure and digestibility. We hypothesized that the pea and hemp proteins would form complexes with blueberry polyphenols and that the proteins would remain digestible when bound to the compounds. Furthermore, we expected the protein structure to change in the presence of the phenolics and that all three protein sources would exhibit similar binding affinities. This research will guide future studies that determine whether the bioavailability of blueberry polyphenols is improved by its complexation with these proteins in a transepithelial cell model.

3.2 Materials & Methods

3.2.1 Polyphenol Extraction

3.2.1.1 Crude Blueberry Polyphenol Extraction

Powdered blueberries were purchased from Bulk Supplements (Henderson, NV) and purified. The crude blueberry powder was mixed with 50% ethanol at a ratio of 1:5 (blueberry powder: 50% ethanol). The pH of the solution was lowered to 3 using concentrated sulfuric acid and then mixed for two hours. The ethanol was removed using rotary evaporation at 80°C. Once cooled, the samples were frozen at -20°C for further processing.

3.2.1.2 C18 Solid Phase Extraction

C18 solid phase extraction was performed to remove residual sugars and polysaccharides from solution. The purified blueberry solution was diluted 1:1 using acidified water (1% acetic acid in water). C18 solid phase extraction columns were conditioned with methanol then acidified water, twice for each solution. The diluted blueberry solution was added to the column and the solution was moved through the column using a vacuum. The column was washed thrice with acidified water and then dried in preparation for elution. The column was eluted twice using acidified methanol (1% acetic acid in methanol). The solvent was removed using rotary evaporation at 80°C. The blueberry polyphenol extract (BPE) was diluted with ultrapure water and frozen at -20°C. The samples were freeze dried and stored at -20°C for further analysis.

3.2.1.3 Determination of Total Phenolic Content

Total phenolic content of BPE was determined using the Folin-Ciocalteau assay. Freeze-dried blueberry polyphenol extract was used to create a 1 mg/mL solution. A standard curve was produced using gallic acid with concentrations ranging from 0 to 500 µg/mL. 10 µL of sample and standard solutions were added to 790 µL of ultrapure water and 50 µL of Folin reagent (Sigma-Aldrich, St. Louis, MO) and shaken for 5 minutes. After mixing, 150 µL sodium carbonate solution at a concentration of 200 g/L was added to each sample and the plate was incubated at 37 °C. After incubation, absorbance was measure using a Bio-Tek UV-Vis plate reader (Winooski, VT) at 765 nm.

3.2.2 Protein Purification

3.2.2.1 Fat extraction using the Soxhlet Method

Pea protein powder (Bulk Supplements, Henderson, NV) and hemp protein powder (Fit Hemp LLC, Melbourne, FL) were defatted using Soxhlet extraction with hexane. Whey protein isolate (Nutricost, Vineyard, UT) contained no fat, therefore did not require this procedure. The Soxhlet extraction was performed for 8 hours. Rotary evaporation was used to determined how much fat was extracted.

3.2.2.2 Alkaline Extraction-Isoelectric Point Precipitation

The protein extraction method was adapted from a by Shen et al (Shen et al., 2020). Defatted pea and hemp protein powders underwent an alkaline extraction and isoelectric point precipitation to isolate the protein and remove insoluble fiber. The whey protein isolate contained no other ingredients, therefore did not require this procedure. The defatted protein powders were dissolved in ultrapure water at a ratio of 1:15 (protein powder: water). The pH was adjusted to 10 for the hemp protein and 9.5 for pea using 2 M NaOH. The solutions were then stirred for 2 hours at 600 rpm. After stirring, the solutions were centrifuged at 4150 rpm for 20 minutes and the pellet was discarded. The collected supernatant was adjusted to a pH of 5 for the hemp and 4.5 for the pea using 1.2 M HCl. The adjusted solutions were centrifuged once again for 10 minutes at 4150 rpm. The precipitates were collected and resuspended in ultrapure water. The pH was adjusted to 7 using 2 M NaOH, frozen at -20°C, and then freeze dried for further analysis.

3.2.2.3 Determination of Protein Content

Protein Concentration was determined using the BCA assay with the Pierce BCA Protein Assay kit from Thermo-Scientific (Waltham, MA). All samples were prepared at a concentration of 1 mg/ml and solubilized in 0.1 M NaOH. Assay was performed according to instructions provided with the kit. A standard curve was generated using bovine albumin serum (BSA) with concentrations ranging from 0 to 2 mg/mL. Absorbances were measured using a BioTek UV-Vis plate reader at 562 nm.

3.2.3 Sample Preparation

Samples were prepared to mimic the conditions of a beverage with a single serving of protein supplement (15 g protein) and the polyphenolic equivalent of 175 g of highbush blueberries (1 cup; 490 mg polyphenols from BPE). Samples were prepared to contain pea, whey or hemp protein alone or the forementioned proteins in addition to polyphenols. Samples had a protein concentration of 1 mg/mL protein, unless otherwise stated and subsequently a concentration of 33 µg/mL of blueberry polyphenol extract, when the samples contained polyphenols, to match the desired ratio of protein to polyphenols (30:1). All samples used a pH 6.8 10 mM phosphate buffer for the solvent, unless otherwise stated.

3.2.4 Turbidity Measurements

A Jasco V-730 UV-Vis Spectrophotometer (Talbot County, MD) was used to measure the turbidity of whey, pea and hemp protein solutions and the respective solutions containing blueberry polyphenols. Turbidity measurements were also taken over a range of different pH values (2.0, 4.6, 6.8, 7.4) to assess the changes in complexation at food production and digestion

related values. The values correlated to gastric, yogurt, duodenal/protein shake, and ileac pH, respectively. Absorbance of the solutions were measured at a wavelength of 600 nm.

3.2.5 Circular Dichroism (CD)

A Jasco J-1100 Circular Dichroism Spectrophotometer (Talbot County, MD) was used to measure the changes in secondary protein structure of whey, pea and hemp protein in solution and when bound to blueberry polyphenols. The samples were prepared with a protein concentration of 0.1 mg/mL and the protein and polyphenol. Measurements were taken at a wavelength from 190 to 260 nm. The data was interpreted using the CD Plotting and Analysis tool (CAPITO) (Wiedemann et al., 2013).

3.2.6 Fluorescence Spectroscopy

Fluorescence spectroscopy was performed to determine the binding affinities for blueberry polyphenols binding to pea, whey and hemp proteins. Protein solutions were created at a concentration of 0.5 mg/mL in the presence or absence of polyphenols. BPE controls were produced for all polyphenol concentrations. The protein concentration remained the same in all solutions, but polyphenol concentrations ranged from: 0.066 mg/mL, 0.033 mg/mL, 0.017 mg/mL (ideal ratio, 30:1), 0.008 mg/mL, 0.004 mg/mL. An Edinburgh Instruments Spectrofluorometer FS5 (Livingston, UK) was used to measure the samples and the Flouracle program was used for the data output. An emission scan was run with an excitation wavelength of 280 nm and emission spectrum from 300 to 450 nm. The following emission scan parameters were applied: dwell time 0.2, steps: 1 nm, # of scans: 3, all corrections applied. BPE controls

were subtracted from each spectrum. Data was analyzed using Stern-Volmer plots and calculation of Hill coefficients to determine the number of binding sites for each protein source.

3.2.7 Analysis of the Digestibility of Pea, Hemp and Whey Protein with Blueberry Polyphenol Extract

3.2.7.1 In Vitro Digestion

An *in vitro* digestion was performed with solutions of pea, whey, and hemp protein in the presence and absence of blueberry polyphenol extract. The pH of the solutions was adjusted to approximately 2 using 1 M HCl to mimic the conditions of the stomach. Pepsin was added at a concentration of 0.3 mg/mL and the samples were incubated at 37°C for two hours. After the two hours, the pH was adjusted to 7.4 using 2 M NaOH which mimics the conditions of the small intestine. Trypsin was added at a concentration of 0.3 mg/mL and the samples were incubated at 37°C for another four hours. Once the digestion was complete, the samples were boiled for 30 minutes to inactivate the enzymes. Aliquots of the of the pure solution and supernatant of the solution were taken at the beginning and end of the digestion. The samples were stored at -20°C until further analysis.

3.2.7.2 Determination of Free Amino Acids

The ninhydrin method adapted from Moore and Stein and Reyanaud et al. was used to determine the free amino acid content of the digested samples (Moore & Stein, 1954; Reynaud et al., 2020). Trione ninhydrin reagent (Pickering Laboratories, Mountain View, CA) was mixed with the supernatant of the digested samples in a ratio of 1:1. The samples were heated for 16 minutes at 95°C. Once cooled to below 30°C, 50% ethanol was added at a ratio of 1:5 (ninhydrin

reagent: 50% ethanol). Absorbance was measured using a BioTek UV-Vis Plate Reader at a wavelength of 570 nm. L-leucine with concentrations ranging from 0 to 4 mM was used to produce a standard curve.

3.2.7.3 Sodium Dodecyl Sulphate–Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Digested samples were analyzed using SDS-PAGE. Samples were mixed with 2X Tris glycine loading buffer (Invitrogen, Waltham, MA) containing 5% beta-mercaptoethanol. The prepared loading buffer and sample were added to a tube at a ratio of 1:1 and boiled for 10 minutes. The gel box was loaded with 1X tris glycine running buffer. Tris-glycine gels (Invitogen, Waltham, MA) were used and 5 µL of Novex Sharp Pre-Stained Protein Standard (Invitogen, Waltham, MA) was added to one well. The prepared samples were added at a volume of 15 µL to the other wells. The gel was run at 165 V. After the gel finished running, it was stained using Coomassie brilliant blue for an hour. The gel was then destained for four hours using a solution of 50% methanol and 10% acetic acid. A second destaining step was performed to rehydrate the gel for imaging using a solution of 20% methanol and 10% acetic acid overnight. The gel was imaged using the UVP Bioimaging System (Upland, CA).

