Role of dry beans (*Phaseolus vulgaris* L.) in binding bile salts and modulating lipid digestion: Impact of the bean matrix and high-hydrostatic pressure processing

By

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Role of dry beans (*Phaseolus vulgaris* L.) in binding bile salts and modulating lipid digestion: Impact of the bean matrix and high-hydrostatic pressure processing

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**ABSTRACT**

According to the American Heart Association, cardiovascular disease (CVD) is the leading cause of death in the U.S., representing about 20-30% of all deaths every year in the U.S. Major risk factors for developing CVD include high blood lipid and LDL-cholesterol levels. A large number of heart attacks and strokes could be prevented by controlling these factors through lifestyle modifications and diet interventions. Epidemiological evidence shows that consumption of dry or common beans (*Phaseolus vulgaris* L.) has positive effects on reducing blood LDL-cholesterol and lipid levels. These health benefits are mainly attributed to the high content of dietary fiber (DF) of beans, including soluble and insoluble DF (SDF and IDF). Some proposed mechanisms to explain the cholesterol and lipid-lowering effects of DF are related to the physico-chemical properties (e.g. viscosity) of DF, and involve binding to bile salts (BS) in the small intestinal to prevent BS re-absorption which further promote cholesterol catabolism and delay lipid digestion. Nevertheless, the precise mechanisms are not fully understood yet. In addition, cooking and processing operations, and in particular high-hydrostatic pressure (HHP) processing, can modify the composition, structure and functional properties of foods; however, whether HHP affects the ability of beans to interfere with different aspects of lipid digestion remains unknown. The overall goal of this research is to understand how common beans and HHP processing impact the ability of beans to bind BS and influence lipid digestion *in vitro*. The specific objectives are 1) to evaluate the effect of HHP treatments (and compared it with conventional cooking (HT)) on the thermo-rheological and functional properties of dry beans; 2) to identify the impact of major bean components on the *in vitro* BS-binding ability of beans, the role played by the bean matrix and how this is affected by HHP processing; 3) to investigate how bean (micro)structure and fiber fractions, as well as HHP processing of dry beans, influence lipid digestion *in vitro*. Results showed that HT caused complete starch gelatinization and protein denaturation of beans, while HHP treatments induced partial or no starch gelatinization and a lower degree of protein denaturation, which resulted in enhanced protein solubility and emulsifying activity/stability. It was observed that, while HT treatment reduced the capacity of bean flours to retain BS because of severe disruption of the bean cell wall integrity, protein matrices, and starch granules, HHP treatments maintained or enhanced BS retention, possibly by promoting the formation of starch/protein/fiber networks able to entrap BS. Furthermore, by using an *in vitro* dialysis-based digestion model combined with viscosity measurements and thermal analysis, it was shown that
the interaction between bean tissue materials and primary BS was not only related to viscosity but also involved hydrophobic linkages. The contribution of IDF and proteins (other than SDF) to retain BS was also significant. There was a different binding preference of beans to four primary BS with sodium glycochenodeoxycholate, the more hydrophobic BS, showing the largest retention levels while sodium taurocholate being the least effectively retained BS by beans. Diverse sequences of the same processing operations showed distinct impacts on BS-retention by dry beans. By means of an in vitro digestion model simulating conditions in the upper gastrointestinal tract, bean flours delayed the digestion of extrinsic lipids to a higher extent, compared to isolated IDF and SDF. Furthermore, HHP treatment and less severe mechanical disintegration maintained the ability of beans to modulate lipid digestion, which suggests the importance of bean structural integrity in reducing the lipolysis rate and extent by beans. Overall, this research work shows that HHP processing is a promising minimal processing technology to produce bean flours with improved functionality. It also highlights the importance of considering the structure of foods, and not just their nutrient content, when evaluating potential health impacts. This knowledge could be applied to develop a range of bean-based ingredients and formulations with desirable health benefits. This work can be extended to research the influence of beans on the gut microbiota and profile of secondary BS and short-chain fatty acids, which are also closely related to cholesterol and lipid metabolism.
Role of dry beans (*Phaseolus vulgaris* L.) in binding bile salts and modulating lipid digestion: Impact of the bean matrix and high-hydrostatic pressure processing

Tiantian Lin

GENERAL AUDIENCE ABSTRACT

According to the American Heart Association, cardiovascular disease (CVD) is the leading cause of death in the U.S., representing about 20-30% of all deaths every year in the U.S. Around the world, millions of people are struggling to control the risk of CVD. Major risk factors for developing CVD include high blood lipid and LDL-cholesterol levels. A large number of heart attacks and strokes can be prevented by controlling the major risk factors through lifestyle modifications and diet interventions. Epidemiological evidence shows that consumption of dry beans (*Phaseolus vulgaris* L.) has positive effects on reducing blood LDL-cholesterol and lipid levels. These health benefits are mainly attributed to the high content of dietary fiber (DF) in beans. DF is carbohydrate polymers that are not hydrolyzed by the endogenous enzymes in humans. However, some of them (water-soluble DF) could increase viscosity and retain the absorption of bile salts (BS) in the small intestinal. The BS retention or the binding of BS could promote more cholesterol convert to BS (thus reduce cholesterol levels) and decrease lipid digestion. Therefore, due to the increased viscosity and BS retention ability of DF, dry beans could help to reduce the blood cholesterol and lipid levels and further help to prevent CVD. Moreover, different cooking and processing method could also affect the composition, microstructure and functional properties of foods. The purpose of this research was to determine how common beans and high hydrostatic pressure (HHP) (compared with hydrothermal (HT)) processing, a non-thermal processing, influence the ability of dry beans to retain bile salts and modulate lipid digestion *in vitro*. This study showed that severe HT treatment disrupted the bean cell wall integrity severally and reduced the BS retaining the efficiency of dry beans, while HHP treatment, produced minimally processed beans, improved the application properties of dry beans and maintained/enhanced BS-retention by dry beans. It also showed that the whole bean matrix (other than soluble DF) also contributes to retain BS and modulate lipid digestion, indicating the importance of retaining intact food structures. The integrity of bean structures through HHP treatment and less severe mechanical treatment could help to retain the ability of dry beans to reduce lipid digestion. These findings suggest that dry beans, with a high content of dietary fiber and resistant starch, have significant health benefits related to lowering cholesterol and lipid levels. Increasing the consumption of dry beans would definitely help to improve overall health. HHP, as a non-thermal processing technology, showed the potential to produce minimally processed bean products with enhanced health benefits and
diverse application properties. This study could be extended through continuing research into the influence of beans on the gut microbiota, which are also closely related to the cholesterol and lipid metabolism regulation.
Dedication

To my family and friends.
Acknowledgements

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Attributions

Several co-authors contributed to the completion of Chapter 3-6 and their contributions are briefly described here.

**Chapter 3:** Effect of thermal and high-pressure processing on the thermo-rheological and functional properties of common bean (*Phaseolus vulgaris* L.) flours
Cristina Fernández-Fraguas, PhD, a current assistant professor in the Department of Food Science and Technology at Virginia Tech, assisted with the study design, compilation and completion of the manuscript.

**Chapter 4:** Manipulation of the dry bean (*Phaseolus vulgaris* L.) matrix by hydrothermal and high-pressure treatments: Impact on *in vitro* bile salt-binding ability
Sean F. O’Keefe, PhD, a current professor in the Department of Food Science and Technology at Virginia Tech assisted with the completion of the manuscript.
Susan E. Duncan, PhD, a current professor in the Department of Food Science and Technology at Virginia Tech assisted with the completion of the manuscript.
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**Chapter 5:** Retention of primary bile salts by dry beans (*Phaseolus vulgaris* L.) during *in vitro* digestion: Role of bean components and effect of food processing
Sean F. O’Keefe, PhD, a current professor in the Department of Food Science and Technology at Virginia Tech assisted with the HPLC analysis of bile salts and the completion of the manuscript.
Susan E. Duncan, PhD, a current professor in the Department of Food Science and Technology at Virginia Tech assisted with the completion of the manuscript.
Cristina Fernández-Fraguas, PhD, a current assistant professor in the Department of Food Science and Technology at Virginia Tech, assisted with the study design, compilation and completion of the manuscript.

**Chapter 6:** Dry bean (*Phaseolus vulgaris* L.) modulate the kinetics of lipid digestion *in vitro*
Sean F. O’Keefe, PhD, a current professor in the Department of Food Science and Technology at Virginia Tech assisted with the GC-MS analysis of fatty acids.
Susan E. Duncan, PhD, a current professor in the Department of Food Science and Technology at Virginia Tech assisted with the completion of the manuscript.
Cristina Fernández-Fraguas, PhD, a current assistant professor in the Department of Food Science and Technology at Virginia Tech, assisted with the study design, compilation and completion of the manuscript.
Chapter 1: Introduction and Justification

Metabolic syndrome is a metabolic disorder that involves a batch of body abnormalities such as obesity, hypertension, hyperlipidemia, hypercholesterolemia and insulin resistance. Among them, obesity is one of the most serious health issues in the world and also identified as a national priority in the U.S. Additionally, hyperlipidemia and hypercholesterolemia are major physiological risk factors related to cardiovascular diseases (CVD), the leading cause of death in U.S. and worldwide. Consequently, viable and sustainable lifestyle modifications and dietary strategies for the prevention of fat excess, high cholesterol levels, and CVD are urgently needed.

Dietary fiber (DF), including soluble (SDF) and insoluble dietary fiber (IDF), is a dietary component that has shown to have positive effects on reducing hyperlipidemia and hypercholesterolemia as well as promoting satiety. These health benefits are mainly linked to the ability of DF to generate viscosity and involve prevention of bile salt (BS) re-absorption possibly by sequestering/retaining BS or by decreasing the rate of lipid digestion and absorption in the small intestinal. The retention of BS could promote more cholesterol converted to BS and delay lipid digestion thus reducing cholesterol and lipid levels. However, the mechanisms behind the BS-binding ability of DF are not fully understood. Despite these clear health benefits, the intake of DF in the U.S. (15g/day) is only half of the recommended level (25-32g/day). Consequently, strategies are needed to increase fiber consumption in the American diet and thus to improve the overall health of the population.

DF is naturally present in a variety of plant foods including cereals, legumes, fruits, and vegetables. Particularly, dry beans (Phaseolus vulgaris L.), a major grain legume, are a complex food matrix with a high level of fiber compared to the other commonly recognized fiber-rich foods mentioned above. The consumption of dry beans has been shown to reduce blood cholesterol and lipid levels and help to prevent CVD. Therefore, incorporating dry beans in a daily diet is a promising strategy to increase DF intake in the U.S. However, the consumption of dry beans per capita has seen a slow but steady decline in developed countries; specifically, 80% of the population do not consume the recommended daily intake (~3 cups per week) of dry beans in U.S. Besides, formulations aimed at including dry beans in processed foods are not widely developed or used in the U.S. There is, therefore, an opportunity for the food industry to process dry beans into various products and ingredients, which could provide people with more choices to consume fiber-rich products and ultimately improve the public health.

Many food processing and cooking techniques are shown to manipulate the composition, structure and functional properties of foods. Understanding how food processing modifies the microstructural, physicochemical, and physiological properties of dry beans could inspire us with new ideas of developing beans into palatable products with desirable health benefits. Hydrothermal
(HT) processing, such as boiling and canning, is widely used to manufacture beans into edible foods. However, high cooking temperature and/or prolonged heating time has reportedly caused negative changes in the sensorial quality and nutritional value of legumes. Consumers' demand for clean label foods (e.g., minimally processed foods) drives the need for the validation of new generation processing technologies. High hydrostatic pressure (HHP), one of the most promising non-thermal processing technologies, has been applied mainly to improve food safety for the last decade. Recently, it has also shown encouraging potential to manipulate the microstructure of foods and functionality of food components. To date, how HT and HHP affect beans to retain BS and impact lipid digestion remains unknown. Moreover, the functionality and physiological properties of foods are controlled by the complexity and structure of the food matrix. Considering the high complexity of the dry bean matrix, it is worth understanding how the structure and the integrity of bean matrices affect the efficiency of dry beans/bean DF to retain BS and modulate lipid digestion. Also, some in vitro studies have shown that whole bean seeds showed superior effects in reducing cholesterol and lipid levels than isolated fiber. Whether and how other components besides DF within the bean matrix have an effect on retaining BS and reducing lipid digestion has not been well understood and need further investigation.

Therefore, the overall goal of this research is to understand how bean matrix and bean components contribute to binding/retaining BS and reducing lipid digestion and how processing affects the ability of dry beans to retain BS and modulate lipid digestion. Our central hypothesis is that the bean matrix plays an essential role in retaining BS and interfering with lipid digestion and HHP processing will manipulate the microstructure and physicochemical properties of dry beans, and further affect the ability of dry beans to retain BS and modulate lipid digestion. In order to meet the overall goal, we proposed the following objectives:

1. Compare the effect of HT and HHP on the thermo-rheological and functional properties of dry beans. We hypothesize that HT and HHP treatments will have a different effect on the rheological, thermal, pasting, and functional properties of beans.

2. Understand how bean fractions, bean matrix and HHP processing impact the BS-binding ability of beans.
   1) Determine how HHP (compared to HT) affects the DF composition and microstructure of dry bean matrix and further impacts BS-binding ability of dry beans. We hypothesize that HHP and HT will differently modify bean DF composition and microstructure, which will have an impact on the ability of beans to retain BS in vitro.
2) Determine which and to what extent the bean fractions and processing of beans contribute to the retention and retention kinetics of primary bile salts. We hypothesize that bean components other than DF could contribute to the BS retention ability of dry beans and food processing-mechanical treatment will affect the viscosity of digested bean matrix and further impact the BS retention kinetics.

3. Investigate how dry bean fiber, bean structure and HHP processing influence the ability of dry beans to modulate in vitro lipid digestion. We hypothesize that the various integrity of bean structures generated by different processing will significantly impact the ability of beans to modulate in vitro lipolysis.

References


Chapter 2: Literature Review

2.1 Metabolic syndrome, Obesity and Cardiovascular disease

Metabolic syndrome is the metabolic disorder including a batch of body abnormalities such as obesity, hypertension, hyperlipidemia, hypercholesterolemia and insulin resistance\(^1\). Among them, obesity is one of the most critical public health issues worldwide and nationwide in U.S. For the last 30 years, a significant increase in obesity was observed, from below 15% to more than 30% in adults and even from 5% to more than 20% in youth\(^2\). In 2016, no state in the U.S. had a prevalence of obesity less than 20%\(^3\). CVD, highly related to the occurrence of hyperlipidemia and hypercholesterolemia, is the No. 1 cause of death globally and in U.S. About 31% of global death (17.7 million people) was because of CVD in 2015. In 2016, it took an estimated 0.85 million lives, representing about 20% of all deaths, in the U.S. The CVD not only takes a heavy toll on the health of Americans, but also induces enormous economic burden. According to the data from the American Heart Association, the health cost for CVD was $555 billion in U.S. in 2016, and by 2035, the cost is predicted to increase to $1.1 trillion\(^4\).

**Table 2.1** National Cholesterol Education Program Cholesterol Guidelines for adults\(^5\).

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<th>Total cholesterol</th>
<th>LDL</th>
<th>HDL</th>
<th>Triglycerides</th>
</tr>
</thead>
<tbody>
<tr>
<td>Desirable</td>
<td>&lt;200 mg/dl</td>
<td>&gt;60 mg/dl</td>
<td>&lt;200 mg/dl</td>
</tr>
<tr>
<td>Borderline</td>
<td>200-240 mg/dl</td>
<td>130-160 mg/dl</td>
<td>200-400 mg/dl</td>
</tr>
<tr>
<td>High CVD risk</td>
<td>&gt;240 mg/dl</td>
<td>&gt;160 mg/dl</td>
<td>&gt;400 mg/dl</td>
</tr>
</tbody>
</table>

LDL: high density lipoproteins, are called “good” cholesterol.
HDL: high density lipoproteins, are called “bad” cholesterol.
mg/dl: milligrams per deciliter of blood.

Obesity, hyperlipidemia, and hypercholesterolemia (Table 2.1) are three major factors that cause CVD, a group of disorders of heart and blood vessels. When elevated blood cholesterol or lipid levels occur in the human body, some cholesterol and lipids accumulated (called plaques) on the inner wall of arteries, which is termed atherosclerosis or coronary arteries. These plaques can restrict blood flow to the heart muscle, causing an inadequate supply of oxygen and vital nutrients for the heart, increasing the risk of heart attack and CVDs\(^6\). The risk factors for CVD include non-modifiable risk factors (ages, gender, family history, and race) and modifiable factors (smoking, high blood cholesterol and triglycerides, high blood pressure, physical inactivity, unhealthy diet). Numerous studies show that CVD is closely related to elevated blood lipids and cholesterol levels, overweight and obesity\(^4\). The risks of heart disease increase steadily with an increasing BMI (Body
Mass Index), especially being overweight (BMI 25-29 kg/m$^2$) and obesity (BMI higher than 30 kg/m$^2$). People with high blood cholesterol (especially high low-density lipoprotein cholesterol, LDL-C (“bad” cholesterol) over 100 mg/dL and high-density lipoprotein cholesterol, HDL-C (“good” cholesterol) under 40 mg/dL) have more than twice the risk of heart disease compared to people with low level. Thus, decreasing blood cholesterol and blood lipid levels are associated with a lower risk of cardiovascular disease.

The current methods to treat and prevent CVD include drug therapy and lifestyle modification. Statins are the most commonly used drugs to prevent CVD by reducing LDL-C levels. They could inhibit the 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, the rate-limiting enzyme involved in the synthesis process of cholesterol, to reduce the de novo synthesis of cholesterol in the liver thus reducing the LDL-C levels. There are six kinds of statins used as drugs to treat CVD world widely, including atorvastatin, simvastatin, lovastatin, pitavastatin, rosuvastatin, Fluvastatin, and pravastatin. The intake of statin drugs is associated with some side effects includes mild muscular symptoms, myopathy or increased risk of diabetes mellitus. Bile Salts (BS) sequestrants (BS binding resins) like cholestyramine and colestimide are another kind of drug used to reduce LDL-C levels. They are positively charged chemicals and could form an insoluble complex with BS through chemical interactions in the small intestinal, which increases the fecal excretion of BS and hence activate the conversion of cholesterol into BS in the liver, thus reducing the blood cholesterol levels. However, these sequestrants could also induce discomfort on the gastric-intestinal tracts of the human body, causing constipation, bloating and cramping.

Another more natural way to prevent these chronic diseases is to make appropriate changes to diet and lifestyle. Consequently, effective and sustainable dietary strategies for weight control and prevention of excess fat and cholesterol accumulation are urgently needed. The World Health Organization emphasizes the importance of improved nutrition and a healthier lifestyle as a means of controlling the expected rise in global obesity incidence and related diseases (i.e., CVD) over the next decades. Anti-obesity campaigns were held recently, appealing to promote healthy eating habits and a healthy lifestyle. For example, the most recent U.S. dietary guidelines, MyPlate, recommends the public to have a healthier diet rich in vegetables, fruits, grains, and legumes. Such an eating pattern is high in fiber and low in energy density, which is claimed to be particularly crucial for controlling fat and cholesterol levels.

### 2.2 Dietary fiber

Many epidemiologic studies show that DF has the health benefits of promoting satiety, reducing hyperlipidemia and reduce LDL-cholesterol levels, thus is a candidate to reduce the risk of CVD. Research studies suggest that a consumption of 14 g of DF per 1000 kcal of meal or
25 g for female and 28 g for male per day can significantly reduce the risk of CVD\textsuperscript{14}. Thus, incorporating dietary fiber in the daily diet may help to fight against the challenge of CVD.