3.2.8 Statistical Analysis

All results are expressed as an average of triplicate measurements, unless otherwise stated. Values are presented as the mean ± standard deviation. Variance analysis was performed using two-way ANOVA with Šídák's multiple comparisons to compare the protein samples without polyphenols to those containing polyphenols. One-way ANOVA with Tukey's multiple comparisons was used to compare the protein types to one another. GraphPad Prism v 9.3.1

(GraphPad Software, Inc., San Diego, CA, USA) was used to perform all statistical analysis and significant statistical difference was calculated at a $p<0.05$ level.

3.3 Results

3.3.1 Physical and Chemical Characterization of the Protein-Polyphenol Complexes

The total polyphenol content of the blueberry extract was determined to be 260.1 ± 1.8 mg gallic acid equivalents (GAE) per g extract. The protein content of the hemp, pea and whey protein powders were determined to be 73.8%, 50.4% and 69.7% respectively. The first analysis that was performed were turbidity measurements to determine whether the proteins did form complexes with the blueberry polyphenols. The addition of blueberry polyphenols to purified proteins resulted in the formation of protein-polyphenol aggregates. The absorbance of all three protein sources increased significantly in presence of blueberry polyphenol extract indicating complexation. The difference in absorbance between the proteins alone and the proteins in the presence of polyphenols is not equivalent to the polyphenol blank alone indicating that the differences observed are as result of the polyphenols binding to the proteins. Moreover, hemp protein increased the turbidity of the solutions when bound to polyphenols to a greater degree than the pea and whey proteins.

Aggregate formation was found to be pH dependent for the pea and hemp proteins. At pH 4.6, pea protein exhibited the highest increase in turbidity indicating increased complexation with BPE. At pH 6.8, the turbidity of the pea protein-BPE complexes decreased significantly due to decreased complex formation. In contrast, the hemp protein exhibited the highest levels of complexation at pH 6.8 and 7.4. Whey protein did not exhibit any pH-dependent changes in complex formation.

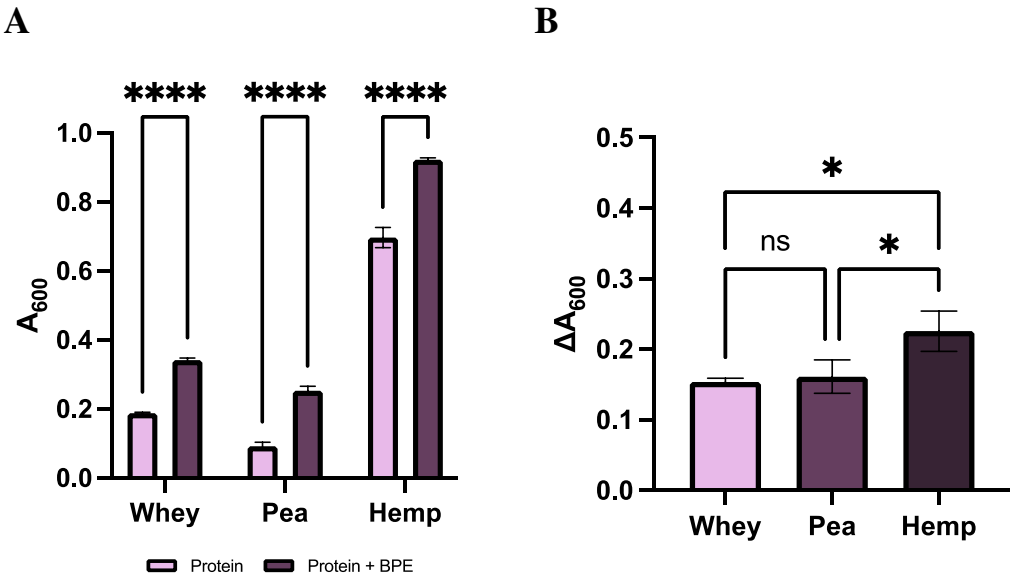


Figure 1. (A) Turbidity of pea, whey and hemp protein in the presence and absence of BPE expressed as average absorbance at 600 nm. Values are expressed as mean ± SD. Asterisks indicate significant differences ($p<0.05$) based on 2-way ANOVA with Šídák's multiple comparisons. (B) Difference in turbidity between the whey, pea and hemp samples with or without BPE added. Asterisks indicate significant differences ($p<0.05$) based on one-way ANOVA with Tukey's multiple comparisons.

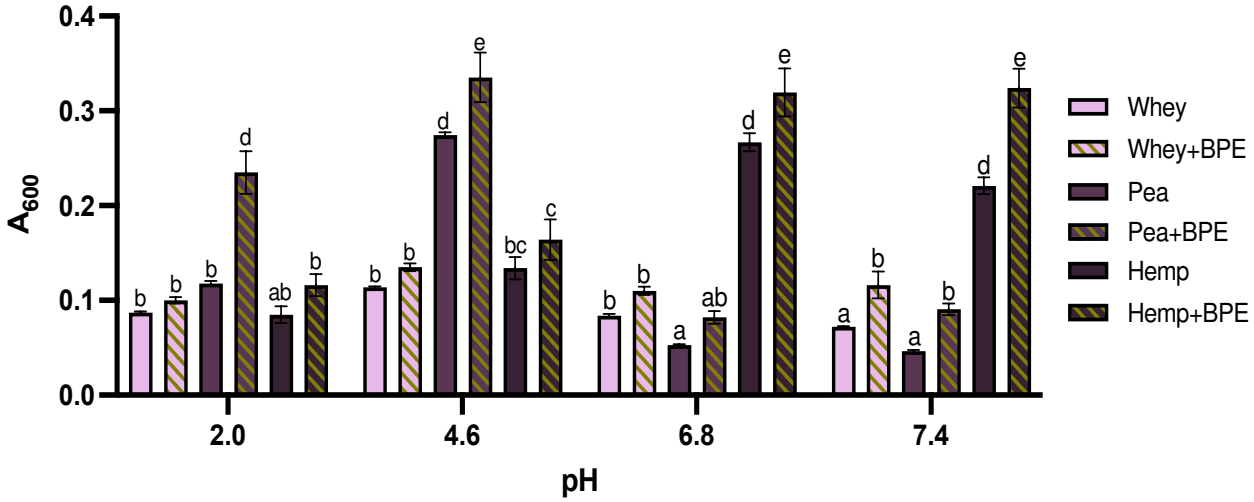


Figure 2. Turbidity of pea, whey and hemp protein in the presence and absence of BPE at pH values related to digestion and food production expressed as average absorbance at 600 nm. Values are expressed as mean ± SD. Different letters in a column indicate significant differences ($p<0.05$) based on one-way ANOVA with Tukey's multiple comparisons.

3.3.2 Polyphenol Binding Affects Hemp Protein Structure

Far UV-Vis circular dichroism was used to determine the secondary protein structures of pea, hemp and whey protein isolates and how it changed when bound to blueberry polyphenols. The relative helicity of the hemp protein increased significantly when bound to polyphenols. Also, hemp protein exhibited a higher relative helicity when compared to pea and whey protein. This indicates that hemp protein has a greater proportion of alpha-helices in its secondary structure than the other protein types. In contrast, both pea and whey proteins did not show significant changes in relative helicity when bound to BPE. The content of other secondary protein structures such as beta sheets, beta-turns, and random coils was not measured but it can be implied that other secondary structures were more prevalent in the whey and pea proteins. Whey, pea and hemp proteins were found to adopt primarily unfolded structures based on comparisons to the CAPITO circular dichroism database. This information could give more insight on how the protein-polyphenol interactions could occur.

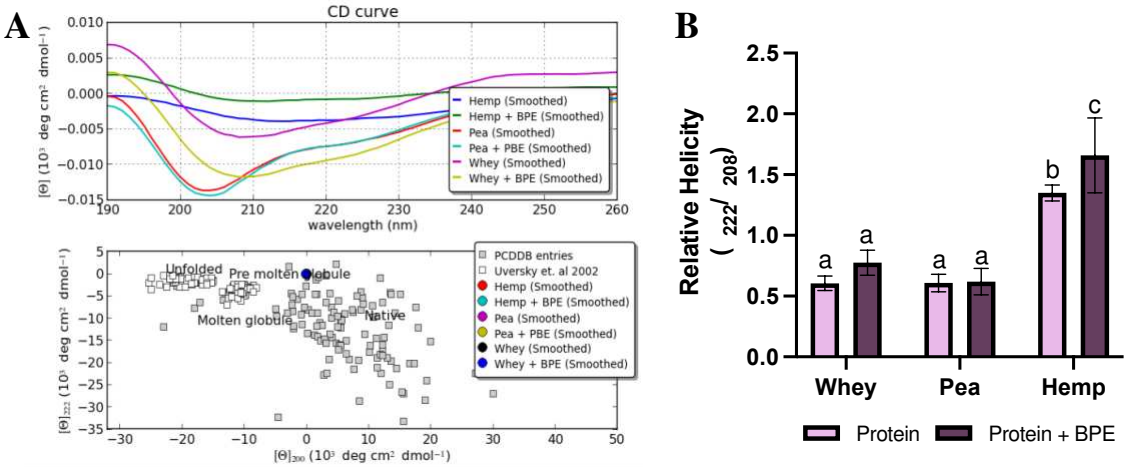
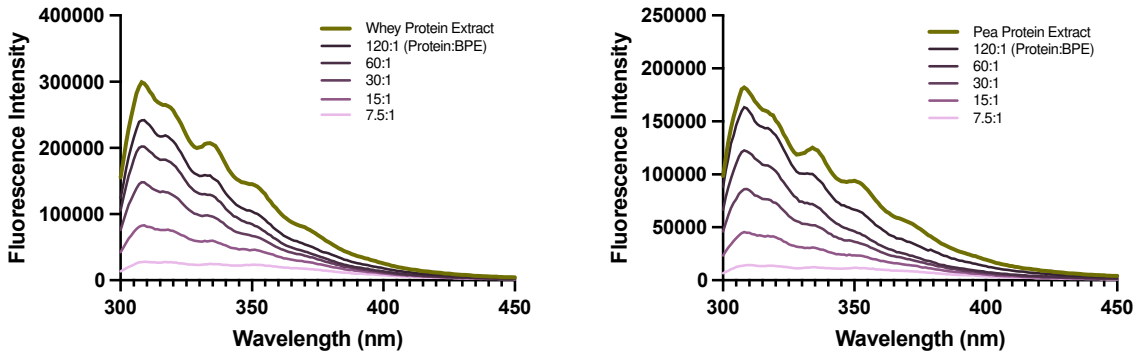


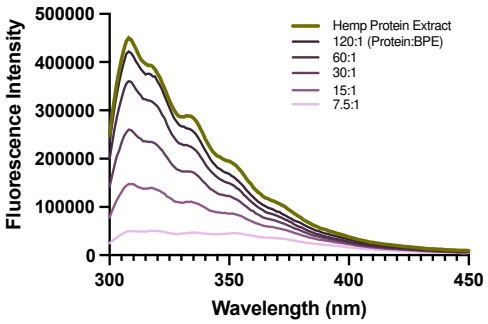
Figure 3. (A) Smoothed CD spectra for hemp, pea, and whey protein in the presence and absence of BPE and the comparisons of the protein secondary structures to the other proteins in the CAPITO database (B) Differences in relative helicity between the protein types and the proteins in the presence of BPE. Different letters in a column indicate significant differences ($p<0.05$) based on one-way ANOVA with Tukey's multiple comparisons.

3.3.3 Binding Affinities Do Not Differ Between Protein Sources

Fluorescence spectroscopy was used to determine the binding affinities for all three protein sources and whether any significant differences could be observed in their affinity for binding blueberry polyphenols. All three protein sources showed fluorescence quenching when in the presence of BPE. Fluorescence intensity decreased as the ratio of BPE to protein decreased (figure 3) indicating increased binding of the extract to the protein. No shifts in peak fluorescence emission were observed due to the binding of the blueberry polyphenols to the proteins. Figure 4 shows the Stern-Volmer plot used to calculate the Stern-Volmer coefficient (K_{sv}), modified Stern-Volmer to calculate binding affinity (K_a), and a plot to calculate the hill coefficient (n). The binding affinity, hill coefficient and Stern-Volmer coefficient for pea, whey and hemp proteins are displayed in table 2. There are no significant differences in binding affinity or in the hill coefficient which indicates number of binding sites for the polyphenols to the proteins.



A **B**



C

Figure 4. Quenching effect of BPE on protein fluorescence intensity in whey-BPE (A), pea-BPE (B) and hemp-BPE (C) complexes as a function of BPE concentration.

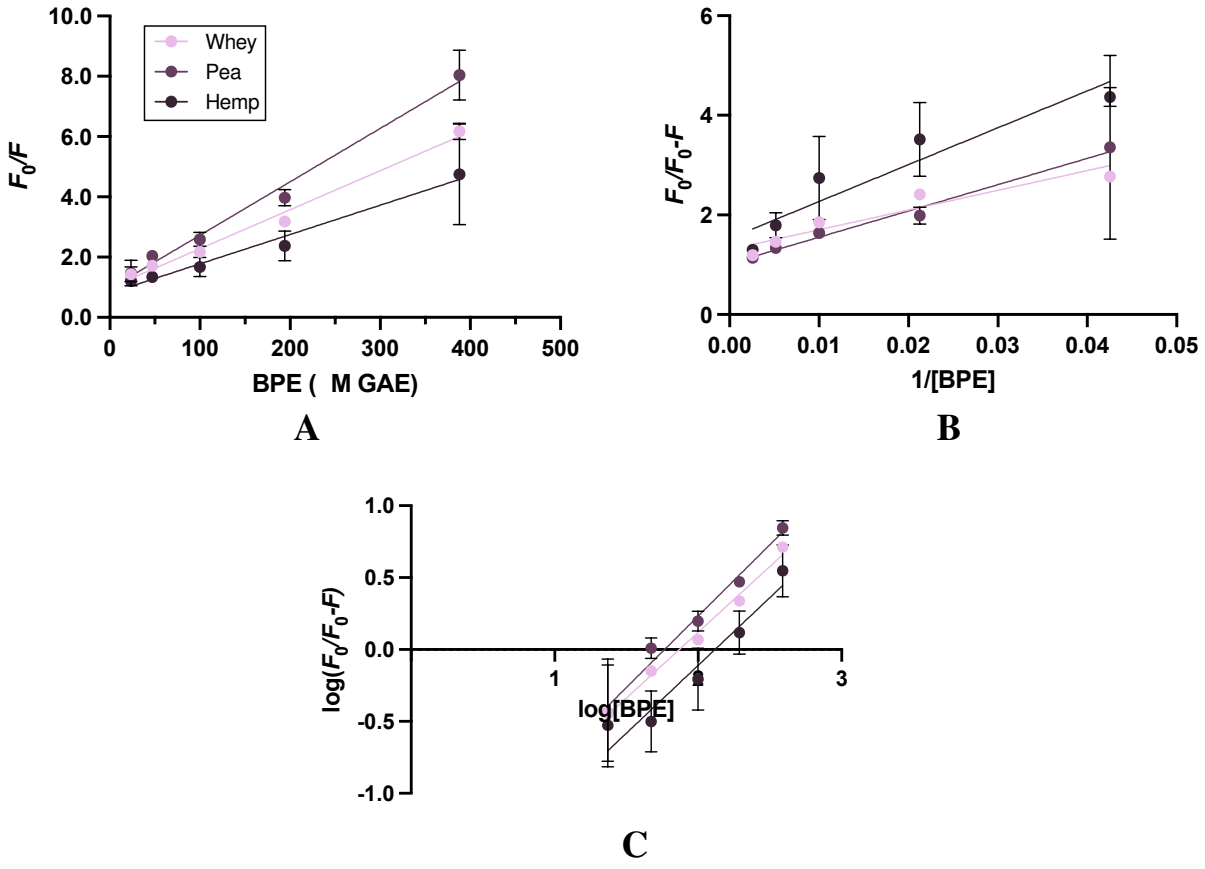


Figure 5. (A) Stern-Volmer plot for the fluorescence quenching of each protein by BPE. (B) Modified form of a Stern-Volmer plot to calculate Ka (C) Calculation of Hill coefficient reveals the number of binding sites for each protein.

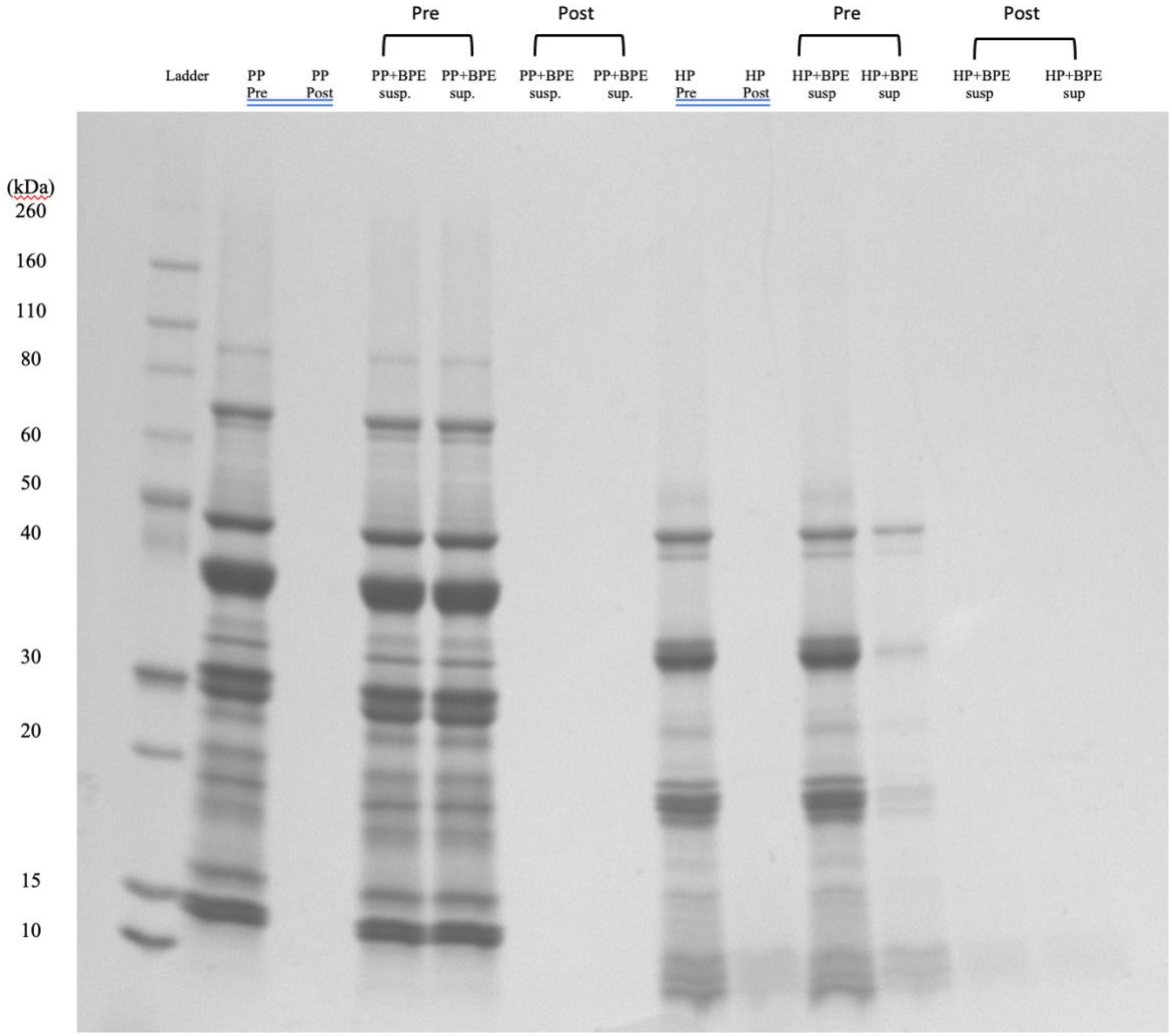
Table 1. Stern-Volmer constant (K_{sv}), binding affinity (K_a) and Hill coefficient (n) for interactions between each protein and BPE, calculated based on Figure 4 graphs. Different letters in a column indicate significant differences ($p<0.05$) based on one-way ANOVA with Tukey's multiple comparisons.