2.2.1 Definition, categories, and sources of dietary fiber

Codex Alimentarius Commission (CAC) gave the most updated and acceptable definition of DF: “Dietary fiber consists of carbohydrate polymers with ten or more monomeric units, which are not hydrolyzed by the endogenous enzymes in the small intestine of humans and belong to the following categories: edible carbohydrate polymers naturally occurring in the food as consumed; carbohydrate polymers which have been obtained from food raw material by physical, enzymatic or chemical means and which have been shown to have a physiological effect of benefit to health as demonstrated by generally accepted scientific evidence to competent authorities, and; synthetic carbohydrate polymers which have been shown to have a physiological effect of benefit to health as demonstrated by generally accepted scientific evidence to competent authorities”\textsuperscript{15}. According to this definition, McCleary et al. developed the lab analysis method to measure the content of total dietary fiber by analyzing the content of insoluble dietary fiber (IDF), soluble dietary fiber (SDF), and non-digestible oligosaccharides (OLIGO)\textsuperscript{16}. Resistant starch (RS) was also included in the fiber content in McCleary’s method.

SDF refers to fibers that cannot be digested or absorbed in the human gastrointestinal tract but is partly soluble in water. SDF includes polysaccharides (PS) such as pectin, gum arabic, guar gum, and \(\beta\)-glucan\textsuperscript{17}. Pectins are those extracts from plant material by hot water, essential members of which are galacturonan. They are key elements in the primary wall hydrogel continuous phase (matrix) in plants. Pectin fragments also play roles in plant defense and signaling. The ability of pectin to form gels in aqueous solution makes it an important ingredient in jams, jellies, yogurts, and other food products. Gelling properties may also provide pectin hypoglycemic properties by delaying the gastric emptying and the small intestinal transmitting time\textsuperscript{18}. Gums and mucilages are secreta from secretory plant cells\textsuperscript{19}. They are polysaccharides that are highly branched and could form a gel and bind with other chemicals. Gums mainly includes guar gum and gum arabic. Guar gum is a polysaccharide gum with a galactomannan structure, while gum arabic is an arabinogalactan PS linked with a glycoprotein\textsuperscript{17}. \(\beta\)-glucan, referred to as \((1\rightarrow3), (1\rightarrow4)-\beta-D\)-glucan, is one of the most critical soluble dietary fibers studied. The benefits of \(\beta\)-glucan to decrease cholesterol level in the blood and induce satiety have been well proven, and its cholesterol lowering effect is related to its ability to bind bile salts and further lower the reabsorption of bile salts. Due to this benefit, the U.S. Food and Drug Administration (FDA) has claimed that having a daily diet with 3g \(\beta\)-glucan per day may help to reduce cholesterol levels and the risk of CVD\textsuperscript{20}. 
### Table 2: Dietary fiber content from various food sources

<table>
<thead>
<tr>
<th>Source</th>
<th>Dietary fiber (g/100g edible portion)</th>
<th>Total</th>
<th>Insoluble</th>
<th>Soluble</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grains</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Barley</td>
<td>17.3</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Corn</td>
<td>13.4</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Oats</td>
<td>10.3</td>
<td>6.5</td>
<td>3.8</td>
<td></td>
</tr>
<tr>
<td>Rice (dry)</td>
<td>1.3</td>
<td>1</td>
<td>0.3</td>
<td></td>
</tr>
<tr>
<td>Rice (cooked)</td>
<td>0.7</td>
<td>0.7</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Wheat (whole grain)</td>
<td>12.6</td>
<td>10.2</td>
<td>2.3</td>
<td></td>
</tr>
<tr>
<td>Wheat germ</td>
<td>14</td>
<td>2.9</td>
<td>1.1</td>
<td></td>
</tr>
<tr>
<td>Legumes &amp; pulses</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Green beans</td>
<td>1.9</td>
<td>1.4</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>Soy</td>
<td>15</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Peas, green frozen</td>
<td>3.5</td>
<td>3.2</td>
<td>0.3</td>
<td></td>
</tr>
<tr>
<td>Kidney beans, canned</td>
<td>6.3</td>
<td>4.7</td>
<td>1.6</td>
<td></td>
</tr>
<tr>
<td>Lentils, raw</td>
<td>11.4</td>
<td>10.3</td>
<td>1.1</td>
<td></td>
</tr>
<tr>
<td>Lima beans, canned</td>
<td>4.2</td>
<td>3.8</td>
<td>0.4</td>
<td></td>
</tr>
<tr>
<td>White beans, raw</td>
<td>17.7</td>
<td>13.4</td>
<td>4.3</td>
<td></td>
</tr>
<tr>
<td>Vegetables</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Potato, no skin</td>
<td>1.3</td>
<td>1</td>
<td>0.3</td>
<td></td>
</tr>
<tr>
<td>Tomato, raw</td>
<td>1.2</td>
<td>0.8</td>
<td>0.4</td>
<td></td>
</tr>
<tr>
<td>Carrot, raw</td>
<td>2.5</td>
<td>2.3</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>Broccoli, raw</td>
<td>3.29</td>
<td>3</td>
<td>0.29</td>
<td></td>
</tr>
<tr>
<td>Fruits</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Apple, unpeeled</td>
<td>2</td>
<td>1.8</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>Kiwi</td>
<td>3.39</td>
<td>2.61</td>
<td>0.8</td>
<td></td>
</tr>
<tr>
<td>Nuts and seeds</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Almonds</td>
<td>11.2</td>
<td>10.1</td>
<td>1.1</td>
<td></td>
</tr>
<tr>
<td>Coconut, raw</td>
<td>9</td>
<td>8.5</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>Sesame seed</td>
<td>7.79</td>
<td>5.89</td>
<td>1.9</td>
<td></td>
</tr>
</tbody>
</table>

IDF is indigestible fiber fraction in the human gastrointestinal tract which is insoluble in water. IDF is the main component of plant cell wall structure including cellulose, hemicellulose, and lignin. Cellulose is a primary structural polymer of plants. It has a long chain of glucose units with repeating linear β-1, 4 glucosidic linkages; it is classified as a no branching homopolysaccharide, with no other monosaccharides, and no substituents in its structure. Hemicelluloses, regarded initially as cellulose precursors, are non-cellulosic plant polysaccharides.
with $\beta-(1\rightarrow4)$-linked, equatorial polypyranoose main chains\textsuperscript{21}. Lignin exists between cellulose, hemicellulose, and pectin in vascular and support tissues of plants. Lignin is not a polysaccharide, but a heterogeneous cross-linked polymer containing many oxygenated phenylpropane\textsuperscript{22}. Due to strong intramolecular bonding, lignin is more rigid and shows a greater resistance than other natural insoluble dietary fiber.

Dietary fiber naturally exists in plant foods, such as cereals, legumes, fruits, vegetables, and nuts, as shown in Table 2.2. Fruits and vegetables mainly contain soluble fiber like pectin from orange peel and apple peel and gum from seaweed. Cereals, legumes, and nuts mainly contain insoluble fiber like cellulose and hemicellulose in the cell wall of the seeds or hull\textsuperscript{23}.

### 2.2.2 Fiber intake in U.S.

The fiber intake recommendations were firstly established in the first decade of the twenty-first century because of its benefit in preventing heart disease and laxation\textsuperscript{24}. Currently, the recommended fiber intake are from 30–35 g/day for male and 25–32 g/day for female, according to the evidence that enough fiber intake could help to reduce the risk of obesity, type 2 diabetes, and CVD\textsuperscript{25}. However, in the U.S., the actual fiber intake is only half of the recommended intake level, ranging from 15-17 g/day. Only about 5% of populations meet the adequate intake level\textsuperscript{26}. Consequently, strategies are needed to increase fiber content in the American diet and then to improve the overall health of the population. The increase of fiber intake can be met by consuming at least one whole or “minimally processed” plant food (e.g., whole grains, legumes, vegetables and fruits) to replace a “highly processed” or high sugar, high fat, or low fiber food at each meal\textsuperscript{27}.

### 2.2.3 Physiological properties of dietary fiber

#### 2.2.3.1 Evidence of preventing CVD and obesity of dietary fiber

Many articles documented that adequate consumption of dietary fiber could significantly decreases the LDL-C levels and reduces the risk of CVD\textsuperscript{1,13,28–30}. A dose-response study suggests an intake of 7–10 g fiber increment/day was associated with a 9–11% reduction of the risk of CVD\textsuperscript{13}. In 2008, the Academy of Nutrition and Dietetics Evidence Analysis Library Committee stated that ability of DF to “attenuate elevated serum lipid levels and cholesterol levels, blood pressure, and systemic inflammatory markers” was an important mechanisms to explain the CVD protective properties of DF \textsuperscript{24}. Many scientific studies have proven that adequate intake of dietary fiber can help to regulate body weight and to prevent the risk of CVD\textsuperscript{31–34}. According to an analysis of NHANES (National Health and Nutrition Examination Survey) 1999–2010 (Figure 2.1), the fiber intake is inversely corelated with the occurrence of obesity among US adults\textsuperscript{35}. Increasing the consumption of fiber or fiber rich foods also contributes to reduced BMI and body fat in children and adolescents\textsuperscript{24}.
Figure 2.1 Relationship between increasing fiber intake and adult obesity risk from the US National Health and Nutrition Examination (NHANES) Survey (1999-2010).

The physiological properties of reducing the blood lipid level and cholesterol concentration by dietary fiber are related to its physiochemical properties demonstrated in the human gastrointestinal tract, especially its effects in modulating lipid digestion and cholesterol level. These physicochemical properties include solubility, viscosity, fermentation property and binding ability with molecules. However, the mechanism(s) underlying the physiological effects of dietary fiber is not well understood. It is not clear yet which aspects of lipid digestion are affected by slowly digestible carbohydrates and fiber and, therefore, which molecular properties are crucial for their functionality. Therefore, it seems a single mechanism cannot explain how dietary fiber impacts on lipid digestion and other gastrointestinal functions.

2.2.3.2 Dietary fiber and modulation of lipid digestion

Some proposed mechanisms of modulating lipid digestion by DF involve its prevention of bile salt (BS) re-absorption, possibly by sequestering BS and cholesterol, which is linked to its capacity to generate high viscosity in the gastrointestinal tract, promoting enhanced fecal excretion of steroids. However, inconsistent results have been found in the literature. Other mechanisms could involve a decrease in the rate of lipid digestion in the duodenum.

1) General introduction of lipid digestion and absorption

Dietary lipids usually include triacylglycerols (TAG), cholesterol, cholesterol esters, and phospholipids. Generally, lipid digestion mainly occurs in the small intestine (70-90%) by the hydrolysis of pancreatic lipase, with a small part in the stomach by the hydrolysis of gastric
lipase\textsuperscript{39}. In the mouth and stomach, lingual and gastric lipase hydrolyze parts of TAG into \textit{sn}-1,2 diacylglycerols (DAG) and free fatty acids (FFA), respectively. After the gastric phase, the food bolus becomes smaller particles and turns into chime\textsuperscript{36}. The duodenum, as the first part of the small intestine, is the major site for lipid digestion. When chime enters the duodenum, this stimulates the pancreas to secrete pancreatic, a mixture of enzymes including a-amylase, proteases, and lipase, to hydrolyze starch, proteins, and fat, respectively\textsuperscript{40}. Most TAGs are hydrolyzed into FFAs (at the \textit{sn}-1 and \textit{sn}-3 positions) and 2-monoglycerides (MAGs) by pancreatic lipase in the duodenum\textsuperscript{41}. For the other two common dietary lipids, cholesterol and phospholipids, they are hydrolyzed by cholesterol esterase and phospholipase \textit{A}_{2}\textsuperscript{38}. The TAGs must be hydrolyzed into these small molecules such as FFAs and MAGs, which are less insoluble in water, so that they can be easily transferred through the intestinal epithelium and distributed to the body for metabolism. The key to achieving this is the digestion of lipids by the enzymatic hydrolysis of emulsified lipids by lipase. Lipids are poorly water-soluble molecules in the digestive tract, while pancreatic lipase is water-soluble and can only work at the surface of emulsified lipid droplets\textsuperscript{42}. Bile salts (BS) are amphipathic molecules with both hydrophilic and hydrophobic ends, acting as effective emulsifying agents and playing a key role in this process. BS could arrange themselves on the surface of lipid droplets, with their hydrophobic ends turned inward and their hydrophilic regions turned outward toward the water phase\textsuperscript{39}. This colloidal phenomenon together with the help of peristaltic agitation converts lipids into smaller droplets with a greatly increased surface area, which facilitates more efficient attachment of pancreatic lipase to lipid droplet\textsuperscript{38}. Another important co-factor associating with pancreatic lipase is colipase. Colipase possesses distinctly hydrophobic regions that act as lipid-binding sites, thus working as anchor or linking point for the attachment of enzymes to the bile salts-stabilized lipid micelles to facilitate the hydrolysis action of lipase on the lipid surface. Under the assistance of BS and colipase, pancreatic lipase hydrolyzes lipids into MAGs and FFAs. The accumulation of FFAs and MAGs at the interface would decrease the surface area for lipase to absorb on the lipid droplets, therefore decreasing lipolysis\textsuperscript{43}. Some polysaccharides or proteins could act as an interfacial barrier and impact the lipase absorbed to the surface of lipid droplets. BS also helps to transfer the lipid hydrolysis products through the intestinal membrane by forming mixed micelles vesicles consisting of BS and phospholipids. The formation of bile salts micelles only occurs at a critical micelle concentration (CMC). BS at concentrations lower than CMC cannot form micelles\textsuperscript{39}.

The impact of DF on lipid digestion and absorption may depend on the type of fibers that exhibit different physiochemical properties and depend on the different food matrix that fiber exits. Generally, it mainly includes the following factors. Dietary fiber could increase the bulk volume of dietary lipids through their solubility and water/oil holding ability, thus delaying gastric emptying\textsuperscript{44}. Besides, viscous water-soluble DF could limit the transmural transport of lipids. On the other hand, dietary fiber is reported to reduce the availability of bile salts for lipid absorption,
possibly by viscously trapping the bile salts or by direct chemical binding with bile salts, like bile salts sequestrants. Since the bile salts are essential components of lipid digestion and absorption, dietary fibers that decrease the availability of bile acids for the lipolysis process may interfere with the lipid digestion most significantly.

2) Cholesterol lowering effect of dietary fiber

Cholesterol is a C27 sterol and a necessary constituent for eukaryotic cell growth and development. Humans obtain cholesterol mainly from two sources: de novo synthesis in the liver (700-900 mg/day) and directly from the daily meals (~300-500 mg/day)\(^{28}\). Cholesterol is removed from the body by direct excretion from GIT tract (~600 mg/day), conversion to bile acids/salts (BA/BS) (~400 mg/day), loss of dead skin cells (~85 mg/day), synthesis of steroid hormones (~50 mg/day), and by transportation into membranes for cells dividing\(^{45}\). The catabolism of hepatocyte cholesterol mainly comes from the BS synthesis which involves 14 enzymes among which, cholesterol 7a-hydroxylase is the rate-limiting enzyme. In humans, the bile acid pool consists of 80% of primary BA (cholic acid, CA and chenodeoxycholic acid, CDCA) and 20% of secondary BA (deoxycholic acid, DCA, derived from CA) and lithocholic acid, LCA, derived from CDCA\(^{46}\). Primary BA is synthesized from cholesterol exclusively in the liver and excreted into small intestinal. Secondary BS are derived from primary BA by bacterial enzymes primarily in the large intestinal and the terminal ileum\(^{47}\). More than 98% of primary BA are conjugated with amino acids through N-acyl linkage in the human liver before their active secretion from the liver into the gallbladder and the small intestinal\(^{48}\). Most of the bile acids that exist in the human body are conjugated with amino acids taurine or glycine with increased water solubility and decreased cellular toxicity\(^{40}\). In the liver, the conjugated BS will further form sodium salts, called bile salts (BS). In the human body, there are mainly four different kinds of BS, as shown in Table 2.3. Even though various BS have similar physiological functions, BS with different numbers, positions and stereochemistry of hydroxyl groups as well as the conjugation of amino acids, have quantitively different properties\(^{49,50}\). Dihydroxyl bile salts (-CDC) are more hydrophobic than trihydroxy bile salts (-C), and glycine-conjugated bile salts (G-) are more hydrophobic than taurine-conjugated bile salts (T-)\(^{48}\).

Bile salts show detergent-like properties since their structure includes a steroid nucleus (hydrophobic side) and a hydroxyl group and an ionic head (hydrophilic side). After each meal, BS is released into the small intestinal and participates in lipid digestion and absorption. Almost 95% of BS are resorbed in the ileum, the last section of the small intestine, and they are returned to the liver via portal blood and re-excreted into the bile, referring to as hepatic circulation. Around 5% of BS will escape the reabsorption and are eliminated in the feces. The loss of BS will be compensated by the de novo synthesis from cholesterol in the liver, thus maintaining a constant BS pool in the human body\(^{51}\). The bile salts pool is maintained at concentrations of 2-6.4 mM or
10-15mM, depending on fasted or feeding states, with 4-12 cycles of biliary excretion per day\textsuperscript{51}. Beyond their classic functions in regulating lipid digestion and cholesterol metabolism, BS also act as modulators of metabolism by activating the farnesoid X receptor (FXR) and G-protein coupled bile acid receptor 1 (also known as TGR5), both of which regulate various pathways related to the glucose, lipid, and energy metabolism\textsuperscript{52}. BS metabolism is also related to gut microbiota. It could control the bacteria overgrowth and the inflammation in the gut\textsuperscript{53}. The gut microbiota also influences the size and composition of the BS pool by alleviating FXR in the small intestine\textsuperscript{54}.

**Table 2.3** Chemical and structural differences of four primary bile salts in human bile.

<table>
<thead>
<tr>
<th>Conjugating group</th>
<th>Primary bile salts in humans</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cholate (-C)</strong></td>
<td>Sodium taurocholate (TC)</td>
</tr>
<tr>
<td></td>
<td>CMC: 3-18, pKa: 2</td>
</tr>
<tr>
<td><strong>Chenodeoxycholate (-CDC)</strong></td>
<td>Sodium taurochenodeoxycholate (NaTCDC)</td>
</tr>
<tr>
<td></td>
<td>CMC: 0.9-7, pKa: 5.6</td>
</tr>
<tr>
<td><strong>Glyco- (G-)</strong></td>
<td>Sodium glycocholate (NaGC)</td>
</tr>
<tr>
<td></td>
<td>CMC: 4, pKa: 3.5</td>
</tr>
<tr>
<td><strong>Chenodeoxycholate (-CDC)</strong></td>
<td>Sodium glycochenodeoxycholate (NaGCDC)</td>
</tr>
<tr>
<td></td>
<td>CMC: 1-2, pKa: 5.6</td>
</tr>
</tbody>
</table>

Not only showing the effect of slowing lipid digestion and absorption but also, dietary fiber displays the effect of lowering blood serum cholesterol level. Many studies have reported the intake of SDF or foods rich in SDF is positively related to the reduction of blood total cholesterol levels and LDL-C levels\textsuperscript{28}. These SDF includes oat/barley \( \beta \)-glucan (bG), pectin from fruits, guar
gum and psyllium. Currently, there are three major potential mechanisms for the cholesterol-lowering ability of SDF: SDF could reduce the glycemic index to further lower the insulin stimulation of hepatic cholesterol synthesis; SDF could sequester BS to reduce the BS re-absorption from the small intestine back into liver, thus promoting more cholesterol converted to BS; and some fermentation products of SDF (i.e. propionate) also exhibit the physiological effects of lowering blood cholesterol levels. The first mechanism, glycemic response to SDF, has been well studied. Generally, the viscous DF could modulate the digestion of macronutrients like sugars by delaying gastric emptying and reducing the transport speed of digestive enzymes. The decrease of glucose level induces a reduction of insulin level, which potentially inhibits the synthesis of cholesterol through hormone regulation. Regarding the second mechanism, many in vivo and in vitro studies have supported that fiber intake could increase the excretion of bile salts in the feces, but how bile salts interact with SDF or even probably IDF, further leading to the incomplete bile salts reabsorption is still poorly understood. As mentioned, cholesterol produces the BS in the liver, 95% of which are reabsorbed in the ileum through enterohepatic circulation. Thus, the physical elimination of BS or the prevention of BS re-absorption by SDF will stimulate the synthesis of BS, which in turn promotes more cholesterol converted into bile salts, lowering the blood cholesterol level. For the third mechanism, the cholesterol-lowering effect comes from the microbial fermentation of dietary fibers in the colon. Fibers are indigestible in the human small intestine; instead, they could be fermented by the colonic macrobacteria in the large intestine. Bacterial fermentation of dietary fibers throughout the large intestine generates short-chain fatty acids (SCFAs), which play a role in appetite regulation and cholesterol metabolism. The BS binding by dietary fiber could also promote more BS delivered to the gut microbiota.