Protein Source	K_{sv} (10^4 L·mol^{-1})	K_a (10^4 L·mol^{-1})	n
Whey	1.319 ± 0.0002	3.026 ± 0.516	0.92 ± 0.008
Pea	1.860 ± 0.0004	5.141 ± 2.163	0.99 ± 0.009
Hemp	1.211 ± 0.0006	4.842 ± 1.543	0.94 ± 0.001

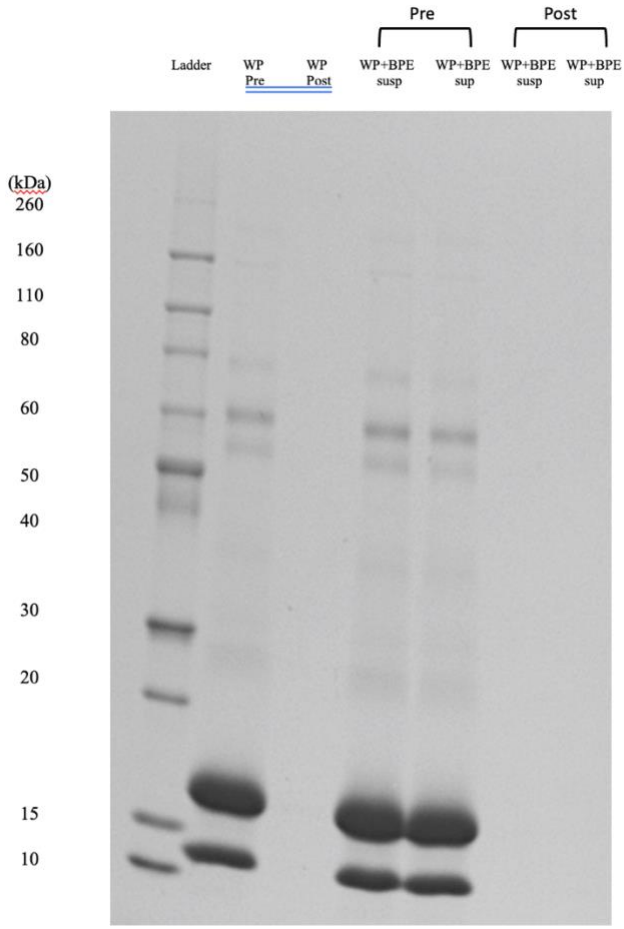
3.3.4 Assessment of the Digestibility of Protein-Polyphenol Complexes

SDS-PAGE was used to identify the protein profile of the hemp, pea and whey purified protein powder with and without BPE. Also, this technique was used to determine how the protein profile changed after an *in vitro* digestion and if it was similar to when the proteins are digested with polyphenols. The pea and hemp proteins digestion displayed a wide array of different sized proteins ranging from 100 kDa to less than 10 kDa. The pea protein isolate had bands for legumin (40-50 kDa), convillicin (~ 70 kDa) and lipoxygenase (~ 100 kDa) which are some of the main types of proteins typically present in peas (Gao 2020). Similarly, the SDS-PAGE of hemp protein isolate showed bands for edestin (20-35 kDa), villicin (40-50 kDa) and albumin (~20 kDa) which are distinctive protein subunits typically observed (Mamone 2019). The whey protein displayed characteristic bands for α-lactalbumin, β-lactoglobulin, and bovine serum albumin between 60 and 70 kDa, slightly above 15 kDa, and between 10 and 15 kDa respectively (Xu 2019). When polyphenols were added to the samples, they exhibited same SDS-PAGE protein profile. After the digestion, there were only faint bands for low molecular weight proteins visible for all three proteins between 10 to 15 kDa. The samples containing BPE appeared to have similar bands to the samples without. The supernatant of the samples

containing BPE were also run on the gel to see how much of the protein formed complexes with the blueberry polyphenols. It appears that there is much more protein available than can complex with BPE considering that the supernatant and suspension appear to have similar band density. Hemp protein appears to have more complexation due to the fainter image of the supernatant but this could also be due to the low solubility of the protein.



A



B

Figure 6. SDS-PAGE of pea, hemp (A) and whey (B) proteins in the presence and absence of BPE after in-vitro digestion. Abbreviations: Pre: pre-digestion, Post: Post digestion, PP: Pea Protein Isolate, HP: Hemp Protein Isolate, WP: Whey Protein Isolate Susp: protein-polyphenol suspension, Sup: protein-polyphenol supernatant. Samples containing only protein are taken from the suspension.

The free amino acid concentration of the protein and protein-polyphenol samples were measured before and after *in vitro* digestion to quantitatively assess whether digestion was successful and how BPE affected the digestibility of the proteins. After digestion, the free amino acid content of the protein and protein-polyphenol complexes increased significantly. The pre-digestion free amino acid concentration was approximately 0.5 mM leucine equivalents for the pea and whey solutions and around 0.4 mM leucine equivalents for the hemp solutions. After

digestion, the free amino acid ranged between 1.5 mM and nearly 3 mM leucine equivalents. The pea protein solutions in the presence and absence of blueberry polyphenols experienced the greatest release of amino acids after incubation with the digestive enzymes while hemp protein experienced the least. Furthermore, the free amino acid content is not significantly different between the protein with or without polyphenols, demonstrating that the presence of polyphenols does not affect digestion when applied at these ratios.

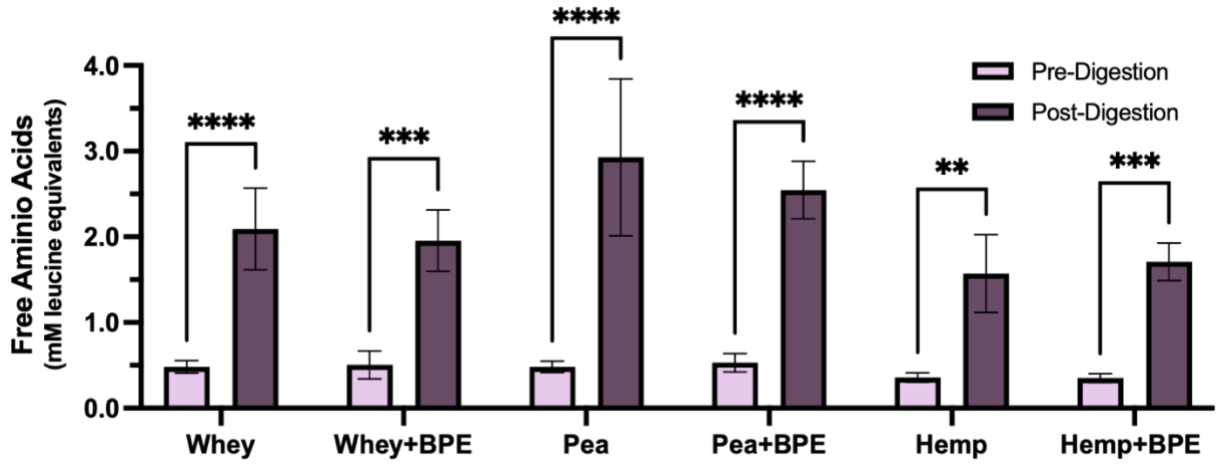


Figure 7. Free amino acid content expressed as mM leucine equivalents of protein and protein-polyphenol complexes before and after in vitro digestion. Asterisks indicate significant differences ($p<0.05$) based on 2-way ANOVA with Šídák's multiple comparisons. Number of asterisks indicate significance of: **** ($p<0.0001$), ***($p<0.001$), ** ($p<0.01$).

3.4 Discussion

The aim of this study was to establish and characterize whether pea and hemp proteins can interact with blueberry polyphenols similarly to whey proteins. Also, the digestibility of all three proteins in the presence of blueberry polyphenol was assessed to determine whether these proteins could successfully act as delivery matrices to improve the bioavailability of these compounds. Pea and hemp protein were found to successfully form complexes with BPE using UV-Vis spectroscopy. The digestibility of the proteins was not impacted by the complexation

with blueberry polyphenols because the release of free amino acids after in vitro digestion were not significantly different between the samples with or without polyphenols.

To begin, powdered blueberry and commercially available pea and hemp protein powders were extracted to create the polyphenol extract and protein isolates. The polyphenol content in other papers that utilized solvent extraction to purify blueberry powder ranged greatly. Total phenolic content was reported to be as low as 1.22 mg GAE per g and up to 702 mg/g GAE (Grace et al., 2009; Jiao et al., 2018). The blueberry polyphenol extract used in this experiment falls within this range at approximately 260 mg per gram GAE. The discrepancies in total phenolic content may be due to slight changes in extraction protocol such as using microwave or ultrasound assisted extraction and the use of different SPE or resin columns. Additionally, the region, variety and season in which the blueberries were harvested in could contribute to the diversity of the values. Moreover, the determined values for the pea and hemp protein powders were lower than what was reported in the studies in which the extraction method this was adapted from. However, there has been a wide range of protein yield reported in studies that have used this method. For the hemp protein, isoelectric point precipitation has been reported to produce isolates with a protein content of as low as 44.3% and as high as 94.6% (Malomo et al., 2014; Teh et al., 2014). The pea protein also has had values reported protein isolate prepared using this method to be around 80 to 85% (Boye et al., 2010; Gao et al., 2020; Stone et al., 2015). These differences may be attributed to different sources of the proteins being used to perform the extractions as well as different protein determination techniques. In this paper, commercially available protein powders were used rather than the raw plant ingredient which could have impacted yield. Also, many other studies used the Khejdhal method to determine total

nitrogen in the protein isolates and therefore the percent protein whereas in this study a colorimetric protein assay was used for protein determination.

To determine whether protein-polyphenol complexation occurred, turbidity of the solutions was measured and found to increase when the proteins were in the presence of BPE. Increased turbidity of a solution containing protein and polyphenols has been associated with the formation of protein-polyphenol complexes numerous accounts (Seczyk et al., 2019; Ye et al., 2013). In this study, the same phenomenon was observed. The hemp protein exhibited a greater degree of change in turbidity when the polyphenols were bound. This may be due to the hemp proteins forming a greater number of complexes with BPE in comparison to the other protein sources. Although this may be possible, it does not appear to be the case considering that all three proteins had similar affinities for binding the blueberry polyphenols as determined in the fluorescence spectroscopy experiments.