3) Ileal break and Satiety

The slowing effect of lipid digestion by DF is also related to satiety, which accounts for the health benefits of preventing obesity or weight control. Most of the fiber-rich foods are low in fat and energy, and usually, soluble fiber could increase viscous intestinal contents with gel-like properties that may delay gastric emptying and promote satiation. Another important impact is through fiber’s intrinsic effect and hormonal responses to promote satiety. If DF delays the absorption of fat or lipid long enough to reach to the distal ileum, it could stimulate a series of metabolic responses termed as the “ileal brake” phenomenon. Specifically, “Ileal brake” refers to the stimulation of satiety hormones such as glucagon-like peptide-1 (GLP-1) and peptide YY (PYY). GLP-1 is reported to be able to control appetite by delaying the gastric emptying and slowing down the small bowel transit, promoting the glucagon secretion and insulin sensitivity, while PYY is shown to suppress appetite by delaying the gastric emptying. There are also some studies showing that high fiber food could inhibit the release of plasma ghrelin, a stomach hunger promoting hormone and increase the secretion of the hormone cholecystokinin (CCK), a brain
neuropeptide able to reduce the food intake\textsuperscript{57–59}. Overall, the promotion of satiety through lowering lipid absorption by dietary fiber may help to decrease the willingness of the next meal intake, thus helping to prevent obesity or to control the weight of obese people. In turn, the prevention of obesity is associated with reducing the risk of CVD.

4) BS binding of DF

To better understand the mechanisms on DF modulating lipid digestion and lowering cholesterol levels, many studies focus on the interaction between bile salts and dietary fiber considering the pivotal role of bile salts in lipid digestion and cholesterol absorption. Some studies reported that the bile salts binding ability of water-soluble dietary fibers is mainly due to its binding with water, resulting in increased viscosity\textsuperscript{60}. This could slow down the diffusion of bile salts to be reabsorbed by the body, thus increasing the excretion of BS\textsuperscript{61}. Some studies indicate that there are also direct molecular binding interaction between soluble dietary fibers and bile acids, similar to the mechanisms of the cholesterol lowering effect by insoluble DF\textsuperscript{30}. But most of the studies indicate that the bile salt-binding of dietary fiber is not solely depend on their viscosity but also influenced by other direct chemical binding forces\textsuperscript{60,62,63}. Currently, three hypotheses are proposed to explain the prevention of BS re-absorption of BS and the increasing of BS excretion through the intake of DF: 1) the generated viscosity by SDF may promote the barrier properties of the unstirred layer between BS stabilized micelles and intestinal absorptive cells thus preventing the absorption of the BS; 2) SDF may interact with BS to form complex with BS through molecular and chemical binding; 3) SDF may form a gel/matrix that could entrap BS micelles.

It is generally considered that the ability of dietary fiber to increase the viscosity in the GIT is the common factor in preventing BS recycling. Other studies show that fiber from cereal flours eliciting different viscosities has cholesterol-lowering effects independently of their ability to increase intestinal content viscosities, which suggests that the role of viscosity should be further investigated. Consequently, the entrapment of BS by dietary fibers is co-contributed by the viscosity generated by DF and the molecular binding between DF and BS. Moreover, soluble fibers may not be the only components responsible for the prevention of BS recycling, and insoluble fibers may play a role\textsuperscript{63}. Animal studies also show the diet with IDF could increase the excretion of BS in the feces\textsuperscript{30}. Zacherl et al. studied BS-binding ability of both soluble and insoluble fibers and found that a significant correlations between the viscosity and BS-binding ability of oat and psyllium but no clear correlation was observed in water-insoluble lupin\textsuperscript{60}. It seems that the main factors for binding BS depend on DF types since different DF show different structures and chemical composition. It is also influenced by the nature and structure of the food matrix that fibers are embedded in. Currently, many \textit{in vitro} studies have proved the BS-binding ability of isolated fibers such as b-glucan\textsuperscript{28,64,65}. Few studies have investigated the impact of the food matrix on the BS-binding ability of fiber-rich foods such as cereal grains and legume seeds. Considering the
different structures, physicochemical properties, and physiological functions among the primary BS, it is necessary to further research the binding ability of fibers or fiber-rich foods to different primary BS. It is especially important to understand how different types of dietary fiber in the food matrix can affect the possible interactions between BS and fibers. Many factors should be considered, including the fiber nature and composition, the microstructure and the physical state of the fiber source, and the food processing conditions.

2.3 Dry beans

Dry beans are valuable sources of dietary fiber. The USDA has ranked dry beans as the top dietary fiber source to increase the level of dietary fiber in the human diet. A study comparing 70 different food items in the U.S., showed that dry beans were found to have the highest dietary fiber content. Epidemiological evidence shows that consumption of dry beans, mainly because of its high fiber content, has many beneficial physiological effects in preventing and alleviating the above mentioned chronic diseases and then contributes to overall health and wellness. Thus, studying the dietary fiber from dry beans and its physicochemical properties in whole beans could help us to understand the physiological mechanisms of DF in foods better, and further to develop food products that incorporated with beans or beans DF as ingredients to promote public health.

2.3.1 Categories and composition of dry beans

Dry beans or common beans (Phaseolus vulgaris L.) belong to pulses, the dry edible seeds of plants in the legume family (Figure 2.2). They mainly include kidney beans, haricot beans, pinto beans, and navy beans, etc. as shown in Table 2.4. Dry beans are excellent sources of protein, carbohydrates, dietary fiber, vitamins, minerals, and phytochemicals. Generally, they contain 55-65% of carbohydrates, extraordinarily rich in dietary fiber, which ranges from 15-35% and also starch, which ranges from 22-45% depending on different varieties. A serving of beans (half-cup) provides 5.2-7.8 g of total fiber while a half-cup of whole grains provides 1.7-4 g of fiber. The protein content of dry beans is generally around 16-33%, almost two to three times that of cereals. Table 2.5 compares the nutrition composition of dry beans and cereal grain crops. In comparison to these cereal grains, beans are relatively high in proteins and dietary fiber while low in fat. Dry beans are also good sources of B-vitamins, namely, riboflavin, thiamin, niacin, pyridoxine, and folic acid, and contain many important minerals, including potassium, magnesium, iron, and zinc. They also provide ample amounts of phenolic compounds such as tannins, phenolic acids, and flavonoids.
**Table 2. 4 Common pulses consumed worldwide.**

<table>
<thead>
<tr>
<th>Genus</th>
<th>Species</th>
<th>Common Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cajanus</td>
<td>Cajan</td>
<td>Pigen pea, cajan pea, Congo bean</td>
</tr>
<tr>
<td>Cicer</td>
<td>arietinum</td>
<td>Chickpea/hgarbanzos, Bangal gram</td>
</tr>
<tr>
<td>Lens</td>
<td>culinaris</td>
<td>Lentils</td>
</tr>
<tr>
<td>Lupins</td>
<td>albus</td>
<td>White lupin</td>
</tr>
<tr>
<td>Lupins</td>
<td>mutabilis</td>
<td>Lupin</td>
</tr>
<tr>
<td>Phaseolous</td>
<td>vulgaris</td>
<td>Kidney, black, avy, pinto, great northern, pink, haricot bean</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Lima, butter bean</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Scalet runner bean</td>
</tr>
<tr>
<td></td>
<td>lunatus</td>
<td>Rice bean</td>
</tr>
<tr>
<td></td>
<td>coccineus</td>
<td>Tepary bean</td>
</tr>
<tr>
<td>Phaseolous</td>
<td>calcaratus</td>
<td></td>
</tr>
<tr>
<td></td>
<td>acutifolius</td>
<td></td>
</tr>
<tr>
<td>Pisum</td>
<td>sativum</td>
<td>Dry pea</td>
</tr>
<tr>
<td>Vicia</td>
<td>faba (var. quina)</td>
<td>Horse bean</td>
</tr>
<tr>
<td></td>
<td>faba (var. major)</td>
<td>Board bean</td>
</tr>
<tr>
<td></td>
<td>faba (var. minor)</td>
<td>Field bean</td>
</tr>
<tr>
<td>Vigna/Dolichos</td>
<td>sinensis</td>
<td>Cowpea, blackee pea/bean</td>
</tr>
<tr>
<td></td>
<td>radiate</td>
<td>Mung bean, green gram</td>
</tr>
<tr>
<td></td>
<td>angularis</td>
<td>Adzuki/red bean</td>
</tr>
<tr>
<td></td>
<td>aconitifolius</td>
<td>Moth bean</td>
</tr>
<tr>
<td></td>
<td>mungo</td>
<td>Black bean</td>
</tr>
<tr>
<td>Vocandzeia</td>
<td>subterranean</td>
<td>Bambara groundnut, earth pea</td>
</tr>
</tbody>
</table>

*aNot all inclusive. Minor pulse genera and species not listed.*
Table 2.5 Comparison of nutritional profiles of dry beans with grains (per 100g)\textsuperscript{73}

<table>
<thead>
<tr>
<th></th>
<th>Dry beans</th>
<th>Wheat</th>
<th>Oat</th>
<th>Corn</th>
<th>Sorghum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Energy (Kcal)</td>
<td>333</td>
<td>339</td>
<td>389</td>
<td>361</td>
<td>339</td>
</tr>
<tr>
<td>Protein (g)</td>
<td>23.58</td>
<td>13.7</td>
<td>16.89</td>
<td>6.93</td>
<td>11.3</td>
</tr>
<tr>
<td>Carbohydrate (g)</td>
<td>60.01</td>
<td>72.57</td>
<td>66.27</td>
<td>76.85</td>
<td>74.66</td>
</tr>
<tr>
<td>Dietary fiber (g)</td>
<td>24.9</td>
<td>12.2</td>
<td>10.6</td>
<td>7.3</td>
<td>6.3</td>
</tr>
<tr>
<td>Fat (g)</td>
<td>0.83</td>
<td>1.87</td>
<td>6.9</td>
<td>3.86</td>
<td>3.3</td>
</tr>
<tr>
<td>Iron (g)</td>
<td>8.2</td>
<td>3.88</td>
<td>4.72</td>
<td>2.38</td>
<td>4.4</td>
</tr>
<tr>
<td>Potassium (g)</td>
<td>1406</td>
<td>405</td>
<td>429</td>
<td>315</td>
<td>350</td>
</tr>
<tr>
<td>Folate (g)</td>
<td>394</td>
<td>44</td>
<td>56</td>
<td>25</td>
<td>0</td>
</tr>
</tbody>
</table>

2.3.2 Bean seed structure and composition

Dry bean seeds are made up of two cotyledons and a seed coat, which comprise 80-90% and 8-20% of total seed weight, representatively\textsuperscript{74}. Figure 2.3 showed a cross-section of dry bean seed with one cotyledon. The different markets of bean seeds have different seed sizes, colors and shapes. The seed coat is the outer layer of a dry bean seed which protects the inner part of the seeds from the external environment. Seed coat contains rich minerals (i.e. calcium and iron) and a high amount of dietary fiber\textsuperscript{74}. It is reported to have about 60% of cellulose, 20% of hemicellulose, 15% of pectin substances and 2% of lignin\textsuperscript{75}. The bean seed coat also contained phenolic compounds, especially for colored dry beans, such as red and black beans. Each bean seed coat also has a micropyle, which is a tiny pore where water enters through for germination. Cotyledons are the main storage unit for bean’s nutrients such as protein and starch\textsuperscript{76}. Each cotyledon cells contain about 15-25% of protein and 50-75% of carbohydrates\textsuperscript{74}. It is reported that cotyledon cell walls also contain certain amounts of dietary fiber including 25-30% of cellulose and 28-42% of pectin substances\textsuperscript{77}.

Figure 2.3 A cross-section of a common bean seed with one cotyledon\textsuperscript{74}. 
### 2.3.3 Dietary fiber in beans cell wall

Among total dietary fiber, about 2/3 of dietary fiber in dry beans is insoluble fiber and 1/3 is soluble fiber. For insoluble fiber fractions, cellulose is the major component in dry beans. The component extract by alkaline solution and to isolate. In dry beans, the second principal component is especially hemicellulose A rather than hemicellulose B. Hemicellulose A and B are hemicellulose fractions extracted by alkaline solutions and precipitated with acetic acid and ethanol, respectively. Certain amounts of lignin also exist in dry beans such as pinto beans. Xyloglucan is the main hemicellulose in the cotyledon of common beans. The hull of common beans contains a high amount of cellulose. High amounts of long-chain branchless arabinans and low-branched xyloglucan were also found in the insoluble fractions of beans hull. For the soluble fiber fractions, the cell wall polymer of common beans contains arabinose-rich pectin, b-glucans, and galacturonan. The primary compositions of galacturonan are xylogalacturonan (XG) and rhamnogalacturonan (RG) rather than homogalacturonans (HG). Bean hulls contain high amounts of xyloses and arabinose, probably derived from xylan and large unbranched linear arabinans.

#### Table 2.6 Composition of different types of fiber and resistant starch in raw and processed beans.

<table>
<thead>
<tr>
<th>Bean class/treatment</th>
<th>SDF $^2$</th>
<th>IDF $^3$</th>
<th>TDF $^4$</th>
<th>RS $^5$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Common</td>
<td>2.42-2.60</td>
<td>19.9-22.6</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Pinto, raw</td>
<td>3.5</td>
<td>19.8</td>
<td>23.3</td>
<td>9.1</td>
</tr>
<tr>
<td>Pinto, soaked-blanced</td>
<td>4.9</td>
<td>15.2</td>
<td>20.1</td>
<td>3.1</td>
</tr>
<tr>
<td>Pinto, soaked-blanced-cooked</td>
<td>4.7</td>
<td>17.5</td>
<td>22.2</td>
<td>5.7</td>
</tr>
<tr>
<td>Dark red kidney, raw</td>
<td>5.02</td>
<td>14.95</td>
<td>19.97</td>
<td>29.73</td>
</tr>
<tr>
<td>Dark red kidney, cooked</td>
<td>5.83</td>
<td>18.73</td>
<td>24.56</td>
<td>3.59</td>
</tr>
<tr>
<td>Small red, raw</td>
<td>3.64</td>
<td>17.97</td>
<td>21.61</td>
<td>33.33</td>
</tr>
<tr>
<td>Small red, cooked</td>
<td>5.65</td>
<td>19.61</td>
<td>25.25</td>
<td>4.31</td>
</tr>
</tbody>
</table>

$^1$g/100g, dry-basis  
$^2$Soluble dietary fiber  
$^3$Insoluble dietary fiber  
$^4$Total dietary fiber  
$^5$Resistant starch

According to the definition of DF in Codex, resistant starch (RS) and oligosaccharides also belong to dietary fiber, since both of them are resistant to digested by enzymes in human intestinal. RS is the starch fractions that cannot be hydrolyzed in the GIT. RS showed a similar physiological properties with dietary fiber in the large intestinal. It can be taken as probiotic and can be fermented by bacteria to produce short-chain fatty acids (SCFAs), which further impacts the lipid and glucose metabolism. Dry beans have higher RS than whole grains, most likely because of their high ratio of amylose to amylopectin. There are around 1.7-5g RS in 100g cooked beans. Table 2.6 shows
the composition of fiber and resistant starch in raw and processed beans. Dry beans also contain α-galactosides, belong to the raffinose family of oligosaccharides, which include raffinose, stachyose, and verbascose. Kleintop et al.\(^8\) analyzed the oligosaccharides ranged from 2.36% to 4.65% in dry edible beans from 31 different commercial varieties including pinto beans, navy beans, cranberry beans, pink bean, and black beans.

### 2.3.4 Health benefits of dry beans

2016 was declared as the "International Year of Pulses" (Food and Agriculture Organization of the United Nations 2016), since pulses include dry beans provide a rich source of nutrition in the diets of people throughout the world, thus promoting human health and food security. Epidemiological studies have proved that dry beans (Phaseolus vulgaris species) have many health benefits in alleviating metabolic syndromes and preventing CVD. The primary physiological properties include lowering cholesterol levels, reducing food intake, and inhibiting the intestinal lipid absorption. The role of pulses in lowering lipid and cholesterol levels and then help to prevent CVDs has been studied for several decades\(^{86,87}\). Abeysekara, et al.\(^8\) investigated the impacts of a pulse-based diet in individuals older than 50 years old for reducing the risk of CVD and found that a 2-month diet with dry beans, chickpeas, peas or lentils reduced total cholesterol and LDL-C. Compared with the regular diet, the pulse-based diet decreased the total cholesterol level by 8.3% and LDL-C by 7.9%, which is estimated to reduce the risk of CVD by 17 to 25%. This study also indicated that the cholesterol-lowering effects are possible as a result of the increased intake of dietary fiber from a pulse-based diet. There was approximately 36% higher fiber during the pulse-based compared with the regular diet, whereas no significant differences in other compositions between both diets. Even though it hasn’t been well understood, many clues from studies suggested that dry beans consumption could influence the appetite regulation, notably increasing satiety and therefore help to control weight\(^8\). The effects of promoting satiety are mainly attributed to the non-digestible fractions in dry beans, including dietary fiber, resistant starch or other bioactive components like lectins and phytohemagglutinin in dry beans through gastric distention, the delayed rate of gastric emptying, regulation of gut hormones and stimulation of specific receptors in the gastrointestinal tract\(^7\).

The specific mechanisms on the physiological effects and the health benefits of dry beans are not well understood, particularly the physicochemical and digestive behaviors of dry beans matrices when dry beans pass through the GI tract (i.e. mouth, stomach, and intestine). A better understanding on the structure changes of the bean tissue as digestion progresses and the mechanisms by which they impact the rate of release of lipids from different food matrices is of crucial importance to elucidate their hypocholesterolemia and satiating effects\(^9\).
2.3.5 Production and consumption of dry beans in the U.S.

The United States is the sixth-leading producer of dry edible beans, behind Brazil, India, China, Burma, and Mexico\textsuperscript{91}. Each year, U.S. farmers plant 1.5-2 million acres of edible dry beans. In 2017, 35845 (1000Cwt./US hundredweight) dry beans were produced in U.S\textsuperscript{92}. Among them the largest dry bean production is the pinto bean, the second one is the navy bean, and the third one is the black bean, as shown in Table 2.7. Figure 2.4 summarizes all the states growing dry beans and their major dry bean categories.

Even though dry beans are widely grown in the U.S., the consumption of dry beans is quite low. More than 80% of Americans do not meet the recommended intake of dry beans (\textasciitilde 3 cups per week)\textsuperscript{72}. The weighted average consumption of legumes based on the available studies is about 0.15 cup/day, which is 58\textendash 65\% lower than the recommended amount\textsuperscript{72}. The factors that influence dry beans consumption are likely physiological effects (digestibility issues), cultural factors, habits (e.g. vegetarianism/veganism), palatability or taste preferences, and knowledge about how to incorporate them into daily meals\textsuperscript{73}. A strong motivation to promote dry beans consumption is their low cost, low fat but high fiber content. There is a tremendous potential for us to take advantage of the nationally wildly growing dry beans and the health benefit of its high dietary fiber content to develop food products with palatability and healthy function, and further promote the health in the U.S.

\begin{table}[h]
\centering
\begin{tabular}{lll}
\hline
Class & Year of 2017 & 5 Year Average (2013-2017) \\
\hline
Small White & 171 & 109 \\
Cranberry & 186 & 108 \\
Baby Lima & 217 & 225 \\
Large Lima & 264 & 240 \\
Pink & 331 & 364 \\
Blackeye & 437 & 538 \\
Small Red & 438 & 691 \\
Other & 597 & 1,150 \\
Light Red Kidney & 899 & 919 \\
Dark Red Kidney & 1,099 & 1,160 \\
Great Northern & 1,403 & 1,413 \\
Pea Bean, (Navy) & 4,161 & 3,982 \\
Black & 5,120 & 4,291 \\
Garbanzo & 6,905 & 4,248 \\
Pinto & 13,617 & 10,236 \\
Total & 35,845 & 29,674 \\
\hline
\end{tabular}
\caption{In 2017, Dry edible beans, U.S. production by commercial class (1,000Cwt.)\textsuperscript{92}}
\end{table}

\textit{Source: USDA, NASS.}
2.4 Food processing of dry beans

If we want to obtain a preventive benefit from bean consumption, particularly for the population at higher risk, it is necessary to achieve adequate levels of intake and consume increased amounts of beans. However, this can be a challenge for most people. Therefore, there is an urgent need to provide functional food ingredients from beans in a form that facilitates their incorporation into various commonly consumed food products, such as cookies, pizza dough, and soups while minimizing negative characteristics derived from food processing.

2.4.1 Impact of processing on the composition of dry beans

Dry beans are hard to cook (HTC) food, because of its tight cell wall structure and high content of dietary fiber. To make dry beans more edible and palatable, and to facilitate their effective utilization as human food, food researchers and engineers try to seek different appropriate processing methods on dry beans, such as soaking, cooking, canning, fermentation, germination, extrusion, and infrared heating. Among all of them, heat treatments (cooking and canning) are the most common ways to process beans to be edible.

Heating treatment either through the application of steam or the use of boiling water could increase beans’ plasticity and water absorption and also inactivate undesirable enzymes. A study of canning in common beans showed that canning (cooking of canned beans) could increase the protein and starch content slightly, which may be due to the loss of soluble solids during canning.
that increases the concentration of protein and starch\textsuperscript{71}. Some studies report that there is a heat-induced aggregation of protein during the heating process, and this denaturation of protein may result in low \textit{in vivo} and/or \textit{in vitro} digestibility of proteins\textsuperscript{95}.

Heating generally changes the ratio of soluble to insoluble fiber. Cooking could increase the amount of resistant starch (RS) because of the starch retrogradation after gelatinization but decrease both soluble and insoluble fiber content\textsuperscript{80}. However, some studies report that heating decreases the amount of resistant starch and oligosaccharides and increases the soluble and insoluble fiber content\textsuperscript{71,96}. The reduction in RS in cooked beans would also be due to the thermal inactivation of amylase inhibitors\textsuperscript{97}. Inconsistent results about the effect of cooking on fiber content have been found in the literature. These differences may due to the different cooking process conditions, beans varieties and analytic methods used\textsuperscript{97}.

\textbf{2.4.2 Impact of food processing on the physiochemical properties of beans and dietary fiber}

The physicochemical properties of dry beans and dietary fiber fraction are influenced during food processing, which further influences the physiological properties of beans and DF in the human GIT. The main physicochemical properties include particle size, hydration properties, solubility, and viscosity.

Particle size plays a vital role in DF in behaving in digestion transit, fermentation, and fecal excretion. It also could affect other physiochemical properties of dietary fiber including hydration, viscosity, and interaction with organic molecules. Mechanical processes like grinding can change the particle size of dietary fiber as well as the hydration properties due to the increased surface and pore volume\textsuperscript{98}. However, Cadden found that the reduction of particle size of wheat bran would decrease its water hydration capacity (WHC), which may due to the collapse of its fiber matrix\textsuperscript{99}. Another study found that there is no influence of particle size on the WHC of lime fiber residues\textsuperscript{100}. The different results may because particle size and hydration properties depend on the primary type of dietary fiber predominant in various food sources and on the food matrix.

Solubility and viscosity play an essential role in the behavior of DF in the gastrointestinal tract. SDF can generate viscosity for food chyme and slow the digestion and absorption of some food components. Insoluble fiber is indigestible in the small intestine but can be fermented in the large intestine to produce fatty acids for human utilization. The viscosity of the fluid is defined as its resistance to deformation by shear stress or tensile stress. Soluble dietary fiber dissolves in water or digestion fluids to form viscous fluid or gel, which could impede the movement of fluid thus delay the gastric emptying and contribute to entrapping bile salts. According to whether the viscosity change depends on shear stress, viscosity or dynamic viscosity can be classified as Newtonian fluids and non-Newtonian fluids. Non-Newtonian fluids exhibit a variety of correlations between shear stress and shear rate, including four different types, which are Bingham plastic fluid, Bingham pseudoplastic fluid, dilatant fluid and pseudoplastic fluid (Figure 2.5).
Factors that affect the viscosity of DF include the molecular weight, chain length, solubility and ionic strength of the fiber inside, fiber concentration, temperature, and pH\textsuperscript{17}. Increased viscosity in high molecular-weight SDF may delay the gastric emptying and the feeling of fullness, thus the nutrient digestion and absorption in the small intestinal\textsuperscript{37}. Studies reported that the heating process could decrease the viscosity of plant-based food by breaking down the chain between polysaccharides like pectin\textsuperscript{101}.

![Figure 2.5 Classification of fluids with shear stress as a function of shear rate.](image)

Dry beans also contain some bioactive compounds such as polyphenols that are sensitive to heating. Many studies have reported that thermal treatment could decrease the polyphenols, thus reducing the antioxidant ability in food such as common beans\textsuperscript{71}, fruit and vegetables\textsuperscript{101,102}.

2.4.3 Clean-label Processing Technologies

2.4.3.1 Introduction of non-thermal processing and HHP

In recent years, concern has grown about ultra-processed food and its health outcomes. For example, certain kinds of soluble dietary fibers, such as β-glucans, in “non-processed or minimally processed forms of grains” has been accepted as a valid health claim of lowering cholesterol levels and prompting satiety, which means over processing of foods may reduce the efficacy of SDF. The USDA recommends a fiber intake ranging from 25–40 g/day, but the fiber is deficient in US diets with a fiber intake of 15g/day, so the daily recommended intake of fiber needs to be highly increased. Most consumers would find some fiber-rich foods unpalatable, therefore there is a need for new and more efficient methods to process fibers to retain their bioactivity and at the same time to improve the sensorial attributes of fiber-containing foods.

The consumers’ demand for clean label foods drives the need for the development and
validation of new generation processing technologies. Hence, in recent years, there has been increasing interest in processing food by non-thermal technologies, which are effective to inactivate microorganisms at ambient temperatures and at the same to avoid the negative effects on heating sensitive bioactive compounds like polyphenols and sensory qualities like flavors. Non-thermal processing, includes high hydrostatic pressure (HHP), high-intensity pulsed electric field, high-pressure carbon dioxide, and radiation sterilization processing, has been applied to ensure product safety and quality of vegetables and fruits juices and other plant-based foods.

High hydrostatic pressure (HHP) treatment, one kind of non-thermal processing methods, has been applied in the food industry as preservation technology, providing food with fresh-like and natural quality. HPP usually processes foods to 100~1000 MPa at room or mild process temperatures with the water being the pressure transmitting medium. HHP, with little or none temperature change during treatment, is reported to preserve small molecules (vitamins, polyphones and free amino acids) because of little effect on covalent bonds and potentially be able to modify structures and affect non-covalent bonds. Numerous studies have shown that compared to thermal processing, HHP treatment could better minimize the loss of bioactive compounds and maintain the physicochemical attributes including color, viscosity, and flavor in plant food including fruits, vegetables and legumes.

2.4.3.2 The effect of HHP on dietary fiber and dry beans

Recently, HHP has shown encouraging potential to manipulate the functionality of food components like starch, protein and dietary fiber in plant food like soybeans, cereals, and vegetables and fruits. But this kind of literature is still insufficient, and most of these research studies are more focused on the study of the effect of HHP on the properties of isolated starch and protein from dry beans. The studies on the HHP-treated whole dry beans are very limited. Most of them studied the effect of HPP on the proximate composition and the hydration, water/oil binding ability of beans. A few studies have researched the effect of HHP on the physiological properties of dry beans, such as antioxidant capacity and trypsin inhibitor activity. However, none of them studied the cholesterol-lowering effect or BS-binding ability of dry beans as affected by HHP processing. Some studies showed that HHP could modify the microstructure of beans thus influencing the starch/protein digestibility and physicochemical of beans. These microstructure and physicochemical modifications by HHP may also affect the health benefit, i.e. cholesterol-and lipid-lowering effects of dry beans. The study on the effect of HPP on the physicochemical and physiological properties of dry beans is rare and the underlying principles and mechanisms of how HHP modifies the function are not yet fully understood and consequently warrant further investigation.

Studies investigating the effect of HHP on dietary fiber in dry beans are also limited. There are some studies of HHP on dietary fiber from soybean by products-fiber residuals and vegetable
or fruit residuals like tomato, cabbage, and orange fiber residuals\textsuperscript{112,113,126,127}. A study on the combination of HHP and controlled temperature (200 and 400 MPa at 30 and 60 °C ) on okara (soybean byproduct) shows that the amount of SDF went up by more than 8-fold and total dietary fiber increased from 38.1% to 64.8% at 400 MPa, and no significant change was observed at 200MPa\textsuperscript{112}. An adverse effect was seen in the distribution between soluble and insoluble fiber in cabbage treated at increased pressure\textsuperscript{113}. These differences may because the architecture of soybeans cell walls are different and more complex than the cell wall of cabbage and cumin. Resistant starch, as one part of IDF fractions, the content of which was also reported to be affected by HHP in legumes and pulses. A study on the HHP treated legume and wheat-based bread showed that HHP-treated (350MPa for 10min) bread showed more RS than DS (digestible starch)\textsuperscript{108}. Another study on special legumes, \textit{prospopis chilensis} seed, also reported that the RS content was increased with HHP processing time (2-8min) at 500MPa\textsuperscript{128}. Considering HHP shows the potential to modify the ratio of SDF and IDF and the content of RS in different plant foods, HHP may also affect the physicochemical properties and the dietary fiber distribution in dry beans, thus it is necessary to investigate how HHP influence the fiber content and its properties in dry beans.

Overall, from these studies, we can speculate that food processing and cooking techniques have a significant impact on the physicochemical properties of beans. This will further affect the physiological roles of common beans and the fiber fractions during digestion, which ultimately impact their biological activities and their potentials to prevent obesity and CVD (if detrimental processing conditions are not avoided). Therefore, a deeper understanding of how the unique chemical and physiological properties of whole beans and dietary fibers are affected during food processing and digestion is urgently needed.

References


15. Codex nutrition labeling 2013e.pdf.


Chapter 3: Effect of thermal and high-pressure processing on the thermo-rheological and functional properties of common bean (Phaseolus vulgaris L.) flours

Submitted to LWT-Food Science and Technology

Abstract

The effect of hydrothermal (HT) (boiling for 15 or 120 min) and high-hydrostatic pressure (HHP) (150, 300, 450, and 600 MPa for 5, 10 or 15 min) processing on the rheological, pasting, thermal and functional properties of bean flours was investigated. HT and HHP treatments differently affected these properties. HT120 led to maximum values of elastic and viscous moduli \((G', G'')\), and gel strength of bean flours. HHP enhanced \(G', G''\) and gel strength as the pressure and holding time increased. The viscoelastic properties of HT120 and HHP600/5-treated bean flours correlated with the increased viscosity of these samples. The pasting profiles and thermograms indicated a full, partial, and limited starch gelatinization in HT120, HHP600/5 and HHP \(\leq 450\) MPa samples, respectively. Enthalpy values showed that HT120 caused a higher degree of protein denaturation than HHP, with protein denaturation increasing as pressurization and time increased. This had an impact on protein solubility and emulsifying activity of flours which were significantly diminished by HT15/HT120, but maintained or slightly decreased by HHP. Nevertheless, HHP-treated samples showed enhanced emulsifying stability with increased pressure and holding time. These results demonstrate that HHP has the technological potential to manufacture bean flours with a range of functionalities into diverse food products.

Keywords: Common bean flour, Rheological properties, Thermal properties, Functional properties, High-Hydrostatic Pressure

3.1 Introduction

Dry or common beans (Phaseolus vulgaris L.), one of the most important legume crops worldwide, are valuable and inexpensive source of functional food ingredients. Due to their high content in fermentable and low-digestible carbohydrates as well as micronutrients (Ai, Cichy, Harte, Kelly, & Ng, 2016; Hall, Hillen, & Garden Robinson, 2017), consumption of dry beans offers an underexploited solution to fight against several cardiovascular disorders (Padhi & Ramdath, 2017). In addition, due to their high content in protein, common beans are promising ingredients for developing meat-substitutes and gluten-free food products of high nutritional value (Foschia, Horstmann, Arendt, & Zannini, 2017). However, despite food nutritional quality has gained a significant importance among consumers, consumption of dry beans per capita has seen a slow but steady decline in developed countries (Siddiq & Uebersax, 2012); specifically, 80% of
the population do not consume the recommended daily intake of dry beans (USDA, 2015). There is, therefore, an opportunity for the food industry to exploit the health benefits of common beans in the development of attractive, new, ready-to-use bean ingredients. The use of common beans in the form of flours or pastes could increase their application range and stimulate bean consumption while improving the nutritional quality of a variety of processed food products.

The development of bean-based foods requires characterization of the physico-chemical and functional properties of bean flours as model systems before their incorporation to food products. In this regard, the rheological and thermal behavior of bean dispersions are of major importance when designing and modelling manufacturing processes of semi-solid foods. In addition, the viscosity and viscoelastic properties of bean dispersions can play a role on the textural and sensorial attributes of formulated foods (J. Ahmed, Ptaszek, & Basu, 2017), and therefore are of practical significance for acceptance and consumption. Both, the composition (i.e. proteins, starch, cell wall polysaccharides and minor components) and the microstructure of bean flours, contribute to these properties and functionalities (Kaur & Singh, 2005).

Processing technologies can diversify the use of bean flours as ingredient in manufactured food products by altering their functional properties. Hydrothermal (HT) processing, such as boiling and canning, is widely used to manufacture beans into edible foods. However, high cooking temperature and/or prolonged heating time has reportedly caused negative changes in the sensorial quality and nutritional value of legumes (Lee et al., 2018). The concern about ultra-processed food and health outcomes that has grown in recent years, has driven the need for development and validation of new generation processing technologies. High-hydrostatic pressure (HHP) is a non-thermal technology that has been mainly applied to increase the microbiological safety of foods during the last decade (Xu, Lin, Wang, & Liao, 2015). This technology has also shown encouraging potential to manipulate the physicochemical properties of plant foods with minimum loss of sensory and nutritional quality (Zou et al., 2016). Specifically, HHP has shown to modify the conformational arrangement of proteins affecting protein functionalities such as protein solubility, water/oil binding ability and emulsifying properties (M. D. Alvarez et al., 2014). In addition, gelatinization and gelation of starch, a primary component of beans, occurs during pressure treatment, and the extent of these phenomena depends on the starch type, pressure level, time and temperature of pressurization, water amount and matrix surrounding the starch. Hence, in contrast to most processing techniques, HHP can be easily applied to obtain a range of functionalities by controlling pressure conditions. Most of the studies found in the literature are focused on the effect of HHP on the properties of isolated starch and protein from pulses (Jasim Ahmed et al., 2018; Garcia-Mora, Peñas, Frias, Gomez, & Martinez-Villaluenga, 2015) or chickpea, lentil and pea flours (Jasim Ahmed, Varshney, & Ramaswamy, 2009; Leite, de Jesus, Schmiele, Tribst, & Cristianini, 2017). Additionally, most of these studies were performed by applying thermal or high-pressure treatment to the whole seeds before applying mechanical
treatment. However, to our knowledge, a comprehensive physico-chemical characterization of HHP-processed flour systems from pinto beans, the most highly consumed type of bean in the US (Gittlein, 2018), is not described in the literature. Therefore, this study aimed to develop pinto bean flours using mechanical and HHP processing, and to systematically compare how HT and HHP treatments affect the rheological, thermal, pasting and resulting functional properties of bean flours.

3.2 Materials and Methods

3.2.1 Materials

Pinto beans were chosen as representative common variety of the market class of *Phaseolus vulgaris*. Raw bean seeds were donated from ADM (Archer Daniels Midland Company, IL, USA) and stored at room temperature. Beans were grown in different regions of North Dakota and harvested in the same crop year. All the lab chemicals were purchased from Fisher Scientific (Hampton, USA).

3.2.2 Hydrothermal and High-Hydrostatic Pressure treatments

The preparation of bean samples was done as described previously (Lin, O’Keefe, Duncan, & Fernández-Fraguas, 2020) with the incorporation of an additional Hydrothermal (HT) treatment. Briefly, whole beans were ground by a grinder (Waring commercial, USA) and the flour had a mean particle size in the range 450~550 μm as measured by a LS13320XR laser diffraction particle size analyzer (Beckman Coulter, Inc., USA). The effect of High-hydrostatic pressure (HHP) was studied on flours, as a function of pressure level (150, 300, 450 and 600 MPa, 25°C) and time (5, 10 and 15 min) at a 1:2 flour-to-water ratio. Treatment at 600MPa was only operated for 5min due the maximum capacity limit of the equipment. HT treatment consisted of a cooking treatment in boiling water at atmospheric pressure for 15min or 120min. The convention for naming samples is to list the type of processing method, with the remainder being the processing conditions (pressure and/or time). For example, HHP-treated samples at 150MPa for 5min is referred to as HHP150/5, and HT-treated samples for 15min is referred to as HT15.

3.2.3 Rheological characterization

Rheological properties of bean samples were determined using a DHR3 rheometer (TA instruments, Waters Co., UK) using a plate-plate measuring system (40mm diameter, 1mm gap) and a solvent trap to minimize moisture loss. The test temperature was set at 25 °C by using a circulating water system. An amount of sample was loaded into the geometry and allowed to rest for 5min. The viscoelastic behavior of samples was studied using small-amplitude oscillatory shear (SAOS) measurements. Amplitude sweeps at a constant angular frequency (ω) of 1 rad/s and shear strain increasing from 0.001 to 10 (0.1%-1000%) were initially performed to determine the linear viscoelastic region (LVR) of samples. Frequency sweeps were then performed from 0.1 to 100 rad/s at a constant shear strain within the LVR. The evolution of the elastic modulus (G’), viscous
modulus ($G''$) and complex viscosity ($\eta^*$) with frequency was determined. A power law model (Eq. (3.1) and Eq. (3.2)) was used to fit frequency sweep data:

$$\ln G' = \ln G'_0 + n' \ln \omega \quad (3.1)$$
$$\ln G'' = \ln G''_0 + n'' \ln \omega \quad (3.2)$$

Where $G'_0$ and $G''_0$ are related to the strength of the network and, $n'$ and $n''$ provide insight into the viscoelastic behavior. The value of ($G'_0 - G''_0$) was used to evaluate gel strength (Lapasin, 2012). Steady shear measurements were carried out at shear rate varying from 0.1 to 120 1/s. Data were fitted to different rheological models including Newtonian, Bingham, Power Law, and Herschel-Bulkley. The Herschel-Bulkley model (Eq. 3.3) showed the best fitting, and was therefore used to describe the flow behavior of bean dispersions:

$$\sigma = \sigma_0 + K \dot{\gamma}^n \quad (3.3)$$

Where $\sigma$ is shear stress (Pa), $\sigma_0$ is yield tress (Pa), $K$ is consistency coefficient (Pa s$^n$), $\dot{\gamma}$ is share rate (1/s), and $n$ is flow behavior index.

### 3.2.4 Pasting properties

Pasting properties were determined using the same rheometer, geometry and gap as described in section 2.3. A temperature (heating + cooling) ramp was performed from 30-95 °C, and from 95°C-30 °C, respectively, at a rate of 5°C/min, and at constant $\omega$ (1 rad/s) and stress (25 Pa). Silicon oil and solvent trap were used to prevent the evaporation of water. The pasting profile of samples was obtained by monitoring the evolution of the complex viscosity $\eta^*$ with temperature, and the peak viscosity, heat-paste viscosity and cold-paste viscosity of bean samples were recorded.