Various factors can contribute to the likelihood for proteins and polyphenols to form complexes. In this study, it was found that pea and hemp protein complex to BPE in a pH dependent manner. This phenomenon was not observed for whey which is supported by other studies which found that binding of green tea polyphenols to whey protein was not affected by pH (Belščak-Cvitanović et al., 2015). Pea protein and other legumes such as soy have been shown to have the strongest protein-polyphenol interactions near, usually slightly above their isoelectric point (Cuevas-Bernardino et al., 2018; Kosińska et al., 2011; X. Zhang et al., 2020). The isoelectric point for pea protein is approximately 4.5 which may explain why increased complexation was visible at pH 4.6 (Gao et al., 2020). The isoelectric point of hemp protein is closer to pH 5 which might explain why the highest levels of complexation were observed above this pH (Shen et al., 2020). Additionally, differences in amino acid composition and protein

structure can result in various effects of pH on different proteins (Ozdal et al., 2013). The secondary protein structures of pea and hemp were not similar according to the CD analysis which may also be responsible for the differences in complexation at the varying pH values. This data provides preliminary evidence on how polyphenol complexation with these proteins may be altered throughout digestion and effect the delivery of the compounds as well as how they may act functionally in a food product.

The proteins all exhibited a change in secondary structure as a result of binding of the polyphenols also indicating complexation. Alpha helices appear to be involved in the binding of blueberry polyphenols to hemp proteins, but other secondary structures seem to be involved for pea and whey. This is supported by other papers which have reported that whey and pea protein isolates contain a higher percentage of beta-sheets than alpha-helices which may be the main structures responsible for binding polyphenols as well has a high proportion of random coils (Abd El-Maksoud et al., 2018; Yan et al., 2020). Also, legumes such as pea and hemp, have been found to have predominantly beta-sheets in their secondary structure (Malomo & Aluko, 2015; Shen et al., 2020) although this has been reported to be pH-dependent for hemp protein (Malomo et al., 2014) which could be why the hemp proteins in this study exhibited a change in relative helicity in the presence of BPE. Furthermore, the presence of more beta sheets may indicate the presence of a more open protein structure. Based on the CAPITO database, all three proteins exhibited an unfolded protein structure. This supports the idea that all three proteins contain more beta sheets in their secondary structures leading to a more open protein conformation. The unfolded structure can increase the potential for proteins to bind to other molecules which may be why these proteins tend to spontaneously bind to polyphenols (Garbuzynskiy et al., 2004).

Additionally, due to the highly alkaline environment present when purifying the pea and hemp proteins, there is potential that this structural conformation is due to denaturation.

The intrinsic fluorescence of all three proteins was quenched by the blueberry polyphenols which is indictive of complexation. Intrinsic fluorescence of the proteins has been attributed to the major fluorophore tryptophan (Cao & Xiong, 2017) and the binding of the polyphenols to this amino acid is likely responsible for the quenching affect. No shifts were visible mostly likely because the protein structure was already unfolded as determined by the CAPITO analysis. Therefore, the tryptophan residues were already exposed allowing the polyphenols to bind easily without requiring the protein structures to change. Traditionally, shifts are observed due to the fluorophores being exposed as a change in polarity of the environment alters the protein structure exposing those residues. Environmental factors such as pH and temperature can cause these changes to occur (S. A. Abbas et al., 2013). The number of binding sites for all three protein sources were close 1 indicating that there is most likely one type of binding site responsible for binding BPE (Dai et al., 2019). Moreover, all three proteins appear to have similar abilities to bind blueberry polyphenols and can bind a large range of concentrations of blueberry polyphenols. Studies that have been conducted on the binding affinity of EGCG to bind whey protein isolate, have reported slightly higher K_{sv} values (2.62 and 1.9 L·mol−1) (Cao & Xiong, 2017; Chen et al., 2019) than these findings, so these proteins may have even higher affinities for binding other types of phenolics as well. Lastly, at a ratio lower than 7.5:1 (protein: polyphenols) the proteins may not be able to bind more polyphenols considering the fluorescence intensity was extremely low at this ratio.

Whey, hemp and pea proteins were found to be fully digested after in-vitro digestion visually through the analysis of SDS-PAGE which was later confirmed quantitatively. The

decrease in higher molecular weight bands for all three proteins indicates that the proteins were successfully digested. Additionally, the similarity between the protein profiles of the protein samples in the presence and absence of BPE indicates that the polyphenols are not inhibiting digestion at the ratio that they are being used in this paper. Moreover, comparable amounts of free amino acids were released after digestion of the proteins in the presence and absence of BPE. It was imperative that the protein digestibility was not affected by the polyphenols considering polyphenols have been shown to inhibit digestion of other proteins. This is believed to be as a result of the ability of polyphenols to inhibit the activity of digestive enzymes (Cirkovic Velickovic, 2018). Other studies have shown that whey protein digestibility decreases significantly when bound to polyphenols. In these studies, a ratio of 1:0.5 (whey protein: polyphenols) is used which may be why these effects observed (de Morais et al., 2020; Xu et al., 2019). This ratio of protein to polyphenols would be much greater than how much of these compounds are customarily consumed together. In this study, a ratio of 30:1 (protein: polyphenols) was used which is more similar to how these compounds would be consumed in a food matrix.

Pea protein seems to have a comparable digestibility to whey protein whereas hemp protein may require more time to be fully hydrolyzed. One study found that in beverages formulated with milk or pea proteins, both proteins were digested to similar amounts. The release of free amino acids from both protein drinks after in-vitro digestion were not significantly different from one another (Štreimikytė et al., 2020). Similarly, Hernandez et al. found that garden pea and grass pea isolates had a similar free amino acid release after in vitro digestion to whey protein. Furthermore, hemp protein which was found to be less digestible than pea protein in this study has been reported to have a protein digestibility–corrected amino acid score

(PDCAAS) value of 0.5 to 0.6. This is much lower than the score for animal proteins such as whey which is about 1 (Shen et al., 2021). Despite this, there is potential for the protein digestibility to be increased. When hemp and pea protein were used together in a protein beverage, the protein digestibility increased significantly from the control indicating that the use of various proteins could enhance its digestibility and nutritional value (Manus et al., 2021). The results are promising that polyphenol consumption at levels present in a serving of berries would not impact digestibility of the proteins which could allow for the proteins to be used as a delivery mechanism for blueberry polyphenols.

CHAPTER IV

CONCLUSIONS & FUTURE DIRECTIONS

In this study, the physical and chemical characteristics of whey, hemp and pea protein and blueberry polyphenol complexes were assessed. Firstly, the hemp and pea protein isolates were found to successfully formed complexes with the blueberry polyphenol extract similarly to whey protein which has been established to occur in many other studies. Then, the change in secondary structure in the presence of BPE was examined. Pea and whey protein displayed similar changes in secondary structure due to complexation with BPE whereas hemp protein exhibited a shift in its alpha helical structure. Furthermore, the binding affinities for the polyphenols to all three proteins were not significantly different which indicates that they would all have similar affinities for the compound. After the in-vitro digestion of the proteins with or without polyphenols, the free amino acid content of all the solutions increased significantly compared to before the digestion. This confirmed the success of the in-vitro digestion which also visualized using SDS-PAGE. Also, release of free amino acid of the protein-polyphenol complexes were similar to the proteins alone indicating that the polyphenols were not hindering digestion, which provides evidence that these proteins can be used to improve polyphenol bioavailability.

One major limitation of this study is that the purification of the protein powders and blueberry powder do not fully represent how these compounds would interact as a part of a whole food matrix. For future studies, it would be important to assess the whole protein powder and the whole berry to see if the effects would be the same but for these preliminary studies it was helpful to see how the protein and the polyphenols alone interact. Also, further processing of

the circular dichroism would give more information on how the secondary structures change for the protein as a result of polyphenol binding. Beta-sheets are a major structural component of all three proteins so it would be important to assess how those structures change as well.

All in all, these results indicate that novel plant proteins may be effective delivery mechanisms for blueberry polyphenols in whole food matrices. These current findings suggest that due to the spontaneous nature of these interactions, when these foods (plant proteins and blueberry polyphenols) are consumed together as a part of a meal or snack, there is potential for these interactions to occur and in turn more polyphenols could be delivered to the small intestine and absorbed. Future directions include assessing the bioavailability of blueberry polyphenols in transepithelial cell model since this study has provided evidence that the delivery of polyphenols to the small intestine could be enhanced as a result of binding to plant proteins. Eventually, bioavailability will also be assessed in clinical trials by providing the protein/polyphenol complexes as a part of a whole food such as a smoothie rather than through supplementation.