### 3.2.5 Thermal properties

A multicell differential scanning calorimeter (MC-DSC, TA Instruments, USA) was used to determine the thermal properties of samples. Samples were prepared by mixing bean flour with water (1:2 w/v) at room temperature. 150±5mg sample was weighed directly into Hastelloy ampoules and sealed. D/I water in the same range of weight was used as baseline of heat change in each ampoule. An empty ampoule was used as reference. The instrument was equilibrated for at least 60min before starting the heating/cooling scanning cycle from 25 °C-150 °C at 2°C/min. Thermal parameters including onset ($T_o$), peak ($T_p$) and conclusion ($T_c$) temperature, as well as enthalpy change ($\Delta H$) of the transition were calculated using the Nanoanalyze software (TA Instruments).

### 3.2.6 Functional properties

#### 3.2.6.1 Protein solubility

Protein solubility was measured by the Bradford method (Bradford, 1976), according to the method of (Boye et al., 2010) using the Coomassie Brilliant Blue G-250 reagent and a
GENESYS™ 10S UV-Vis Spectrophotometer. Protein solubility was calculated as the ratio of soluble protein in the supernatant to that of total protein in the original mixture dispersions.

### 3.2.6.2 Water-holding capacity and Oil-binding capacity

The water-holding capacity (WHC) and oil-binding capacity (OBC) of samples were determined according to (Setia et al., 2019). The WHC and OBC were calculated as the weight of retained water or bound oil (g), respectively, by sample (g).

### 3.2.6.3 Emulsifying properties

The emulsifying properties of bean flours were analyzed according to the method of Pearce & Kinsella, 1978 and Shen, Fang, Gao, & Guo, 2017 by the turbidimetric technique, and were expressed as EAI (Emulsifying activity index, m²/g) and ESI (Emulsifying stability index, min). EAI measures the maximum surface area occupied by surface active molecules and their capacity to form an emulsion, while ESI measures the stability of an emulsion over a specific time (Kiosseoglou & Paraskevopoulou, 2011).

### 3.2.7 Data and statistical analysis

All the tests were performed in triplicate and values were reported as means and standard deviation. The data were analyzed using Analysis of Variance (ANOVA). The Tukey HSD comparison test were conducted to evaluate the significant differences among experimental mean values at the significance levels of 0.05 ($p<0.05$). All the statistical analyses were conducted and plotted by JMP Pro13 (SAS Institute, USA) and Graphpad Prism8 (GraphPad Software Inc., USA).

### 3.3 Results and discussion

#### 3.3.1 Rheological characterization of bean flour dispersions

##### 3.3.1.1 Viscoelastic behavior

The frequency-dependent behavior of raw, HT- and HHP-treated samples is shown in Figure 3.1. Control and processed samples showed a predominantly more elastic response with magnitudes of $G'$ higher than $G''$ through the angular frequency range studied. Both moduli were relatively independent of the oscillation frequency without crossover of $G'$ and $G''$, indicating that bean dispersions can be characterized as weak gels (Rao, 2010). Similar weak gel-like behavior was reported in thermal-treated chickpea flours (M. Alvarez, Fuentes, & Canet, 2015) and HHP-treated lentil flour slurries (Ahmed, Varshney, & Ramaswamy, 2009). Frequency sweep data were fitted by Eqs. (1) and (2) and the obtained magnitudes of estimated slopes ($n'$ and $n''$) and intercepts ($G'\theta$ and $G''\theta$) also support the weak gel character observed (Table 3.1). While slopes provide insight into the viscoelastic and gelling behavior, the intercepts are related with the network strength. All bean dispersions had similar magnitudes of slope $n'$ (0.11-0.14) and thus a similar frequency dependence of $G'$, implying that the weak gel character of bean dispersions was not modified by thermal or pressure processing. However, both processing treatments had a
significant effect on the network strength, with oscillatory data indicating an increase of rigidity of the gels after processing.

Figure 3.1 Viscoelastic properties of bean flours treated by hydrothermal and high-hydrostatic pressure processing as a function of (A) treatment type, (B) treatment pressure at 5 min, (C) treatment pressure at 15 min and (D) treatment time at 450 MPa

(G': storage or elastic moduli; G'': loss or viscous moduli; ω: frequency)

After HT treatment, both HT15 and HT120-treated samples exhibited a significant increase in both G' and G'' moduli (Figure 1A) reflecting the creation of a more solid-like structure compared to untreated samples. HT15 and HT120-treated beans showed also the highest values of intercepts (G'₀ and G''₀) as well as gel strength (G'₀ - G''₀) (Table 3.1), supporting the fact that a high entanglement density three-dimensional network of cross-linked protein and starch is formed when gelatinization occurs during thermal treatment. It is also possible that an increased stiffness of the swollen starch granules consequence of starch retrogradation occurred during storage, contributing to the large gel strength observed (Keetels, Vliet, & Luyten, 1995). All HHP treatments increased G' and G'', with both moduli gradually increasing with increasing pressure (Figures 3.1A & B) an
Table 3.1: Power law parameters of Eq. (3.1) and Eq. (3.2) from mechanical spectra of bean flour dispersions treated by hydrothermal and high-hydrostatic pressure processing

<table>
<thead>
<tr>
<th>Treatment</th>
<th>$\ln G' = \ln G'_0 + n' \ln \omega$ (Eq.3.1)</th>
<th>$\ln G'' = \ln G''_0 + n'' \ln \omega$ (Eq.3.2)</th>
<th>$G'_0$-$G''_0$ (Pa s$^n$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\ln G'_0$ (Pa s$^{n'}$)</td>
<td>$n'$</td>
<td>$R^2$</td>
</tr>
<tr>
<td>Raw</td>
<td>9.27±0.03$^b$</td>
<td>0.12±0.01$^{ab}$</td>
<td>99.56±0.16$^a$</td>
</tr>
<tr>
<td>HT15</td>
<td>9.98±0.02$^{de}$</td>
<td>0.12±0.00$^{ab}$</td>
<td>99.62±0.33$^a$</td>
</tr>
<tr>
<td>HT120</td>
<td>10.83±0.12$^a$</td>
<td>0.11±0.02$^b$</td>
<td>98.37±1.55$^a$</td>
</tr>
<tr>
<td>HHP150/5</td>
<td>9.72±0.11$^{ef}$</td>
<td>0.13±0.002$^{ab}$</td>
<td>99.69±0.07$^a$</td>
</tr>
<tr>
<td>HHP300/5</td>
<td>9.76±0.02$^{ef}$</td>
<td>0.13±0.009$^a$</td>
<td>99.64±0.04$^a$</td>
</tr>
<tr>
<td>HHP450/5</td>
<td>10.19±0.05$^{bde}$</td>
<td>0.13±0.006$^{ab}$</td>
<td>99.46±0.06$^a$</td>
</tr>
<tr>
<td>HHP600/5</td>
<td>10.60±0.02$^{ab}$</td>
<td>0.13±0.002$^b$</td>
<td>98.85±0.01$^a$</td>
</tr>
<tr>
<td>HHP150/10</td>
<td>9.44±0.06$^{fg}$</td>
<td>0.13±0.006$^{ab}$</td>
<td>99.65±0.09$^a$</td>
</tr>
<tr>
<td>HHP300/10</td>
<td>10.12±0.15$^{cde}$</td>
<td>0.14±0.001$^a$</td>
<td>99.72±0.04$^a$</td>
</tr>
<tr>
<td>HHP450/10</td>
<td>10.45±0.20$^{abc}$</td>
<td>0.12±0.001$^{ab}$</td>
<td>99.83±0.10$^a$</td>
</tr>
<tr>
<td>HHP150/15</td>
<td>9.87±0.03$^{de}$</td>
<td>0.13±0.004$^{ab}$</td>
<td>99.82±0.02$^a$</td>
</tr>
<tr>
<td>HHP300/15</td>
<td>10.11±0.08$^{cde}$</td>
<td>0.13±0.01$^a$</td>
<td>99.65±0.18$^a$</td>
</tr>
<tr>
<td>HHP450/15</td>
<td>10.56±0.09$^{ab}$</td>
<td>0.14±0.00$^a$</td>
<td>99.61±0.22$^a$</td>
</tr>
</tbody>
</table>

$G'$: storage or elastic moduli; $G''$: loss or viscous moduli; $\omega$: oscillation frequency (rad/s); $n'$ and $n''$ illustrate the dependence degree of both moduli on oscillation frequency.

Data are expressed as the mean values (n=2) ± standard deviation (SD). Comparisons for all pairs were made using Tukey-Kramer HSD; Along the column, mean values with different letters are significantly different (P < 0.05).
holding time (Figure 3.1C). This might indicate an increase in molecular interactions and a strengthening of bean microstructure compared to raw samples, which was in agreement with previous findings reported in flour dispersions (Ahmed et al., 2009). Values of intercepts and gel strength revealed differences between pressure levels. Whereas low pressures (150 MPa) were not enough to cause any significant change in the network strength of bean dispersions at any of the holding times, higher pressures (600 MPa) resulted in the formation of a stronger network dominated by the presence of a cross-linked arrangement of partially gelatinized starch and/or denatured protein, even at short holding time (Jiang, Li, Hu, Wu, & Shen, 2015). Pressure-induced starch gelatinization seems to occur over a range of pressures and a critical level of pressure needs to be reached for gelatinization to occur effectively (Bauer & Knorr, 2005; Oh et al., 2008). During treatment at low pressures, the compression force exerted on starch granules might predominate over granule swelling; as the pressure increases the granule rigidity increases but also more water penetrates into the granule promoting swelling and gelatinization (Jiang et al., 2015). Particularly, HHP 600MPa/5 min resulted in the formation of network structures of slightly lower strength to that created after HT treatment. The effect of HHP on complex viscosity ($\eta^*$) (Figure 3.1A) clearly shows an increase in viscoelasticity with increased pressure. $\eta^*$ values of HT120- and HHP600/5-treated samples were very similar and significantly higher than the corresponding to raw samples which supports the more viscoelastic character of processed gels under these conditions. Overall, the greater viscoelastic moduli, $\eta^*$ and gel strength observed in HT- and HHP600/5-treated beans indicated molecular interactions and major structural modifications under those conditions.

3.3.1.2 Flow behavior

Figure 3.2 shows the flow behavior of raw, HT- and HHP-treated bean samples. The apparent viscosity ($\eta_a$) of all bean samples decreased with increasing shear rate ($\gamma$), indicating a shear-thinning behavior, characteristic of most non-Newtonian foods (Rao, 2010). Similar shear-thinning behavior was also reported in previous studies on pulse dispersions (M. D. Alvarez, Herranz, Campos, & Canet, 2017). Among the flow models used in this study, the Herschel–Bulkley model (Eq. 3.3), which is widely used to describe pseudoplastic behavior, showed the best fitting of flow parameters in bean samples (R >99%) (Table A1). Therefore, only the fitting parameters ($\sigma$, $\sigma_0$, K and n) obtained with this model are discussed (Table 3.2). All samples showed $n$ values <1 confirming the shear-thinning behavior of bean dispersions. Raw and processed samples did not show a large variation of $n$ (0.40-0.53), which were comparable to values reported in chickpea slurries (Alvarez et al., 2017). HT treatment had a significant effect on the flow behavior of bean dispersions (Figure 3.2A). Compared to HHP, HT-treated samples were less stable to shear, representative of a greater orientation and disentanglement of polymer chains under shear. In particular, K exhibited a maximum value in HT120 samples, ~25 times higher than for raw samples, in accordance with the highest $\eta_a$ observed over the whole range of $\gamma$. This could indicate a significant extent of swollen starch granules, consequence of starch gelatinization.
Figure 3.2 Flow behavior of bean flours treated by hydrothermal and high-hydrostatic pressure processing as a function of (A) treatment type, (B) treatment pressure at 5 min, (C) treatment pressure at 15 min and (D) treatment time at 450 MPa.

Regarding HHP treatments, the effect of pressure level was dependent on the length of treatment. Whereas at 5 min, $\eta_a$ and $K$ increased with increasing pressure (Figure 3.2B), at 10/15 min, $K$ decreased with increasing pressure (Figure 3.2C), even if no clear effects on $\eta_a$ were noticed. For a specific pressure (e.g. 450 MPa), $K$ and $\eta_a$ decreased with increased holding time (Figure 3.2D). The decreased $K$ and $\eta_a$ could be due to the presence of pores on the surface that increased in size with increasing holding time, as we previously reported (Lin et al., 2019). Other studies also reported an increase of $K$ with increasing pressure in mung starch (Jiang et al., 2015) and a decrease of $K$ with holding time in chickpea dispersions (Alvarez et al., 2017). In any case, HT600/5 samples showed the highest $K$ and $\eta_a$ values within HHP-treated samples. Yield stress ($\sigma_0$) is defined as the minimum shear stress required to initiate flow (Steffe, 1996). Raw samples showed a low $\sigma_0$ that sharply and significantly increased after HT-treatment (Figure 3.2A and table 3.2) reflecting the elevated resistance of heated bean dispersions to flow, which is consequence of the...
high cross-linked network that must be broken down (Duran & Costell, 1982; Qiu & Rao, 1988).

Although \( \sigma_0 \) values exhibited a non-identifiable trend with HHP treatment, 600MPa considerably decreased the \( \sigma_0 \), which was consistent with studies on cereal starch (Li & Zhu, 2018). From these observations, it can be concluded that the rheological response of bean dispersions can be attributed to the degree of gelatinized starch and their cross-linking with proteins.

**Table 3.2** Flow behavior parameters of bean flour dispersions treated by hydrothermal and high-hydrostatic pressure processing estimated by the Herschel-Bulkley model

<table>
<thead>
<tr>
<th>Treatment</th>
<th>( \sigma_0 ) (Pa)</th>
<th>K (Pa \cdot s^n)</th>
<th>n</th>
<th>( R^2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raw</td>
<td>2.83 ±0.61^b</td>
<td>18.74±0.64^k</td>
<td>0.53±0.01^ab</td>
<td>99.99%</td>
</tr>
<tr>
<td>HT15</td>
<td>65.72±3.19^b</td>
<td>119.09±9.98^b</td>
<td>0.22±0.01^f</td>
<td>98.97%</td>
</tr>
<tr>
<td>HT120</td>
<td>186.72±15.94^a</td>
<td>522.79±163.95^s</td>
<td>0.13±0.01^g</td>
<td>99.87%</td>
</tr>
<tr>
<td>HHP150/5</td>
<td>2.84±0.62^h</td>
<td>19.31±0.68^i</td>
<td>0.53±0.08^ab</td>
<td>99.85%</td>
</tr>
<tr>
<td>HHP300/5</td>
<td>6.01±0.73^e</td>
<td>27.20±0.88^g</td>
<td>0.54±0.01^a</td>
<td>99.82%</td>
</tr>
<tr>
<td>HHP450/5</td>
<td>3.61±0.4^f</td>
<td>40.36±2.52^e</td>
<td>0.47±0.01^c</td>
<td>99.59%</td>
</tr>
<tr>
<td>HHP600/5</td>
<td>2.17±0.49^j</td>
<td>68.1±3.13^c</td>
<td>0.43±0.01^d</td>
<td>99.79%</td>
</tr>
<tr>
<td>HHP150/10</td>
<td>6.60±1.18^d</td>
<td>43.20±1.41^d</td>
<td>0.40±0.01^c</td>
<td>99.83%</td>
</tr>
<tr>
<td>HHP300/10</td>
<td>7.54±0.89^c</td>
<td>24.86±1.64^b</td>
<td>0.53±0.02^ab</td>
<td>98.60%</td>
</tr>
<tr>
<td>HHP450/10</td>
<td>2.08±0.72^i</td>
<td>23.85±0.99^i</td>
<td>0.52±0.01^ab</td>
<td>99.42%</td>
</tr>
<tr>
<td>HHP150/15</td>
<td>5.62±1.84^f</td>
<td>37.68±1.46^f</td>
<td>0.40±0.01^c</td>
<td>99.94%</td>
</tr>
<tr>
<td>HHP300/15</td>
<td>2.03±0.58^l</td>
<td>23.78±1.11^i</td>
<td>0.46±0.01^c</td>
<td>99.68%</td>
</tr>
<tr>
<td>HHP450/15</td>
<td>2.81±0.39^i</td>
<td>19.31±0.62^j</td>
<td>0.51±0.01^b</td>
<td>99.66%</td>
</tr>
</tbody>
</table>

^a \( \sigma = \sigma_0 + Ky^n \) (Eq.3.3), \( \sigma \): the shear stress (Pa); \( \sigma_0 \): Yield stress (Pa); \( K \): Consistency coefficient (Pa s^n); \( n \): Flow behavior index; \( \dot{\gamma} \): shear rate (s^-1); \( R^2 \): Linear regression of Herschel-Bulkley model.

Data are expressed as the mean values (n=3) ± standard deviation (SD). Comparisons for all pairs were made using Tukey-Kramer HSD; Along the column, mean values with different letters are significantly different (P < 0.05).
3.3.2 Pasting properties of bean flour dispersions

The pasting profile of bean dispersions is showed in Figure 3.3, and the pasting parameters (i.e. initial viscosity, peak viscosity (PV), hot-paste viscosity (HV) and cold-paste viscosity (CV)) are summarized in Table A2. Significant qualitative and quantitative differences in pasting properties were observed among raw, HT- and HHP-treated flour dispersions during heating and cooling. Raw samples initially showed a viscosity of ~533 Pa.s that increased as the dispersion was heated and starch granules swelled. Starch granules were fully swollen and gelatinized at ~74 ℃ reaching a maximum viscosity of 9970 Pa.s (i.e. PV). This gelatinization temperature is in the range of gelatinization temperature of pinto bean starch reported previously (Du, Jiang, Yu, & Jane, 2014; Rui & Boye, 2012). Pulse starch contains ~30% amylose (~10% higher than cereals starches) which is partly responsible for the high gelatinization temperature showed by common beans compared to cereals (~65℃) (Rebello, Greenway, & Finley, 2014; Sharma & Gujral, 2010). As the temperature was further increased, a sharp decrease in viscosity was observed as consequence of the breakdown and disintegration of swollen granules, reaching hot paste values of ~1869 Pa.s at 85℃ in raw samples (Figure 3.3A&Table A2). This drop in viscosity (~70%) was more evident than the one observed in other pulses, such as pea and chickpeas (Ahmed, Thomas, Taher, & Joseph, 2016; Leite, de Jesus, Schmiele, Tribst, & Cristianini, 2017). Differences in the extent of viscosity breakdown among pulses could be due to differences in amylose content and extent of amylose leaching. Phosphorus content in starch has been also reported to contribute to the viscosity breakdown in different cultivars of pinto bean starch, as the repulsion between anionic phosphate groups could weaken amylopectin clusters, thus leading to a greater susceptibility of the starch granule towards shear (Ambigaipalan et al., 2011).

Regarding HT treatment, due to the fact that the starch was pre-cooked, HT15 and HT120 samples showed a considerably high initial viscosity compared to raw (and HHP-treated) samples, which agrees with their flow behavior (Figure 3.2A). In particular, it can be suggested that HT120 treatment led to a complete starch gelatinization since no PV appeared during heating cycle; however, HT15-treated samples showed a very small peak at ~86-88℃ which could suggest that a fraction of starch was not gelatinized (Figures 3.3A&B). Regarding HHP, the higher initial viscosity and well-defined PV observed in HHP600/5-samples compared to raw samples indicates that these HHP conditions led to partial starch gelatinization. The rest of HHP samples showed a more similar profile, in terms of PV, to the raw samples (Figures 3.3A, B, C &D) which suggested the presence of a low proportion (if any) of pre-gelatinized starch. However, both PV and HV were dependent on the pressurization level and time. While 600MPa increased PV, pressures ≤ 450MPa increased gelatinization temperature and decreased PV values compared to raw samples. Specifically, PV decreased with decreasing pressure from 450-150MPa at holding time of 5min, but increased with decreasing pressure at the longer times (10 and 15min) (although the change...
Figure 3. 3 Effect of hydrothermal (HT) and high-hydrostatic pressure (HHP) treatments on the pasting profile of bean flour dispersions (A). Pasting properties of dispersions treated by HHP during 5min (B), 15min (C) and at 450 MPa (D) obtained during the heating cycle. Pasting properties of dispersions treated by HT (15 and 120 min) and by HHP during 5min (E), 15min (F) and at 450 MPa (G) obtained during the cooling cycle.