REFERENCES

Abbas, M., Saeed, F., Anjum, F. M., Afzaal, M., Tufail, T., Bashir, M. S., Ishtiaq, A., Hussain, S., & Suleria, H. A. R. (2016). Natural polyphenols: An overview. *Http://Dx.Doi.Org/10.1080/10942912.2016.1220393*, *20*(8), 1689–1699. https://doi.org/10.1080/10942912.2016.1220393

Abbas, S. A., Gaspar, G., Sharma, V. K., Patapoff, T. W., & Kalonia, D. S. (2013). Application of Second-Derivative Fluorescence Spectroscopy to Monitor Subtle Changes in a Monoclonal Antibody Structure. *Journal of Pharmaceutical Sciences*, *102*(1), 52–61. https://doi.org/10.1002/JPS.23354

Abd El-Maksoud, A. A., Abd El-Ghany, I. H., El-Beltagi, H. S., Anankanbil, S., Banerijee, C., Petersen, S. v., Pérez, B., & Guo, Z. (2018). Adding functionality to milk-based protein: Preparation, and physico-chemical characterization of β-lactoglobulin-phenolic conjugates. *Food Chemistry*, *241*, 281–289. https://doi.org/10.1016/J.FOODCHEM.2017.08.101

Alcorta, A., Porta, A., Tárrega, A., Alvarez, M. D., & Pilar Vaquero, M. (2021). Foods for Plant-Based Diets: Challenges and Innovations. *Foods 2021, Vol. 10, Page 293*, *10*(2), 293. https://doi.org/10.3390/FOODS10020293

Annunziata, G., Jiménez-García, M., Capó, X., Moranta, D., Arnone, A., Tenore, G. C., Sureda, A., & Tejada, S. (2020). Microencapsulation as a tool to counteract the typical low bioavailability of polyphenols in the management of diabetes. *Food and Chemical Toxicology*, *139*, 111248. https://doi.org/10.1016/J.FCT.2020.111248

Ashwar, B. A., & Gani, A. (2021). Noncovalent Interactions of Sea Buckthorn Polyphenols with Casein and Whey Proteins: Effect on the Stability, Antioxidant Potential, and Bioaccessibility of Polyphenols. *ACS Food Science & Technology*, *1*(7), 1206–1214. https://doi.org/10.1021/ACSFOODSCITECH.1C00103

Baba, W. N., McClements, D. J., & Maqsood, S. (2021). Whey protein–polyphenol conjugates and complexes: Production, characterization, and applications. *Food Chemistry*, *365*, 130455. https://doi.org/10.1016/J.FOODCHEM.2021.130455

Belščak-Cvitanović, A., Dordević, V., Karlović, S., Pavlović, V., Komes, D., Ježek, D., Bugarski, B., & Nedović, V. (2015). Protein-reinforced and chitosan-pectin coated alginate microparticles for delivery of flavan-3-ol antioxidants and caffeine from green tea extract. *Food Hydrocolloids*, *51*, 361–374. https://doi.org/10.1016/J.FOODHYD.2015.05.039

ben Lagha, A., Dudonné, S., Desjardins, Y., & Grenier, D. (2015). Wild Blueberry (Vaccinium angustifolium Ait.) Polyphenols Target Fusobacterium nucleatum and the Host Inflammatory Response: Potential Innovative Molecules for Treating Periodontal Diseases. *Journal of Agricultural and Food Chemistry*, *63*(31), 6999–7008. https://doi.org/10.1021/ACS.JAFC.5B01525/SUPPL_FILE/JF5B01525_SI_002.PDF

Boye, J. I., Aksay, S., Roufik, S., Ribéreau, S., Mondor, M., Farnworth, E., & Rajamohamed, S. H. (2010). Comparison of the functional properties of pea, chickpea and lentil protein concentrates processed using ultrafiltration and isoelectric precipitation techniques. *Food Research International*, *43*(2), 537–546. https://doi.org/10.1016/J.FOODRES.2009.07.021

Burkholder-Cooley, N., Rajaram, S., Haddad, E., Fraser, G. E., & Jaceldo-Siegl, K. (2016). Comparison of polyphenol intakes according to distinct dietary patterns and food sources in

the Adventist Health Study-2 cohort. *British Journal of Nutrition*, *115*(12), 2162–2169. https://doi.org/10.1017/S0007114516001331

Cano-Lamadrid, M., Tkacz, K., Turkiewicz, I. P., Clemente-Villalba, J., Sánchez-Rodríguez, L., Lipan, L., García-García, E., Carbonell-Barrachina, Á. A., & Wojdyło, A. (2020). How a Spanish Group of Millennial Generation Perceives the Commercial Novel Smoothies? *Foods 2020, Vol. 9, Page 1213*, *9*(9), 1213. https://doi.org/10.3390/FOODS9091213

Cao, Y., & Xiong, Y. L. (2017). Binding of Gallic Acid and Epigallocatechin Gallate to Heat-Unfolded Whey Proteins at Neutral pH Alters Radical Scavenging Activity of in Vitro Protein Digests. *Journal of Agricultural and Food Chemistry*, *65*(38), 8443–8450. https://doi.org/10.1021/ACS.JAFC.7B03006

Chandan, R. C., Gandhi, A., & Shah, N. P. (2017). Yogurt: Historical Background, Health Benefits, and Global Trade. *Yogurt in Health and Disease Prevention*, 3–29. https://doi.org/10.1016/B978-0-12-805134-4.00001-8

Chen, W., Wang, W., Ma, X., Lv, R., Balaso Watharkar, R., Ding, T., Ye, X., & Liu, D. (2019). Effect of pH-shifting treatment on structural and functional properties of whey protein isolate and its interaction with (−)-epigallocatechin-3-gallate. *Food Chemistry*, *274*, 234–241. https://doi.org/10.1016/j.foodchem.2018.08.106

Chew, B., Mathison, B., Kimble, L., McKay, D., Kaspar, K., Khoo, C., Chen, C. Y. O., & Blumberg, J. (2019). Chronic consumption of a low calorie, high polyphenol cranberry beverage attenuates inflammation and improves glucoregulation and HDL cholesterol in healthy overweight humans: a randomized controlled trial. *European Journal of Nutrition*, *58*(3), 1223–1235. https://doi.org/10.1007/S00394-018-1643-Z/TABLES/4

Cirkovic Velickovic, T. D. (n.d.). *The Role of Dietary Phenolic Compounds in Protein Digestion and Processing Technologies to Improve Their Antinutritive Properties*. https://doi.org/10.1111/1541-4337.12320

Cuevas-Bernardino, J. C., Leyva-Gutierrez, F. M. A., Vernon-Carter, E. J., Lobato-Calleros, C., Román-Guerrero, A., & Davidov-Pardo, G. (2018). Formation of biopolymer complexes composed of pea protein and mesquite gum – Impact of quercetin addition on their physical and chemical stability. *Food Hydrocolloids*, *77*, 736–745. https://doi.org/10.1016/J.FOODHYD.2017.11.015

Dai, T., Chen, J., McClements, D. J., Hu, P., Ye, X., Liu, C., & Li, T. (2019). Protein–polyphenol interactions enhance the antioxidant capacity of phenolics: analysis of rice glutelin–procyanidin dimer interactions. *Food & Function*, *10*(2), 765–774. https://doi.org/10.1039/C8FO02246A

Das, K., Choudhary, R., & Thompson-Witrick, K. A. (2019). Effects of new technology on the current manufacturing process of yogurt-to increase the overall marketability of yogurt. *LWT*, *108*, 69–80. https://doi.org/10.1016/J.LWT.2019.03.058

de Morais, F. P. R., Pessato, T. B., Rodrigues, E., Peixoto Mallmann, L., Mariutti, L. R. B., & Netto, F. M. (2020). Whey protein and phenolic compound complexation: Effects on antioxidant capacity before and after in vitro digestion. *Food Research International*, *133*, 109104. https://doi.org/10.1016/J.FOODRES.2020.109104

Devcich, D. A., Pedersen, I. K., & Petrie, K. J. (2007). You eat what you are: Modern health worries and the acceptance of natural and synthetic additives in functional foods. *Appetite*, *48*(3), 333–337. https://doi.org/10.1016/J.APPET.2006.09.014

Diaz, J. T., Foegeding, E. A., & Lila, M. A. (2020). Formulation of protein–polyphenol particles for applications in food systems. *Food & Function*, *11*(6), 5091–5104. https://doi.org/10.1039/D0FO00186D

Djuardi, A. U. P., Yuliana, N. D., Ogawa, M., Akazawa, T., & Suhartono, M. T. (2020). Emulsifying properties and antioxidant activity of soy protein isolate conjugated with tea polyphenol extracts. *Journal of Food Science and Technology*, *57*(10), 3591–3600. https://doi.org/10.1007/S13197-020-04391-9/FIGURES/5

Fang, Z., & Bhandari, B. (2010). Encapsulation of polyphenols – a review. *Trends in Food Science & Technology*, *21*(10), 510–523. https://doi.org/10.1016/J.TIFS.2010.08.003

Fernandez, M. A., & Marette, A. (2017). Potential Health Benefits of Combining Yogurt and Fruits Based on Their Probiotic and Prebiotic Properties. *Advances in Nutrition*, *8*(1), 155S-164S. https://doi.org/10.3945/AN.115.011114

Gao, Z., Shen, P., Lan, Y., Cui, L., Ohm, J. B., Chen, B., & Rao, J. (2020). Effect of alkaline extraction pH on structure properties, solubility, and beany flavor of yellow pea protein isolate. *Food Research International*, *131*, 109045. https://doi.org/10.1016/J.FOODRES.2020.109045

Garbuzynskiy, S. O., Lobanov, M. Yu., & Galzitskaya, O. v. (2004). To be folded or to be unfolded? *Protein Science : A Publication of the Protein Society*, *13*(11), 2871. https://doi.org/10.1110/PS.04881304

Grace, M. H., Ribnicky, D. M., Kuhn, P., Poulev, A., Logendra, S., Yousef, G. G., Raskin, I., & Lila, M. A. (2009). Hypoglycemic activity of a novel anthocyanin-rich formulation from lowbush blueberry, Vaccinium angustifolium Aiton. *Phytomedicine*, *16*(5), 406–415. https://doi.org/10.1016/J.PHYMED.2009.02.018

Gundersen, C., Kreider, B., & Pepper, J. (2011). The Economics of Food Insecurity in the United States. *Applied Economic Perspectives and Policy*, *33*(3), 281–303. https://doi.org/10.1093/AEPP/PPR022

Huang, Q., Braffett, B. H., Simmens, S. J., Young, H. A., & Ogden, C. L. (2020). Dietary Polyphenol Intake in US Adults and 10-Year Trends: 2007-2016. *Journal of the Academy of Nutrition and Dietetics*, *120*(11), 1821–1833. https://doi.org/10.1016/j.jand.2020.06.016

Jiang, J., Zhang, Z., Zhao, J., & Liu, Y. (2018). The effect of non-covalent interaction of chlorogenic acid with whey protein and casein on physicochemical and radical-scavenging activity of in vitro protein digests. *Food Chemistry*, *268*, 334–341. https://doi.org/10.1016/J.FOODCHEM.2018.06.015

Jiao, X., Li, B., Zhang, Q., Gao, N., Zhang, X., & Meng, X. (2018). Effect of in vitro-simulated gastrointestinal digestion on the stability and antioxidant activity of blueberry polyphenols and their cellular antioxidant activity towards HepG2 cells. *International Journal of Food Science & Technology*, *53*(1), 61–71. https://doi.org/10.1111/IJFS.13516

Jiao, X., Wang, Y., Lin, Y., Lang, Y., Li, E., Zhang, X., Zhang, Q., Feng, Y., Meng, X., & Li, B. (2019). Blueberry polyphenols extract as a potential prebiotic with anti-obesity effects on C57BL/6 J mice by modulating the gut microbiota. *The Journal of Nutritional Biochemistry*, *64*, 88–100. https://doi.org/10.1016/J.JNUTBIO.2018.07.008

Kang, J., Thakali, K. M., Jensen, G. S., & Wu, X. (2015). Phenolic Acids of the Two Major Blueberry Species in the US Market and Their Antioxidant and Anti-inflammatory Activities. *Plant Foods for Human Nutrition*, *70*(1), 56–62. https://doi.org/10.1007/S11130-014-0461-6/FIGURES/2

Katherine Hoy, M., Clemens, J. C., Martin, C. L., & Moshfegh, A. J. (2020). Fruit and Vegetable Consumption of US Adults by Level of Variety, What We Eat in America, NHANES 2013–2016. *Current Developments in Nutrition*, *4*(3). https://doi.org/10.1093/CDN/NZAA014

Kosińska, A., Karamać, M., Penkacik, K., Urbalewicz, A., & Amarowicz, R. (2011). Interactions between tannins and proteins isolated from broad bean seeds (Vicia faba Major) yield soluble and non-soluble complexes. *European Food Research and Technology*, *233*(2), 213–222. https://doi.org/10.1007/S00217-011-1506-9/FIGURES/6

Kuntz, S., Rudloff, S., Asseburg, H., Borsch, C., Fröhling, B., Unger, F., Dold, S., Spengler, B., Römpp, A., & Kunz, C. (2015). Uptake and bioavailability of anthocyanins and phenolic acids from grape/blueberry juice and smoothie in vitro and in vivo. *British Journal of Nutrition*, *113*(7), 1044–1055. https://doi.org/10.1017/S0007114515000161

Lau, F. C., Joseph, J. A., McDonald, J. E., & Kalt, W. (2009). Attenuation of iNOS and COX2 by blueberry polyphenols is mediated through the suppression of NF-κB activation. *Journal of Functional Foods*, *1*(3), 274–283. https://doi.org/10.1016/J.JFF.2009.05.001

Li, D., Yang, Y., Yang, X., Wang, Z., Yao, X., & Guo, Y. (2022). Enhanced bioavailability and anti-hyperglycemic activity of young apple polyphenols by complexation with whey protein isolates. *Journal of Food Science*. https://doi.org/10.1111/1750-3841.16062

Liu, Y., Wang, D., Zhang, D., Lv, Y., Wei, Y., Wu, W., Zhou, F., Tang, M., Mao, T., Li, M., & Ji, B. (2011). Inhibitory Effect of Blueberry Polyphenolic Compounds on Oleic Acid-Induced Hepatic Steatosis in Vitro. *Journal of Agricultural and Food Chemistry*, *59*(22), 12254–12263. https://doi.org/10.1021/JF203136J

Louis, X. L., Thandapilly, S. J., Kalt, W., Vinqvist-Tymchuk, M., Aloud, B. M., Raj, P., Yu, L., Le, H., & Netticadan, T. (2014). Blueberry polyphenols prevent cardiomyocyte death by preventing calpain activation and oxidative stress. *Food & Function*, *5*(8), 1785–1794. https://doi.org/10.1039/C3FO60588D

Mäkinen, O. E., Wanhalinna, V., Zannini, E., & Arendt, E. K. (2015). Foods for Special Dietary Needs: Non-dairy Plant-based Milk Substitutes and Fermented Dairy-type Products. *Https://Doi.Org/10.1080/10408398.2012.761950*, *56*(3), 339–349. https://doi.org/10.1080/10408398.2012.761950

Malomo, S. A., & Aluko, R. E. (2015). A comparative study of the structural and functional properties of isolated hemp seed (Cannabis sativa L.) albumin and globulin fractions. *Food Hydrocolloids*, *43*, 743–752. https://doi.org/10.1016/J.FOODHYD.2014.08.001

Malomo, S. A., He, R., & Aluko, R. E. (2014). Structural and Functional Properties of Hemp Seed Protein Products. *Journal of Food Science*, *79*(8), C1512–C1521. https://doi.org/10.1111/1750-3841.12537

Mamone, G., Picariello, G., Ramondo, A., Nicolai, M. A., & Ferranti, P. (2019). Production, digestibility and allergenicity of hemp (Cannabis sativa L.) protein isolates. *Food Research International*, *115*, 562–571. https://doi.org/10.1016/j.foodres.2018.09.017

Manus, J., Millette, M., Uscanga, B. R. A., Salmieri, S., Maherani, B., & Lacroix, M. (2021). In vitro protein digestibility and physico-chemical properties of lactic acid bacteria fermented beverages enriched with plant proteins. *Journal of Food Science*, *86*(9), 4172–4182. https://doi.org/10.1111/1750-3841.15859

McAnulty, S. R., McAnulty, L. S., Nieman, D. C., Dumke, C. L., Morrow, J. D., Utter, A. C., Henson, D. A., Proulx, W. R., & George, G. L. (2004). Consumption of blueberry polyphenols reduces exercise-induced oxidative stress compared to vitamin C. *Nutrition Research*, *24*(3), 209–221. https://doi.org/10.1016/J.NUTRES.2003.10.003

McClements, D. J., & Grossmann, L. (2021). The science of plant-based foods: Constructing next-generation meat, fish, milk, and egg analogs. *Comprehensive Reviews in Food Science and Food Safety*, *20*(4), 4049–4100. https://doi.org/10.1111/1541-4337.12771

Mendly-Zambo, Z., Powell, L. J., & Newman, L. L. (2021). Dairy 3.0: cellular agriculture and the future of milk. *Https://Doi.Org/10.1080/15528014.2021.1888411*, *24*(5), 675–693. https://doi.org/10.1080/15528014.2021.1888411

Meng, Y., & Li, C. (2021). Conformational changes and functional properties of whey protein isolate-polyphenol complexes formed by non-covalent interaction. *Food Chemistry*, *364*, 129622. https://doi.org/10.1016/J.FOODCHEM.2021.129622

Ming, Y., Chen, L., Khan, A., Wang, H., & Wang, C. (2020). Effects of tea polyphenols on physicochemical and antioxidative properties of whey protein coating. *Food Science and Biotechnology*, *29*(12), 1655. https://doi.org/10.1007/S10068-020-00824-5

Moore, S., & Stein, W. H. (1954). A MODIFIED NINHYDRIN REAGENT FOR THE PHOTOMETRIC DETERMINATION OF AMINO ACIDS AND RELATED COMPOUNDS. *Journal of Biological Chemistry*, *211*, 907–913. https://doi.org/10.1016/S0021-9258(18)71178-2

Morissette, A., Kropp, C., Songpadith, J. P., Moreira, R. J., Costa, J., Mariné-Casadó, R., Pilon, G., Varin, T. v., Dudonné, S., Boutekrabt, L., St-Pierre, P., Levy, E., Roy, D., Desjardins, Y., Raymond, F., Houd, V. P., & Marette, A. (2020). Blueberry proanthocyanidins and anthocyanins improve metabolic health through a gut microbiota-dependent mechanism in diet-induced obese mice. *American Journal of Physiology - Endocrinology and Metabolism*, *318*(6), E965–E980. https://doi.org/10.1152/AJPENDO.00560.2019/ASSET/IMAGES/LARGE/ZH1005208340 0008.JPEG

Ni, H., Hayes, H., Stead, D., Liu, G., Yang, H., Li, H., & Raikos, V. (2020). Interaction of whey protein with polyphenols from salal fruits (Gaultheria shallon) and the effects on protein structure and hydrolysis pattern by Flavourzyme®. *International Journal of Food Science & Technology*, *55*(3), 1281–1288. https://doi.org/10.1111/IJFS.14394

Oidtmann, J., Schantz, M., Mäder, K., Baum, M., Berg, S., Betz, M., Kulozik, U., Leick, S., Rehage, H., Schwarz, K., & Richling, E. (2012). Preparation and Comparative Release Characteristics of Three Anthocyanin Encapsulation Systems. *Journal of Agricultural and Food Chemistry*, *60*(3), 844–851. https://doi.org/10.1021/JF2047515

Ozdal, T., Capanoglu, E., & Altay, F. (2013). A review on protein–phenolic interactions and associated changes. *Food Research International*, *51*(2), 954–970. https://doi.org/10.1016/J.FOODRES.2013.02.009

Reynaud, Y., Lopez, M., Riaublanc, A., Souchon, I., & Dupont, D. (2020). Hydrolysis of plant proteins at the molecular and supra-molecular scales during in vitro digestion. *Food Research International*, *134*, 109204. https://doi.org/10.1016/j.foodres.2020.109204

Ribnicky, D. M., Roopchand, D. E., Poulev, A., Kuhn, P., Oren, A., Cefalu, W. T., & Raskin, I. (2014). Artemisia dracunculus L. polyphenols complexed to soy protein show enhanced bioavailability and hypoglycemic activity in C57BL/6 mice. *Nutrition*, *30*(7–8), S4–S10. https://doi.org/10.1016/J.NUT.2014.03.009

Rodriguez-Mateos, A., Rendeiro, C., Bergillos-Meca, T., Tabatabaee, S., George, T. W., Heiss, C., & Spencer, J. P. E. (2013). Intake and time dependence of blueberry flavonoid–induced improvements in vascular function: a randomized, controlled, double-blind, crossover

intervention study with mechanistic insights into biological activity. *The American Journal of Clinical Nutrition*, *98*(5), 1179–1191. https://doi.org/10.3945/AJCN.113.066639