(Data are expressed as the mean values (n=3) ± standard error (SE) bar)

was not significative between 300 and 150 MPa). These PV values could be partially explained by the presence of an increased number of small pores in the surface of bean dispersions pressurized at 150-450 MPa (Lin et al., 2019) that contributed to the lesser capacity of starch granules to swell. On the other hand, the HV linearly increased with increasing pressure, which led to a reduced breakdown viscosity in all HHP samples (~38-50%) compared to raw samples (~70%) indicating that HHP enhanced the resistance to shear-thinning. This increased resistance of granules to...
rupture in HHP-treated samples may be related to a reinforced crystalline structure (Tester & Debon, 2000) and to the formation of a cross-linked network of starch-protein/fiber as consequence of high-pressure (Lin et al., 2019). The higher PV and HV showed by HHP600/5 compared to raw and HHP≤450MPa-treated samples (Figure3.3B &Table A2), indicates that high-pressures applied for a short time can improve the thickening properties of bean flours or starch (Leite et al., 2017).

HT and HHP treatments also affected differently the pasting profile of dispersions during cooling (Figures 3.3A, E, F&G). The CV of raw sample was close to its PV values (Figure 3.3A&Table A2). HT treatments increased the CV of bean dispersions (Figure 3.3A&E), with the short-time treatment having a more pronounced effect on CV than the long one, indicating an increased starch retrogradation in HT15 samples. This different gelling capacity could be due to variations in the degree of granule breakdown, extent of gelatinization and hence HV, which was higher in HT15 samples. Regarding HHP, 600MPa increased CV values, whereas at HHP<600MPa/5min, CV dropped linearly with increasing pressure from 150 to 450MPa (Figures 3.3E&F). The decrease in CV was highly pronounced at 300-450MPa applied during longer times (Figures 3.3F&G). The presence of a higher proportion of gelatinized starch in HT and HHP600/5 samples compared to raw and the rest of HHP samples, consequence of a lower degree of starch crystallinity and packing, partly contributed to a greater extent of starch retrogradation, and consequently to a higher CV viscosity. The sharp reduction in CV observed at 300 and 450MPa (Figures 3.3A,E,F,G), could be attributed to the reinforced crystalline structure of starch in these samples and consequent lower amount of leached amylose. These lower pressures may also restrict the rearrangement/re-association of amylose molecules, which are the main contributor to starch retrogradation in short-term (Colussi et al., 2018). Similar effects of HT and HHP processing on the pasting profile of other pulses, such as mung beans and peas, were observed in previous studies (Kaur, Sandhu, Ahlawat, & Sharma, 2015; Leite, de Jesus, Schmiele, Tribst, & Cristianini, 2017).

### 3.3.3 Thermal properties of bean flour dispersions

The effect of HT and HHP processing on the thermal properties of bean flours is shown in Figure 3.4. The T₀, Tₚ, Tc and ΔH corresponding to each endothermic peak are summarized in Table 3.3. Two endothermic thermal transitions are observed in the thermograms of all samples: Peak1 is associated to starch gelatinization and peak 2, observed at higher temperature, is mainly related to protein denaturation and dissociation of lipid-starch complexes (Ahmed, Mulla, Arfat, & Kumar, 2017; Wright & Boulter, 1980). The observed Tₚ values in raw samples are similar to the Tₚ corresponding to starch gelatinization (~84°C) and protein denaturation (~98°C) reported for pinto bean (Chung, Liu, Peter Pauls, Fan, & Yada, 2008) and black/navy bean flours (Ai, Cichy, Harte, Kelly, & Ng, 2016). The beginning of protein denaturation (peak2 T₀) coincided with the end of starch gelatinization (peak1 Tc) as shown by the overlay of peak1 and peak2 in all bean
samples (Figure 3.4). Both HT treatments significantly decreased the enthalpy values ($\Delta H$) of raw samples (Figure 3.4A). At HT120, the bean starch completely gelatinized, and therefore, no glass transition temperature associated with gelatinization ($T_p$) was almost detected. Additionally, while HT120 significantly shift $T_p$ to lower temperatures, HT15 did not shift $T_p$, compared to raw samples. These results agree with the pasting (Figures 3.3A&B) and rheological properties (Figure 3.2A) of HT15 and HT120-treated samples which confirm the partial and full starch gelatinization observed in thermal-treated samples during short and long time, respectively. Both, moderate and severe thermal treatment, did not significantly shift $T_o, T_p, T_c$ of peak2, however severe heating significantly decreased $\Delta H$ values (0.07J/g) indicating a high degree of protein denaturation and microstructural modification (Table 3.3).

![Graph A](image1.png)
![Graph B](image2.png)
![Graph C](image3.png)
![Graph D](image4.png)

Figure 3.4 Thermal properties of bean flours treated by HT processing (A), by HHP processing for 5min (B), HHP processing for 15min (C) and HHP processing at 450MPa (D). 
($T_o$: onset temperature; $T_p$: peak temperature; $T_c$: conclusion temperature)

Regarding HHP-treated samples, while $T_p$ was no significative different from raw samples (Figures 3.3A-D&Table 3.3), the enthalpy values gradually decreased as pressurization (Figures 3.4B&C) and holding time (Figure 3.4D) increased (Table 3.3), indicating an increased degree of starch gelatinization with pressure intensity. Similar trend has been reported by many researchers for starches (M. D. Alvarez, Fuentes, Olivares, & Canet, 2014; Leite et al., 2017)). As observed in
HT samples, HHP had little influence on the transition temperature associated to peak2, whereas $\Delta H$ values dropped with increased pressure (Figure 3.4B&C) and holding time (Figure 3.4D), which could be related to both, breakup of non-polar interactions and aggregation upon unfolding of the native protein (Arntfield & Murray, 1981) as consequence of the elevated pressure and temperature. The extent to which aggregation affects enthalpy depends on the relative proportion of polar to non-polar amino acid residues exposed upon unfolding. Similar results were reported in isolated proteins from kidney beans pressurized at 200-600MPa 15min (Ahmed, Al-Ruwaih, Mulla, & Rahman, 2018). For both peak1 and peak2, HHP-treated samples showed significant higher $\Delta H$ than HT-treated samples, indicating that HHP leads to a lower extent of starch gelatinization and protein denaturation in bean flours (Table 3.3). The gelatinization of starch induced by HHP depends on the pressure level, holding time and the presence of an adequate amount of water (M.D. Alvarez et al., 2014) reported that complete starch gelatinization of chickpea slurries only occurred at 600MPa applied15min at low concentrations of flour (1:5 flour:water ratio). The HHP conditions used in the current study (i.e. 600MPa but shorter holding time and high concentrations of flour) may not be enough to cause complete gelatinization of bean starch, which is in accordance with previous studies on legumes using similar HHP conditions (Ahmed et al., 2016, 2009).

3.3.4 Functional properties of bean flours

3.3.4.1 Protein solubility

The protein content in raw bean flour was 25.35%, and no significant differences were found with respect to processed samples (data not shown). Bean proteins are mainly storage proteins with globulins, considered as “multi-subunits” molecules of high molecular weight and relatively hydrophobic, constituting the main fraction (Rebello et al., 2014). The albumin fraction is of low-medium molecular weight and have a hydrophilic surface that renders this protein fraction water-soluble (Kiosseoglou & Paraskevopoulou, 2011). The effect of HT and HHP treatments on bean protein solubility is shown in Figure 3.5. The protein solubility of raw pinto bean flours was ~61%, similar to other pulse flours (Boye et al., 2010; Setia et al., 2019). HT treatment applied for 15min or 120min dramatically decreased the protein solubility by ~80%. Similar results were reported in boiled chickpea and lentil flours 1h (Ma et al., 2011). The decreased protein solubility of HT-treated beans supports the observed thermal properties of these samples and could be a consequence of protein denaturation during heating which alters the hydrophilicity and hydrophobicity of the globulins and albumins surface in contact with the surrounding water. It is likely that a larger number of surface hydrophobic patches led to
Table 3. Thermal properties of bean flour dispersions treated by hydrothermal and high-hydrostatic pressure processing.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Peak1</th>
<th>Peak2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$T_o$ (°C)</td>
<td>$T_r$ (°C)</td>
</tr>
<tr>
<td>Raw</td>
<td>72.73±0.71</td>
<td>84.03±0.42</td>
</tr>
<tr>
<td>HT15</td>
<td>78.26±0.28</td>
<td>86.84±0.57</td>
</tr>
<tr>
<td>HT120</td>
<td>48.41±0.14</td>
<td>62.53±0.81</td>
</tr>
<tr>
<td>HHP150/5</td>
<td>68.70±0.71</td>
<td>81.64±0.28</td>
</tr>
<tr>
<td>HHP300/5</td>
<td>67.74±0.28</td>
<td>81.16±0.81</td>
</tr>
<tr>
<td>HHP450/5</td>
<td>69.02±0.42</td>
<td>81.40±0.67</td>
</tr>
<tr>
<td>HHP600/5</td>
<td>69.00±0.57</td>
<td>81.25±0.62</td>
</tr>
<tr>
<td>HHP150/10</td>
<td>68.56±0.71</td>
<td>82.10±0.57</td>
</tr>
<tr>
<td>HHP300/10</td>
<td>69.45±0.61</td>
<td>81.27±0.42</td>
</tr>
<tr>
<td>HHP450/10</td>
<td>69.63±0.42</td>
<td>81.57±0.71</td>
</tr>
<tr>
<td>HHP150/15</td>
<td>70.23±0.62</td>
<td>81.97±0.82</td>
</tr>
<tr>
<td>HHP300/15</td>
<td>69.58±0.57</td>
<td>81.44±0.22</td>
</tr>
<tr>
<td>HHP450/15</td>
<td>70.03±0.52</td>
<td>81.01±0.45</td>
</tr>
</tbody>
</table>

$T_o$: onset temperature; $T_r$: peak temperature; $T_c$: conclusion temperature; $\Delta H$: enthalpy change.

Data are expressed as the mean values (n=3) ± standard deviation (SD). Comparisons for all pairs were made using Tukey-Kramer HSD; Along the column, mean values with different letters are significantly different (P < 0.05).
hydrophobic interactions over electrostatic repulsions between charged proteins, which induced protein aggregation and eventually lead to protein precipitation. It is also possible that cross-links between protein and starch molecules induced by heating led to the formation of aggregates, thus decreasing protein solubility (Ma et al., 2011). On the other hand, HHP treatments affected protein solubility in a different manner. Low pressures applied for short times (i.e. 5/10min) did not apparently affect protein solubility, whereas pressures >150MPa even if applied 5min significantly decreased protein solubility. Protein solubility was also reduced when low pressurization levels were applied for longer times. Similarly, Chapleau & de Lamballerie-Anton, 2003 reported that the protein solubility of lupin was only decreased at pressures>400MPa. These results agree with the higher degree of protein denaturation observed at either high pressure (600MPa) or long holding times (15min) (Figures3.4B&D,Table 3.3).

Figure 3. 5 Protein solubility of bean flours treated by hydrothermal and high hydrostatic pressure processing.
(Data are expressed as the mean values (n=3) ± standard error (SE) bar)

3.3.4.2 Water holding capacity and oil binding capacity

Figure 3.6 shows the WHC and OBC of raw, HT- and HHP-treated bean flours. The WHC of raw bean flours was ~1.7g/g (Figure 3.6A) which was in accordance with previous studies (Deshpande, Sathe, Cornforth, & Salunkhe, 1982). The effect of HT-treatment on the WHC of bean flours was dependent on the duration of heating. HT120 significantly increased WHC, whereas HT15 showed no differences compared to raw flours. Similarly, other studies reported a significant increase of WHC of pulse flours after boiling 1h (Aguilera, Esteban, Benítez, Mollá, & Martín-Cabrejas, 2009). The increased WHC in HT120 samples could be partially related to protein denaturation while the less degree of protein denaturation observed in HT15 samples (Figure3.4A) may be not sufficient to show effect on their WHC (Peyrano, Speroni, & Avanza,
Heat-induced starch gelatinization could also increase water-absorption due to the formation of more disordered crystalline structures at extended heating times (Aguilera et al., 2009). Regarding HHP treatments, 5min had no significant effect on the WHC of bean flours, even at 600MPa. However, longer holding times at 300/450MPa significantly decreased WHC which could be explained by the decreased viscosity observed in these samples (Figures 3.2C&D) (Ai et al., 2016).

The presence of enlarged pores on the surface of these bean samples may also contribute to the reduced ability to retain moisture (Lin et al., 2019). The OBC of raw bean flours was ~1.4 g/g (Figure 3.6B), equivalent to an OBC of 140%, which was similar to that reported in other pulse flours (Setia et al., 2019). Overall, both HT and HHP treatments did not significantly affect the OBC of bean flours. Nevertheless, there was a slight increase of OBC in HT120 samples, compared to HT15. A clear tendency could not be identified in HHP-treated samples, but in general terms, while at 5 and 15min, OBC decreased with increasing pressure, at 10 min, OBC seemed to increase slightly with pressure. Moreover, low pressure (150MPa) did not change OBC regardless of holding time, while higher pressure (300MPa and 450MPa) led to more fluctuant OBC values with increasing holding time. The variation in OBC is mainly related to the hydrophobic character of the protein (Aguilera et al., 2009). HT and HHP may affect the composition and profile of polar and non-polar aminoacids thus causing OBC fluctuations. Polar aminoacids are reported to decrease after pressure and thermal processing whereas non-polar amino acids increased in cooked beans (Kim et al., 2014; Mbithi-Mwikya, Ooghe, Van Camp, Ngundi, & Huyghebaert, 2000). A higher proportion of non-polar groups at the protein surface rather than a higher ratio of hydrophobic to hydrophilic residues alone, would be responsible for an enhanced OBC.

Figure 3.6 Water holding capacity (A) and oil binding capacity (B) of bean flours treated by hydrothermal and high hydrostatic pressure processing. (Data are expressed as the mean values (n=3) ± standard error (SE) bar.)
3.3.4.3 Emulsifying properties

The emulsifying properties of bean flours were studied by determining the emulsifying activity index (EAI) and the emulsifying stability index (ESI) (Figure 3.7). The EAI indicates the ability of proteins and other surface-active molecules present in beans to adsorb to the oil-water interface and then contribute to the formation of an emulsion. The ESI indicates the stability of the adsorbed layer in a time period (Pearce & Kinsella, 1978). Raw bean flours showed good emulsifying ability with EAI ~42 m²/g, higher than values reported in chickpea and lentil flours (~20-25 m²/g) (Ma et al., 2011) and close to EAI of bean protein isolates (Karaca, Low, & Nickerson, 2011). HT-treatments significantly decreased the EAI of bean flours by 69-79% (Figure 3.7A) as similarly observed in thermal-treated lentils and chickpea flours. Regarding HHP, pressures<450MPa increased or maintained the EAI of bean flours when applied during 5 and 15 min. For a specific treatment time, EAI decreased with increasing pressure. As a general tendency, the decrease of EAI was proportional to the decrease of protein solubility in all processed samples (Figure 5A), which can be expected as only the soluble protein fraction will contribute to the emulsification capacity (Kiosseoglou & Paraskevopoulou, 2011). This result is consistent with the general correlation between protein solubility and EAI previously reported in thermal-treated pulse flours (Aguilera et al., 2009). The partial protein denaturation observed at 150MPa, which result in solubilization, could have increased EAI of beans, as consequence of improved molecular flexibility and surface hydrophobicity (Damodaran & Parkin, 2017). The ESI of raw beans was about 13min (Figure 3.7B), similar to the ESI of lentil and chickpea flours previously reported (Ma et al., 2011). Although ESI is attributed to the strength of the protein adsorbed layer, bean starch and fiber might also contribute to the emulsion stability by increasing the viscosity of the continuous phase, which would prevent droplet aggregation. HT and HHP treatments considerably

![Figure 3.7 Emulsifying activity index (EAI) (A) and emulsifying stability index (ESI) (B) of bean flours treated by hydrothermal and high hydrostatic pressure processing.](image-url)
enhanced the ESI of flours by approximately 15-17% and 13-50% respectively. As observed from these results, HT and HHP altered the protein surface activity, which is related not only to the ratio of hydrophobic/hydrophilic groups, but mostly to the protein conformation. Despite HT and HHP decreased the emulsification power of flours, both processing methods increased the ability of bean flours to stabilize the emulsion. Emulsification ability and emulsion stability seem to be affected by antagonistic molecular properties. For example, globular proteins, like globulin which represent ~70% of bean proteins, because of more conformational constraints, adsorb slowly and only partially unfold at the interface, hence exhibiting poor emulsification power. However, since the protein retains some degree of folded structure that extends into the sub-surface promoting molecular interactions, it usually forms stable interfacial layers (Aryee, Agyei, & Udenigwe, 2018). Therefore, the decreased EAI might be due to a reduced ability of processed bean proteins to rapidly adsorb, unfold and reorient at the interface, probably because of a change in the distribution pattern of hydrophilic/hydrophobic residues on the protein surface that favored a higher proportion of hydrophilic groups at the surface. On the other hand, processing appeared to lead to protein conformational changes that enhanced interactions among neighbor proteins, resulting in the formation of a stronger, cohesive and viscoelastic layer able to better adapt to environmental changes (Aryee et al., 2018).

3.4 Conclusions

HT and HHP processing of bean flours differently affected starch gelatinization and protein denaturation which translated into differences in gelling behavior and other functional properties. Severe HT treatment resulted in complete starch gelatinization and protein denaturation which drastically reduced the resistance to shear-thinning, protein solubility and emulsifying activity of bean flours, while increasing their water absorption capacity and cold-paste viscosity. The increased tendency to retrogradation observed in HT-treated bean flours would negatively impact the freeze-thaw stability of products to which flours are incorporated. HHP resulted in partial or no gelatinization of starch with the degree of swelling increasing with applied pressure and time. HHP-induced starch gelatinization was proportional to the increase of viscoelastic character of bean flours, and consequently, a range of gels of various strengths could be obtained. Results also showed a positive effect of HHP on pasting properties and resistance to shear-thinning and heating of flours, suggesting their suitability for batter-based products. Additionally, protein denaturation was more effectively preserved with HHP than with HT treatments resulting in superior protein solubility and emulsifying activity/stability, which may be exploited to partially replace emulsifier additives. In conclusion, compared to conventional cooking, HHP processing is a promising non-thermal technology for tailoring the functionality of bean flours and therefore for increasing their use as nutritious ingredients in a range of food formulations while reducing the time and energy required for processing and preparation.
Acknowledgement

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Conflicts of interest
All the authors declare no conflict of interest.

References


Chapter 4: Manipulation of the dry bean (*Phaseolus vulgaris* L.) matrix by hydrothermal and high-pressure treatments: Impact on *in vitro* bile salt-binding ability

*Published in Food Chemistry*

**Abstract**

The capacity of high-fiber foods to sequester BS during digestion is considered a mechanism to lower serum-cholesterol. We investigated the effect of hydrothermal (HT) and high-hydrostatic-pressure (HHP) on the bile salt (BS)-binding ability of dry beans, and how this relates to changes in bean microstructure, fiber content (insoluble-IDF/soluble-SDF), and viscosity. HT and HHP-600MPa led to significant IDF reduction, including resistant starch (RS), whereas 150-450MPa significantly increased RS, without modifying IDF/SDF content. Microscopy analysis showed that heating disrupted the bean cell wall integrity, protein matrix and starch granules more severely than 600MPa; however, tightly-packed complexes of globular starch granules-protein-cell wall fiber formed at HHP≤450MPa. While HT significantly reduced BS-binding efficiency despite no viscosity change, HHP-treatments maintained or enhanced BS-retention. 600MPa-treatment induced the maximum BS-binding ability and viscosity. These results demonstrate that BS-binding by beans is not solely based on their fiber content or viscosity, but is influenced by additional microstructural factors.

**Keywords:** Dry beans, Dietary fiber, Bile salt-binding, Microstructure, Viscosity, High hydrostatic pressure.
4.1 Introduction

Epidemiological evidence shows that consumption of dry or common beans (*Phaseolus vulgaris* L.) has many health benefits including controlling body weight, regulation of postprandial glucose and insulin responses and lowering blood LDL-cholesterol and lipid levels (Rebello, Greenway, & Finley, 2014; Tharanathan & Mahadevamma, 2003; Thorne, Thompson, & Jenkins, 1983). In particular, the observed positive effect of beans on blood cholesterol levels, a well-known risk factor for cardiovascular diseases, is mainly attributed to their high content of indigestible carbohydrates (i.e. dietary fiber, DF). Dry beans have two to three times more fiber-soluble (SDF) and insoluble dietary fiber (IDF), including resistant starch (RS) than other staples like cereals. One of the mechanism associated with the cholesterol-lowering effect of DF involves the prevention of bile salt (BS)-reabsorption in the enterohepatic circulation, possibly by sequestering or binding BS, which promotes enhanced fecal excretion of BS (Gunness & Gidley, 2010; Vahouny, Tombes, Cassidy, Kritchevsky, & Gallo, 1980). Since BS are produced from cholesterol, the liver uses some of the available cholesterol to replenish BS reserves, thus lowering blood cholesterol concentrations. The prevention of BS re-absorption by DF is mainly related to the increased viscosity generated by SDF in the digestive tract which allows SDF to entrap or bind BS, and/or form a barrier with the intestinal mucus between BS and cells in the intestine. DF may also directly bind BS through molecular interactions, so fiber and BS are associated at a molecular level (Gunness & Gidley, 2010). However, this is still controversially discussed and the detailed mechanisms are still unclear.

The functionality and physiological properties of DF depend on the nature and distribution of fiber fractions and are controlled by the complexity and structure of the food matrix. This explains the fact that, for example, not all foods containing DF seem to lead to the same health outcome. While most of *in vitro* studies have focused on the BS-binding ability of purified polysaccharides, isolated SDF fractions from high-fiber foods and cereal flours (Gunness, Flanagan, & Gidley, 2010; Naumann, Schweiggert-Weisz, Bader-Mittermaier, Haller, & Eisner, 2018; Torcello-Gómez et al., 2015), relatively few studies have examined the impact of the food matrix on the BS-binding ability of cereal grains and legume seeds.

In addition, beans need to be processed before consumption, and processing and cooking techniques can promote structural and compositional changes inside the bean matrix that can influence the bioactivity of their components, including the ability of DF to bind BS, and ultimately their potential to reduce cholesterol levels. For example, the cholesterol-lowering activity of some types of SDF, such as β-glucans, has been accepted as a valid health claim in non-processed or minimally processed forms of grains, which means that further food processing may reduce the efficacy of DF to exert this action. H. J. Kim and White (2012) showed that thermal treatment could influence the rheological properties of oat slurries and further affect the ability of oat flour to bind BS. Although the *in vitro* BS-binding ability of diverse common bean varieties (*P. vulgaris*)
has been previously studied (Kahlon & Woodruff, 2002), whether microstructural, rheological and compositional modifications of the bean matrix caused by processing influence their capacity to lower cholesterol is not yet understood and consequently warrant investigation.

High hydrostatic pressure (HHP), one of the new generation non-thermal processing technologies, has been developed in the last decade as alternative to thermal processing to increase the safety of many plant foods. Recently, it has shown to promote structural changes inside the food matrix, affecting cell integrity, starch gelatinization and gelling behavior (Oh, Hemar, Anema, Wong, & Neil Pinder, 2008) as well as modifying DF composition (D. Kim & Han, 2012). HHP has also demonstrated technological improvement in bean processing with no negative effects in the final texture of the seeds (Belmiro, Tribst, & Cristianini, 2018). Nevertheless, the impact of this technology on the biological functionality of legumes has received little attention, and as of yet, the impact of HHP on the BS-binding ability of dry beans has not been investigated. Hence, the aim of this work was to explore how the structural integrity of the bean matrix and distribution of the soluble and insoluble fiber fractions, including resistant starch, are affected by HHP treatments to further evaluate the impact of these modifications on the capacity of beans to retain BS in vitro. HHP processing will be compared with hydrothermal (HT) processing, a conventional boiling treatment at atmospheric pressure.

4.2 Materials and Methods

4.2.1 Materials

Pinto beans were chosen as a representative variety of the market classes of *Phaseolus vulgaris* since Pinto represents one of the most commonly consumed variety worldwide. Dry pinto bean seeds (K1152-P) were kindly provided by ADM (Archer Daniels Midland Company, Decatur, IL, USA) and stored at room temperature. The plant material was grown in different regions of North Dakota and represented one harvesting season as reported by the manufacturer. Bile acid sodium salt sodium taurocholate (NaTC, T4009), pepsin (P-7125) and pancreatin (USPx8, P-7545) were purchased from Sigma-Aldrich (St Louis., USA). α-amylase (100,000 Ceralpha Units/g) as well as the Integrated total dietary fiber (K-INTDF) and Resistant starch (K-RSTAR) kit assays were obtained from Megazyme International (Wicklow, Ireland). The bile acid fluorometric diagnostic kit (MT-5005) was purchased from Cell Biolabs Inc (USA). All other chemicals were purchased from Fisher Scientific (Hampton, USA) and VWR International (Radnor, USA).

4.2.2 Hydrothermal and High hydrostatic pressure treatments

Whole beans were ground with a domestic grinder (51BL30, Waring commercial, Stamford, USA), and passed through a 32-mesh sieve (~500um); samples were then stored at room temperature in dry conditions. The effect of High hydrostatic pressure (HHP) was studied on mechanically disintegrated beans (i.e. bean flour), as a function of pressure level (150, 300, 450
and 600 MPa at 25 °C) and time (5, 10 and 15 min) at a fixed 1:2 flour-to-water ratio. Bean flours were hydrated in D/I water under gentle agitation for 30 min. HHP treatments were carried out using a hydrostatic pressurization unit (Quintus Food Press 35L-600, Avure Technologies, OH, USA) with a capacity of 35L at ambient temperature (≈25°C). Samples (bean slurries) were kept in 400mL plastic bags, vacuum-sealed, put into the pressurization unit vessel and treated at 150MPa, 300MPa, 450MPa, and 600MPa for 5min, 10min and 15min, not considering the pressure build up (2-3 min) and release times (5-10s). Treatment at 600MPa was only operated for 5min due the maximum capacity limit of the equipment. Distilled (D/I) water was used as the pressure-transmitting fluid. The temperature in the high-pressure vessel was monitored by a submerged thermocouple and kept constant by means of a circulating water bath. Pressure and temperature during treatment were recorded with a Lab Tech notebook program. Hydrothermal (HT) treatment was compared with HHP processing by following the same sequence of steps according to Ma et al., (2011), and it consisted of a cooking treatment in boiling water at atmospheric pressure. Ground beans were dispersed in D/I water at a 1:5 flour-to-water ratio under gentle and constant agitation for 30 min and boiled at 90 °C for 2h, using a non-tight lid to avoid evaporation and increase of pressure. After processing treatments, samples were frozen at −80 °C overnight, freeze-dried in a console freeze dry system (Labcono, Kansas, MO, USA) for 48 h, at a vacuum pressure of 28 Pa and moisture collector temperature of -52 °C, and stored in vacuum-sealed plastic bags and airtight containers at room temperature until further analysis. Untreated beans flours (control samples) were also freeze-dried and stored under the same conditions that HT- and HHP-treated samples.

4.2.3 Chemical composition analysis

Samples were analyzed for soluble (SDF), insoluble (IDF) and total dietary fiber (TDF) by following a sequential enzymatic digestion and gravimetric filtration according to the AOAC 2011.25 method developed by (McCleary, 2010) and using the Integrated total dietary fiber assay kit (K-INTDF). Klason lignin content was analyzed according to Martin-Cabrejas et al. (2004) with some modifications. The IDF residue obtained from the fiber analysis was hydrolyzed with 72% (v/v) H2SO4, diluted and then autoclaved for 45min. The insoluble residue was collected by filtration using glass crucibles (Pyrex) and dried at 105°C overnight. Dried crucibles containing the samples were ashed at 525°C for 5h. The weight loss after ashing was calculated as Klason lignin. The resistant starch (RS), digestible or non-resistant starch (NRS) and total starch (TS) were determined according to the AOAC Method 2002.02 (Fabbri, Schacht, & Crosby, 2016), by using the resistant starch assay (K-RSTAR). TS was calculated as the sum of RS and Non-RS. Nitrogen (method 954.01), fat (method 920.39), ash (method 923.03) and moisture (method 925.09) contents of bean samples were determined according to official AOAC procedures and shown in Table 1S. Protein content was calculated as nitrogen×6.25. Dry matter (DM) content was
calculated by subtracting the moisture content to the total weight of the samples. All composition results were expressed as g/100g DM.

### 4.2.4 Microstructure analysis

Freeze dried samples were used to study the microstructure and morphology of beans by Field-emission scanning electron microscope (SEM) (LEO 1550, Zeiss, Germany). All of the test samples were coated with Pt/Pd in a sputter with a thickness of 20nm before being scanned and photographed at various magnifications (100×, 300× and 1K× and 5K× and 10K×) by two different signals (InLens and dSE) according to Lee et al. (2018) and Ma et al. (2011). The accelerating voltage was 3–5 kV.

### 4.2.5 In vitro bile salt binding ability of bean matrices

The *in vitro* determination of BS-binding was performed according to the procedure described by H. J. Kim and White (2012) and Zacherl, Eisner, and Engel (2011) with some modifications. In this study, NaTC was used as a representative BS at a final concentration of 10mM, which is within the range of physiological BS concentrations found in the human intestinal fluid in the fed state. Bean samples were dispersed in 2mM Bis-tris buffer pH 7 containing NaCl 150mM and CaCl₂ 1.5mM, and digested with α-amylase 12mg/mL for 30min at 37 °C in a shaking incubator. In order to simulate gastric digestion, the pH was adjusted to 3 with 1N HCl and pepsin was added to achieve 2000 U/ml. After 1h of incubation, the pH was raised to 7 and the BS solution and 1mL of pancreatin 12mg/ml were added to simulate intestinal digestion. After 90 min of incubation, the digested mixture was centrifuged at 12000g for 15min. The unbound BS in the supernatant extract were analyzed by using a fluorometric assay kit. The concentration of unbound BS was calculated according to a standard curve prepared from the BS at different concentrations. The extracts were diluted within the range of standard BS concentrations of the assay kit. The supernatant of samples without added BS had negligible absorbance. Cholestyramine, a bile acid sequestrant and synthetic drug currently used for blood cholesterol reduction, was used as positive control, and cellulose was used as negative control. BS-binding ability was determined as the difference between the initial amount of BS added and the unbound BS measured in the supernatant and was expressed as μmol BS/100mg DM of samples. To eliminate methodology effects, BS-binding was normalized between 0% (blank sample) and 100% (cholestyramine) (Zacherl et al., 2011) and calculated according to the equation:

\[
\text{Normalized BS-binding ability (％)} = \frac{\text{BS binding ability (sample)} - \text{BS binding ability (blank)}}{\text{BS binding ability (cholestyramine)} - \text{BS binding ability (blank)}}
\]  (4)

### 4.2.6 Viscosity determination

In order to evaluate the viscosity properties of digested bean matrices, their flow behavior was characterized by using a DHR3 rheometer (TA instruments, Waters Co., Ltd., Leatherhead,
UK) with a plate-plate measuring system (40mm diameter, 1mm gap) and a solvent trap to minimize moisture loss. The test temperature was set at 37 °C by using a circulating water system. An aliquot of the digested bean sample, which was simultaneously used for the BS-binding determination, was taken and steady shear measurements were carried out at shear rate varying from 1 to 120 1/s. A fresh sample was loaded into the geometry for each measurement and the rheological parameters were obtained from the manufacturer-supplied software (Rheology Advantage, TA Instruments, Waters Co., Ltd.). Apparent viscosity values were taken at shear rates of 10 1/s. All the tests were performed in triplicate.

4.2.7 Data analysis

All measurements were performed in triplicate using freshly prepared samples, and values are reported as means and standard deviation. The obtained data were analyzed by conducting an Analysis of Variance (ANOVA). The Tukey HSD comparison test was conducted to evaluate significant differences among experimental mean values (p < 0.05). All the statistical analyses were conducted by JMP Pro13 (SAS Institute, Cary, NC, USA) and plotted by MATLAB (R2018a, MathWorks, Natick, MA, USA). The fitting of flow curves was performed by using OriginPro 8.0 (Northampton, MA, USA).

4.3 Results and discussion

One of the goals of this study was to investigate the effect of different processing conditions on the redistribution and composition of fiber and starch fractions and the microstructure of common bean matrices. To this end, a clean label, non-thermal processing technology, high hydrostatic pressure (HHP), was applied and compared with hydrothermal processing (HT).

4.3.1 Effect of HT and HHP processing on bean composition

The soluble (SDF), insoluble (IDF), total dietary fiber (TDF) and resistant starch (RS) content of raw pinto beans is shown in Table 4.1. The IDF and TDF content of raw beans (59 and 66g/100g DM respectively) was 50% higher than those reported in previous studies (20-25g/100g DM) (Kleintop, Echeverria, Brick, Thompson, & Brick, 2013). Similarly, the levels of RS in untreated beans (22.06g/100g DM), which account for about 80% of TS, were higher than those previously reported for Phaseolus vulgaris (4-5g/100g DM) (Yadav, Sharma, & Yadav, 2010). These differences are mainly due to differences in the methods of analysis and pre-treatments applied to the samples. In our study, DF content was determined according to the AOAC Official Method of Analysis 2011.25 based on the definition of dietary fiber given in the Codex (McCleary, 2010) which includes RS as IDF (Kutoš et al., 2003). Thus, the high IDF content reported in the present study was partly due to the high RS levels of common beans. The high content of IDF and RS reported in our study may also be due to the absence of any thermal pretreatment before DF
Table 4.1 Chemical composition (g/100 g DM)* of untreated, hydrothermal (HT) treated and high hydrostatic pressure (HHP) treated dry beans

<table>
<thead>
<tr>
<th>Treatment</th>
<th>SDF</th>
<th>IDF</th>
<th>TDF</th>
<th>RS</th>
<th>Non-RS</th>
<th>TS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>--</td>
<td>6.71±0.53</td>
<td>56.60±0.86</td>
<td>63.31±0.32</td>
<td>22.06±0.12</td>
<td>3.08±0.17</td>
</tr>
<tr>
<td>HT</td>
<td>90°C/2h</td>
<td>6.28±0.07</td>
<td>24.88±0.25</td>
<td>31.16±0.18</td>
<td>4.70±0.24</td>
<td>28.48±0.12</td>
</tr>
<tr>
<td>150MPa/5min</td>
<td>7.39±1.00</td>
<td>57.59±1.84</td>
<td>64.98±0.84</td>
<td>33.31±0.29</td>
<td>3.66±0.12</td>
<td>36.97±0.17</td>
</tr>
<tr>
<td>300MPa/5min</td>
<td>6.29±1.28</td>
<td>54.49±1.92</td>
<td>60.78±0.64</td>
<td>33.85±0.42</td>
<td>4.13±0.01</td>
<td>37.98±0.42</td>
</tr>
<tr>
<td>450MPa/5min</td>
<td>5.81±1.90</td>
<td>56.38±1.91</td>
<td>62.18±0.01</td>
<td>30.44±0.58</td>
<td>4.26±0.06</td>
<td>34.70±0.64</td>
</tr>
<tr>
<td>600MPa/5min</td>
<td>6.30±1.59</td>
<td>46.03±0.86</td>
<td>52.33±0.73</td>
<td>5.03±0.17</td>
<td>32.30±0.58</td>
<td>37.33±0.75</td>
</tr>
<tr>
<td>HHP</td>
<td>150MPa/10min</td>
<td>6.79±0.04</td>
<td>56.15±1.19</td>
<td>62.94±1.22</td>
<td>35.46±0.59</td>
<td>4.31±0.23</td>
</tr>
<tr>
<td>300MPa/10min</td>
<td>6.48±0.24</td>
<td>60.41±0.97</td>
<td>66.89±0.73</td>
<td>36.79±0.96</td>
<td>5.31±0.30</td>
<td>42.10±0.66</td>
</tr>
<tr>
<td>450MPa/10min</td>
<td>6.55±0.60</td>
<td>58.08±0.32</td>
<td>64.64±0.28</td>
<td>34.67±0.12</td>
<td>5.89±0.23</td>
<td>40.55±0.35</td>
</tr>
<tr>
<td>150MPa/15min</td>
<td>6.20±0.04</td>
<td>55.91±0.96</td>
<td>62.11±0.92</td>
<td>37.47±0.29</td>
<td>4.95±0.23</td>
<td>42.41±0.52</td>
</tr>
<tr>
<td>300MPa/15min</td>
<td>6.52±0.56</td>
<td>56.25±2.96</td>
<td>62.77±2.40</td>
<td>38.15±0.36</td>
<td>5.66±0.18</td>
<td>43.81±0.18</td>
</tr>
<tr>
<td>450MPa/15min</td>
<td>7.09±0.87</td>
<td>56.63±0.40</td>
<td>63.72±0.47</td>
<td>36.76±1.57</td>
<td>5.93±0.12</td>
<td>42.69±1.69</td>
</tr>
</tbody>
</table>

* DM: Dry matter.

TDF (Total dietary fiber) was calculated by the sum of SDF (soluble dietary fiber) and IDF (insoluble dietary fiber); TS (Total starch) was calculated by the sum of non-RS (non-resistant starch) and RS (resistant starch).

Data are expressed as the mean values (n=3) ± standard deviation (SD). Comparisons for all pairs were made using Tukey-Kramer HSD; Along the column, mean values with different letters are significantly different (P < 0.05).

and RS analysis. Some studies that applied a thermal or autoclaving pretreatment on bean samples before analysis, showed lower IDF and RS levels (Kleintop et al., 2013) than those without preheating. In fact, the levels of IDF and RS reported in the studies that use heat as sample pretreatment, were close to the IDF and RS levels of our HT-treated samples (25 and 4.7g/100g DM, respectively). The RS content of raw bean samples was similar to that reported in studies using the same RS analysis method used in our study (AOAC Method 2002.02) and that did not apply any thermal pretreatment (Fabbri et al., 2016). Regardless method of analysis and pretreatments applied, the high amount of RS reported in dry beans in the present and previous studies can be explained by several factors, including i) the rigid and thick cell wall of dry beans, ii) the higher proportion of amylose (30-40%) compared to cereals, and iii) the crystallinity of starch.
granules (Madhusudhan & Tharanathan, 1995). Starches from legumes have more ordered crystalline structure (B and C type) which is more resistant to digestion compared to cereal starch (A type) (Tharanathan & Mahadevamma, 2003; Rebello et al., 2014). All of this accounts for the low digestibility of starch (and hence, for the high levels of RS) from legumes, which is approximately 45% lower than that of cereal starches (Thorne et al., 1983).