Roopchand, D. E., Kuhn, P., Krueger, C. G., Moskal, K., Lila, M. A., & Raskin, I. (2013). Concord Grape Pomace Polyphenols Complexed to Soy Protein Isolate Are Stable and Hypoglycemic in Diabetic Mice. *Journal of Agricultural and Food Chemistry*, *61*(47), 11428–11433. https://doi.org/10.1021/JF403238E

Ryan, J. M., White, C. H., Gough, R. H., & Burns, A. C. (1984). Consumer Acceptance of Fruit-Flavored Yogurt Drinks. *Journal of Dairy Science*, *67*(7), 1369–1375. https://doi.org/10.3168/JDS.S0022-0302(84)81450-2

S eczyk, L., Swieca, M., Kapusta, I., & Gawlik-Dziki, U. (2019). Protein–Phenolic Interactions as a Factor Affecting the Physicochemical Properties of White Bean Proteins. *Molecules 2019, Vol. 24, Page 408*, *24*(3), 408. https://doi.org/10.3390/MOLECULES24030408

Samtiya, M., Aluko, R. E., & Dhewa, T. (2020). Plant food anti-nutritional factors and their reduction strategies: an overview. *Food Production, Processing and Nutrition 2020 2:1*, *2*(1), 1–14. https://doi.org/10.1186/S43014-020-0020-5

Satija, A., & Hu, F. B. (2018). Plant-based diets and cardiovascular health. *Trends in Cardiovascular Medicine*, *28*(7), 437–441. https://doi.org/10.1016/j.tcm.2018.02.004

Schneider, M., Esposito, D., Lila, M. A., & Foegeding, E. A. (2016). Formation of whey protein–polyphenol meso-structures as a natural means of creating functional particles. *Food & Function*, *7*(3), 1306–1318. https://doi.org/10.1039/C5FO01499A

Shen, P., Gao, Z., Fang, B., Rao, J., & Chen, B. (2021). Ferreting out the secrets of industrial hemp protein as emerging functional food ingredients. *Trends in Food Science & Technology*, *112*, 1–15. https://doi.org/10.1016/J.TIFS.2021.03.022

Shen, P., Gao, Z., Xu, M., Ohm, J. B., Rao, J., & Chen, B. (2020). The impact of hempseed dehulling on chemical composition, structure properties and aromatic profile of hemp protein isolate. *Food Hydrocolloids*, *106*, 105889. https://doi.org/10.1016/J.FOODHYD.2020.105889

Stone, A. K., Karalash, A., Tyler, R. T., Warkentin, T. D., & Nickerson, M. T. (2015). Functional attributes of pea protein isolates prepared using different extraction methods and cultivars. *Food Research International*, *76*(P1), 31–38. https://doi.org/10.1016/J.FOODRES.2014.11.017

Štreimikytė, P., Keršienė, M., Eisinaitė, V., Jasutienė, I., Lesauskaitė, V., Damulevičienė, G., Knašienė, J., & Leskauskaitė, D. (2020). Formulating protein-based beverages for the dysphagia diets of the elderly: viscosity, protein quality, in vitro digestion, and consumers acceptability. *Journal of the Science of Food and Agriculture*, *100*(10), 3895–3901. https://doi.org/10.1002/JSFA.10431

Tang, H. Y., Fang, Z., & Ng, K. (2020). Dietary fiber-based colon-targeted delivery systems for polyphenols. *Trends in Food Science & Technology*, *100*, 333–348. https://doi.org/10.1016/J.TIFS.2020.04.028

Teh, S. S., Bekhit, A. E. D., Carne, A., & Birch, J. (2014). Effect of the defatting process, acid and alkali extraction on the physicochemical and functional properties of hemp, flax and canola seed cake protein isolates. *Journal of Food Measurement and Characterization*, *8*(2), 92–104. https://doi.org/10.1007/s11694-013-9168-x

Teleszko, M., & Wojdylo, A. (2014). Bioactive compounds vs. organoleptic assessment of 'smoothies'-type products prepared from selected fruit species. *International Journal of Food Science & Technology*, *49*(1), 98–106. https://doi.org/10.1111/IJFS.12280

Thongkaew, C., Gibis, M., Hinrichs, J., & Weiss, J. (2014). Polyphenol interactions with whey protein isolate and whey protein isolate–pectin coacervates. *Food Hydrocolloids*, *41*, 103–112. https://doi.org/10.1016/J.FOODHYD.2014.02.006

Tian, L., Kejing, Y., Zhang, S., Yi, J., Zhu, Z., Decker, E. A., & McClements, D. J. (2021). Impact of tea polyphenols on the stability of oil-in-water emulsions coated by whey proteins. *Food Chemistry*, *343*, 128448. https://doi.org/10.1016/J.FOODCHEM.2020.128448

Tkacz, K., Wojdyło, A., Turkiewicz, I. P., & Nowicka, P. (2021). Anti-diabetic, anti-cholinesterase, and antioxidant potential, chemical composition and sensory evaluation of novel sea buckthorn-based smoothies. *Food Chemistry*, *338*, 128105. https://doi.org/10.1016/J.FOODCHEM.2020.128105

Tresserra-Rimbau, A., Rimm, E. B., Medina-Remón, A., Martínez-González, M. A., López-Sabater, M. C., Covas, M. I., Corella, D., Salas-Salvadó, J., Gómez-Gracia, E., Lapetra, J., Arós, F., Fiol, M., Ros, E., Serra-Majem, L., Pintó, X., Muñoz, M. A., Gea, A., Ruiz-Gutiérrez, V., Estruch, R., & Lamuela-Raventós, R. M. (2014). Polyphenol intake and mortality risk: A re-analysis of the PREDIMED trial. *BMC Medicine*, *12*(1), 1–11. https://doi.org/10.1186/1741-7015-12-77/FIGURES/2

US Yogurt and Yogurt Drinks Market Report 2021. (n.d.). Retrieved December 10, 2021, from https://store.mintel.com/report/us-yogurt-and-yogurt-drinks-market-report

Vanga, S. K., & Raghavan, V. (2017). How well do plant based alternatives fare nutritionally compared to cow's milk? *Journal of Food Science and Technology 2017 55:1*, *55*(1), 10–20. https://doi.org/10.1007/S13197-017-2915-Y

Wang, Y., Beydoun, M. A., Min, J., Xue, H., Kaminsky, L. A., & Cheskin, L. J. (2020). Has the prevalence of overweight, obesity and central obesity levelled off in the United States? Trends, patterns, disparities, and future projections for the obesity epidemic. *International Journal of Epidemiology*, *49*(3), 810–823. https://doi.org/10.1093/IJE/DYZ273

Wang, Y., Li, J., & Li, B. (2017). Chitin microspheres: A fascinating material with high loading capacity of anthocyanins for colon specific delivery. *Food Hydrocolloids*, *63*, 293–300. https://doi.org/10.1016/J.FOODHYD.2016.09.003

Wiedemann, C., Bellstedt, P., & Görlach, M. (2013). CAPITO—a web server-based analysis and plotting tool for circular dichroism data. *Bioinformatics*, *29*(14), 1750–1757. https://doi.org/10.1093/BIOINFORMATICS/BTT278

Wolf, C. A., Malone, T., & McFadden, B. R. (2020). Beverage milk consumption patterns in the United States: Who is substituting from dairy to plant-based beverages? *Journal of Dairy Science*, *103*(12), 11209–11217. https://doi.org/10.3168/JDS.2020-18741

Wu, G., Hui, X., Stipkovits, L., Rachman, A., Tu, J., Brennan, M. A., & Brennan, C. S. (2021). Whey protein-blackcurrant concentrate particles obtained by spray-drying and freeze-drying for delivering structural and health benefits of cookies. *Innovative Food Science & Emerging Technologies*, *68*, 102606. https://doi.org/10.1016/J.IFSET.2021.102606

Xu, H., Zhang, T., Lu, Y., Lin, X., Hu, X., Liu, L., He, Z., & Wu, X. (2019). Effect of chlorogenic acid covalent conjugation on the allergenicity, digestibility and functional properties of whey protein. *Food Chemistry*, *298*, 125024. https://doi.org/10.1016/J.FOODCHEM.2019.125024

Yan, X., Liang, S., Peng, T., Zhang, G., Zeng, Z., Yu, P., Gong, D., & Deng, S. (2020). Influence of phenolic compounds on physicochemical and functional properties of protein isolate

from Cinnamomum camphora seed kernel. *Food Hydrocolloids*, *102*, 105612. https://doi.org/10.1016/J.FOODHYD.2019.105612

Yang, S., Wang, C., Li, X., Wu, C., Liu, C., Xue, Z., & Kou, X. (2021). Investigation on the biological activity of anthocyanins and polyphenols in blueberry. *Journal of Food Science*, *86*(2), 614–627. https://doi.org/10.1111/1750-3841.15598

Ye, J., Fan, F., Xu, X., & Liang, Y. (2013). Interactions of black and green tea polyphenols with whole milk. *Food Research International*, *53*(1), 449–455. https://doi.org/10.1016/J.FOODRES.2013.05.033

Zhang, X., Luo, X., Wang, Y., Li, Y., Li, B., & Liu, S. (2020). Concentrated O/W Pickering emulsions stabilized by soy protein/cellulose nanofibrils: Influence of pH on the emulsification performance. *Food Hydrocolloids*, *108*, 106025. https://doi.org/10.1016/J.FOODHYD.2020.106025

Zhang, Z., Qiu, C., Li, X., McClements, D. J., Jiao, A., Wang, J., & Jin, Z. (2021). Advances in research on interactions between polyphenols and biology-based nano-delivery systems and their applications in improving the bioavailability of polyphenols. *Trends in Food Science & Technology*, *116*, 492–500. https://doi.org/10.1016/J.TIFS.2021.08.009

Zhong, S., Sandhu, A., Edirisinghe, I., & Burton-Freeman, B. (2017). Characterization of Wild Blueberry Polyphenols Bioavailability and Kinetic Profile in Plasma over 24-h Period in Human Subjects. *Molecular Nutrition & Food Research*, *61*(12), 1700405. https://doi.org/10.1002/mnfr.201700405