Processing is required to make some legumes suitable for consumption and promotes compositional changes in their DF fractions and other nutrients (Kutoš et al., 2003). As shown in Table 4.1 and Table B1, HT and HHP processing had a significant effect on the RS and IDF content of beans, whereas the SDF fraction (6.71g/100g DM) and Klason lignin (11.97g/100g DM) did not change significantly after processing. In particular, for HT-treated sample, the RS content was significantly decreased by more than 70% (from 22.06 to 4.7g/100g DM), and the IDF content was reduced almost by half (from 59.13g to 26.26g/100g DM). This agrees with data presented in studies from Fabbri et al. (2016) who, using our same RS analysis method, found that RS content in black and pinto beans sharply decreased after 90 min of cooking achieving a steady content of ~4g/100g DM. The non-significant decrease in SDF levels and the considerable decrease of IDF content observed after HT treatment, is in accordance with previous studies on thermally processed pinto beans (Kutoš et al., 2003). In relation to the content of lignin of beans, other authors also observed that it was not modified by conventional cooking (applied during 1-2h) or pressure cooking (Rehinan, Rashid, & Shah, 2004). The decreased RS levels reported in HT-treated samples could be mainly attributed to the heat-induced starch gelatinization. Starch in legume seeds is surrounded by a protein matrix and cell wall material, and therefore is considered physically inaccessible and classified as type I RS (RSI) (Birt et al., 2013). In ground seeds, the penetration of water into the starch and swelling during gelatinization (as observed in Figure 4.1A,a) exposes the starch molecules to hydrolytic enzymes which improves starch digestibility and hence decreases RSI (Rebello et al., 2014). Another explanation for the decrease of RS is that heating of ground seeds can cause the loss of the B- and C-type crystalline structure, which is highly resistant to enzymatic hydrolysis, decreasing type II RS (RSII) (Rebello et al., 2014).

The effect of HHP on IDF and RS content was dependent on the applied pressure level. At 5min, 600MPa treatment decreased both, IDF and RS, and increased non-RS significantly, while lower pressure levels (150, 300 and 450MPa) significantly increased RS and TS content. The increase of non-RS may be due to the breakdown of the bean cell wall and to the redistribution of soluble pectin molecules as reported by other authors (Briones-Labarca, Muñoz, & Maureira, 2011). The longer the holding time applied (10 and 15 min) at pressures ≤ 600MPa, the higher the increase of RS; however, both, SDF and IDF, did not significantly change at lower pressure conditions regardless of holding time. Previous studies have also shown that HHP treatment at 350MPa for 10min did not change the amount of IDF present in a high legume wheat-based bread (Collar & Angioloni, 2017). Considering HHP treatments, only 600MPa-treated samples showed
a significant reduction in TDF levels, which was due to the decrease of both, IDF and RS. The significant decrease observed in IDF values is probably related to a partial disruption of the cell wall, and to the significant decrease of RS content, which in turn might be caused by pressure-induced starch gelatinization. This will be discussed with more detail in the next section. Shen et al. (2018) reported that HHP-treated high amylose maize at pressures < 600 MPa led to lower starch digestibility (i.e. increased RS content) compared to untreated samples, and in contrast, HHP treatment at pressures ≥ 600MPa significantly increased the starch digestion rate (i.e. decreased RS content), all of which is in accordance with the RS levels that we observed in bean samples treated at low (<600MPa) and high pressures (600MPa). The results presented in this section indicate that different processing conditions changed differently the composition and distribution of DF and starch fractions in dry bean flour.

4.3.2 Effect of HT and HPP processing on bean microstructure

The microstructure of untreated, HT- and HHP-processed bean samples was examined by SEM (Figure 4.1) and used to further explain the compositional changes caused by HT and HHP treatments. Two signal detectors, InLens and SE, were used to better display the morphology of bean flours including the overall structure (Figures 4.1A-O) and the cells surface (Figures 4.1 a-l), respectively. The microstructural characteristics of untreated samples were different to those of processed samples, and were in agreement with the results shown in the previous section. Untreated samples showed spherical and ellipsoidal starch granules with smooth surface and an approximate width and length size range of 20-25 µm, and were surrounded by cell wall material (Figures 4.1A-C and a-c). These size ranges coincide with those observed in common bean flours in previous studies (Hughes & Swanson, 1989). In whole beans, the starch granules are embedded in a protein matrix and both components are enclosed in the cell wall. However, in bean flours, the cell wall integrity has been partially damaged during mechanical disintegration and some protein bodies (average size ~100 µm) attached to or located between the starch granules can be observed (Figure 4.1a, b). Also, part of the protein matrix is disrupted leading to irregular particles and fragments (Figure 4.1A, a) that include components of cell wall material (i.e. DF). Similar microstructure has been reported in previous studies on pulse flours (Lee et al., 2018; Ma et al., 2011).

The bean cell wall integrity and cytoplasmic matrix (i.e. proteins bodies attached to starch granules) were further compromised by the application of HT treatment. Apparent changes were detected in the microstructure of cooked bean flours as can be seen in Figures 4.1D-F and d-f. As expected, a significant proportion of starch granules burst and disappeared (Figures 4.1D, E, d, e) due to the almost complete gelatinization occurred to starch granules in the presence of water and heat. The protein matrix showed a distorted and amorphous structure of irregular size and shape.
Figure 4.1 Scanning Electron Microscopy (SEM) of untreated, HT-treated and HHP-treated beans (through two signals: InLens at magnification of 300×, 1K× and 3K× (A-O) and SE at magnification of 300×, 1K× and 10K×(a-l)) (P: protein or protein matrix; S: starch; C: cell wall or cell wall fiber; Po: pores; S&P: cross-linked network of denatured protein and leached starch polymers; S/P or C/P: tighten or loosened starch/protein or protein/cell wall fiber complexes)
consequence of protein denaturation caused by heat. These phenomena resulted in a cross-linked network of denatured protein and leached starch polymers (Figures 4.1F, E, e), as observed by other authors in chickpea flour after thermal processing (Ma et al., 2011). In addition, cavities and deep holes (Figures 4.1D, F), rough surfaces and a corrugated and fibrous cell wall were noticeable in HT-treated samples (Figures 4.1F, f, e). The high degree of gelatinization (i.e. small proportion of integral starch granule structure remained) and the cross-linking observed after heating of beans could be explained by the fact that, in this study, the application of HT treatment was directly to bean flours, where starch granules and protein molecules are in more intimate contact (Ma et al., 2011).

HHP treatments also affected the microstructure of bean dispersions and these effects were different from those observed after HT treatment, and more significant and evident as the applied pressure increased (Figures 4.1G-O and g-l). Samples treated at lower pressures (150, 300 and 450MPa) showed a high fraction of intact starch granules of similar size and shape to those observed in untreated samples (Figures 4.1G-L and Fig B1. A-C), suggesting that these pressures were not large enough to substantially induce starch gelatinization. On the contrary, treatment at 600MPa partially disrupted the cell wall (Figure 4.1N, O), affecting their permeability (Belmiro et al., 2018) and allowing penetration of water, and swelling of a proportion of granules. Some starch granules lost the spherical shape and some of them ruptured, but some of the granules remained intact (Figure 1M-O) indicating that pressurization at 600MPa only caused partial starch gelatinization. Remnants of the protein matrix adhering to the starch granules were appreciable in some microphotographs (Figures 4.1N, O). The critical pressure and holding time that induce starch gelatinization depend on several factors including, the ratio of starch to water, cell wall material surrounding starch, and source of starch (i.e. degree of crystallinity, amylose/amylopectin ratio and granule size) (Oh et al., 2008). While waxy starch (~100% amylopectin) can fully gelatinize at pressures > 400 MPa, “regular” starch (~20-25 % amylose, 80-75% amylopectin) only partially gelatinize at those pressures, and high-amylose starch (~70% amylose) normally does not gelatinize at pressures< 650 MPa and thereby keeps the entity of their granules (Błaszczyk, Fornal, Valverde, & Garrido, 2005; Oh et al., 2008). As previously stated, starch from common beans have a relatively high proportion of amylose (30-40%) (Hoover & Ratnayake, 2002; Madhusudhan & Tharanathan, 1995) which together with its rigid B- and C-type crystal structure make bean starch considerably “pressure-resistant”, particularly at low pressures (Błaszczyk et al., 2005). These factors could explain the low or absent gelatinization of bean samples treated at 150-450MPa, and the partial starch gelatinization observed in 600MPa-treated samples.

These microstructural observations support and explain the change in RS, and IDF levels, observed in beans dispersions after HT and HHP-treatment (Table 4.1). During cooking and pressurization at 600MPa, the cell wall barrier is compromised allowing swelling of granules and exposing starch molecules to hydrolytic enzymes, which increases starch digestibility and thus
decreases the amount of physically inaccessible RS (Rebello et al., 2014). The modification of the cell wall and the higher degree of starch gelatinization during heating supports the larger RS decrease observed in HT-treated samples. However, without proper swelling, starch molecules in samples treated at 150-450MPa are not well-exposed and consequently starch is not readily susceptible to enzymatic hydrolysis, which increases the amount of RS (Rebello et al., 2014). Although the starch kept their granular shape at these low pressures, it is possible that internal changes occurred in the starch structure (Biaszczak et al., 2005). These modifications were not possible to visualize with SEM, but taking into consideration the discussion presented in the work of Shen et al., (2018) on the effect of HHP on the digestibility of high-amylose starch, it could be hypothesized that the changes observed in RS are also connected with the modification of the more ordered B- and C-type crystalline structure of bean starch. These authors reported that lower pressures < 600 MPa only cause a rearrangement of the ordered crystalline structure (which is highly resistant to enzymatic hydrolysis) leading to an even more organized crystalline structure (Shen et al., 2018) and therefore to a greater RS levels. On the other hand, HHP≥600MPa could partly destroy or modify the crystalline order of starch to a more amorphous structure that facilitates starch gelatinization (Shen et al., 2018), and consequently decreases RS levels.

In addition, compared to untreated samples, pressures from 150MP to 450MPa induced the formation of tightly packed complexes of globular starch granules-protein and/or of starch-protein-cell wall/fiber (Figures 4.1H, K), limiting the surface area between enzyme-substrate and causing less accessibility of α-amylase to starch (Li et al., 2015). Higher pressures (600MPa) resulted in the loosening of the complexes compactness (Figures 4.1N, O & Fig B1.E). Rovalino-Córdova, Fogliano, and Capuano (2018) have been recently reported that in addition to the barrier effect exerted by the cell wall, a compact organization of starch granules and protein matrix represent an additional barrier to starch enzymatic hydrolysis. The cell wall and the cytoplasmic compactness observed in HHP-treated samples at 150, 300 and 450MPa further supports their increased RS content (Table 4.1). For a specific pressure, prolonged holding times resulted in the tightening of the complexes compactness which led to increased RS levels in samples pressurized during 10 min (Figure B1. A) and 15min (Figures 4.1J). It has been suggested that protein stability to denaturation increases under conditions of molecular crowding or confinement (Eggers & Valentine, 2001). An incomplete protein denaturation results in less protein being digested which in turn affects the way that starch is hydrolyzed (Rovalino-Córdova et al., 2018; Rovalino-Córdova, Fogliano, & Capuano, 2019). Therefore, the tight packing observed in samples treated at lower pressures could have probably limited the degree of protein denaturation and degradation, resulting in less starch hydrolysis. This further supports the increase in RS following treatment at low pressures. On the other hand, the protein denaturation caused by heat and 600 MPa made proteins, and thereby, starch more easily digested, decreasing RS content.
Interestingly, a large number of big size pores appeared in HHP-treated samples when the holding time increased from 5 to 15 min (Figures B1. a-d and Figures 4.1i, l), regardless of pressure level. Formation of pores in the bean matrix has been also observed during extrusion of black beans (Berrios, Wood, Whitehand, & Pan, 2004). Since the extrusion process involves both, pressure increase and release, similar to HHP treatment, the formation of the pores observed in our study might have occurred as consequence of the rapid release of pressure. Longer holding times (i.e. 15 min) during HHP treatment possibly made the bean matrix more susceptible to the pressure release effect, thus inducing the formation of lager pores on the surface (Figures 4.1l), compared to short times (Figures B1).

4.3.3 *In vitro* bile salt-binding ability of bean matrices as affected by compositional, microstructural and viscosity factors

Another goal of this study was to establish a relationship between the microstructural characteristics and content in soluble and insoluble fiber of processed bean flours, and their capacity to generate viscosity and retain BS *in vitro* during digestion. Differences in BS-binding ability among the different bean microstructures generated by processing were assessed using cholestyramine as positive control (Table 4.2). One hundred milligrams of cholestyramine bound 9.62 μmol BS, which was in agreement with the amount (10-12 μmol/100mg DM) reported in previous studies (Kahlon & Woodruff, 2002; H. J. Kim & White, 2012). Untreated bean samples bound 1.85 μmol BS/100mg DM, which expressed as normalized BS-binding ability corresponds to 19.27%. This value was comparable to the percentage of BS bound by various bean cultivars and varieties (which was between 20 and 30%) as reported by other authors (Kahlon & Woodruff, 2002). The slightly lower values observed in our study could be due to the different experimental conditions, sensitivity of the assay used for the quantification of BS and/or to a different substrate to BS ratio tested. A comparison with previous studies on cereals, showed that the BS-binding ability of oat flour was 22.1% compared to cholestyramine (H. J. Kim & White, 2012). In the case of isolated fibers such as psyllium and oat fiber, higher values, between 30 and 60% and between 20-50%, respectively, were reported (Zacherl et al., 2011). The capacity of high fiber-foods to sequester BS during intestinal digestion could be attributable to a combination of viscosity and interactive effects between DF components and BS (Gunness & Gidley, 2010; Naumann et al., 2018). The contribution of the viscosity generated by SDF in the small intestine to trap BS is well documented, mainly in studies on oat β-glucans (H. J. Kim & White, 2012) and fruit arabinoxylans (Dongowski, 2007). Other authors have observed a direct molecular interaction between SDF and BS (Gunness et al., 2010). In a complex food matrix, like the bean matrices studied in this work, the contribution of these effects is not clear yet.

HT and HHP treatments influenced differently the viscosity (Figure 4.2) and the capacity of bean flours to bind BS *in vitro* (Table 4.2). In order to better understand the contribution of DF
content of bean samples to their BS-binding capacity, a comparison and correlation between the normalized BS-binding ability (%) and SDF, IDF and RS contents, as well as viscosity of digested

Table 4. 2 In vitro bile salt-binding ability of untreated, hydrothermal (HT)-treated and high hydrostatic pressure (HHP)-treated bean matrices.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>BS-binding ability (μmol/100mg DM&lt;sup&gt;a&lt;/sup&gt;)</th>
<th>BS-Bound %&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Normalized BS-binding ability (%)&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>Untreated</td>
<td>1.85±0.19&lt;sup&gt;ef&lt;/sup&gt;</td>
<td>18.54±1.87&lt;sup&gt;ef&lt;/sup&gt;</td>
</tr>
<tr>
<td>HT</td>
<td>90°C/2h</td>
<td>1.32±0.03&lt;sup&gt;g&lt;/sup&gt;</td>
<td>13.23±0.3%&lt;sup&gt;g&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>150MPa/5min</td>
<td>1.75±0.05&lt;sup&gt;f&lt;/sup&gt;</td>
<td>17.50±0.51%&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>300MPa/5min</td>
<td>2.22±0.03&lt;sup&gt;bcde&lt;/sup&gt;</td>
<td>22.2±0.26%&lt;sup&gt;bed&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>450MPa/5min</td>
<td>2.42±0.06&lt;sup&gt;c&lt;/sup&gt;</td>
<td>24.18±0.63%&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>600MPa/5min</td>
<td>2.48±0.06&lt;sup&gt;b&lt;/sup&gt;</td>
<td>24.81±0.63%&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>HHP</td>
<td>150MPa/10min</td>
<td>1.94±0.01&lt;sup&gt;def&lt;/sup&gt;</td>
<td>19.35±1.03%&lt;sup&gt;def&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>300MPa/10min</td>
<td>2.19±0.03&lt;sup&gt;bcd&lt;/sup&gt;</td>
<td>21.86±0.33%&lt;sup&gt;bed&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>450MPa/10min</td>
<td>2.13±0.03&lt;sup&gt;bde&lt;/sup&gt;</td>
<td>21.33±0.33%&lt;sup&gt;bde&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>150MPa/15min</td>
<td>2.09±0.01&lt;sup&gt;def&lt;/sup&gt;</td>
<td>20.86±0.06%&lt;sup&gt;def&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>300MPa/15min</td>
<td>2.06±0.03&lt;sup&gt;def&lt;/sup&gt;</td>
<td>20.63±0.27%&lt;sup&gt;def&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>450MPa/15min</td>
<td>2.04±0.06&lt;sup&gt;def&lt;/sup&gt;</td>
<td>20.39±0.06%&lt;sup&gt;def&lt;/sup&gt;</td>
</tr>
<tr>
<td>Cholestyramine</td>
<td>9.62±0.001&lt;sup&gt;a&lt;/sup&gt;</td>
<td>96.23±0.04%&lt;sup&gt;a&lt;/sup&gt;</td>
<td>100±0.04%&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> DM: Dry matter.

<sup>b</sup> BS-Bound %: Amount of BS bound compared to the initial amount of BS.

<sup>c</sup> Normalized BS-binding ability (%): BS-binding ability of samples compared to the BS-binding ability of Cholestyramine and blank.

Data are expressed as the mean values (n=3) ± standard deviation (SD). Comparisons for all pairs were made using Tukey-Kramer HSD; Along the column, mean values with different letters are significantly different (P < 0.05)

bean samples are shown in Figure 4.3. HT treatment had a significant impact on BS-binding, with HT-treated samples showing the lowest ability to retain BS (13.23% normalized BS-binding ability). Similarly, Zacherl et al. (2011) reported that heat treatment of oat fiber decreased its BS-binding ability which was attributed to the degradation and decreased solubility of β-glucan, and subsequent reduced viscosity, as consequence of the thermal conditions applied (90°C, 1h). However, heated-oat fiber still showed a considerably high BS-binding capacity even if viscosity
was completely lost. Other authors found that heating (90°C, 10 min) improved the ability of oat flours to bind BS (H. J. Kim & White, 2012) even if the viscosity was reduced. While the HT

![Figure 4.2](image)

**Figure 4.2** Apparent viscosity of *in vitro* digested bean matrices as a function of shear rate. Untreated, HT-treated and A) HHP-treated bean samples at 5min, B) HHP-treated bean samples at 10min and C) HHP-treated bean samples at 15min.

treatment applied in our study was severe (90°C, 2h), the SDF content and viscosity of the digested mixture were not significantly changed but the BS-binding ability was significantly decreased. The significant parallel reduction of IDF and RS content (~56% and ~78% respectively) occurred during HT treatment and the absence of change of viscosity of digested bean matrices and SDF
levels (Figure 4.3A). This indicates that the ability of beans to bind BS is not solely based on the viscosity generated by the SDF fraction, but also IDF, including RS, might contribute to BS retention. Several studies have reported the contribution of IDF (Kahlon & Woodruff, 2003) and RS (Drzikova, Dongowski, Gebhardt, & Habel, 2005) to the retention of BS in cereal grains and have suggested the existence of both, binding forces or molecular interactions (Zacherl et al., 2011), and adsorptive effects (Naumann et al., 2018). Indeed, in our study, non-linear relationships with high correlation coefficients were observed between BS-binding and IDF ($R^2=0.882$) content, and BS-binding and RS ($R^2=0.749$) content (Figure 4.3B).

![Figure 4.3 Normalized BS-binding ability (%), SDF, IDF and RS contents, and viscosity of digested bean matrices at shear rate of 10s$^{-1}$ (left chart). Non-linear correlation between normalized BS-binding ability (%) and SDF, IDF, RS contents and viscosity (at 10s$^{-1}$) of digested bean matrices (right chart).](image)

In contrast to HT, HHP processing maintained or improved the BS-binding ability of bean matrices, and the effect of pressure level was dependent on the length of treatment (Table 4.2). Whereas at 5 min, the BS-binding ability of bean matrices increased with increasing pressure level, BS-binding decreased with increasing pressure during 10 and 15 min-treatments. For a specific pressure, BS-binding decreased with increased holding time with the exception of the lowest applied pressure (150MPa). In fact, 150MPa did not have a significant effect on BS-binding at any of the holding times studied. At pressures $\geq$300MPa, 5 min-treatments were enough to increase the BS binding capacity of bean flours significantly and reach levels one-fourth the BS-binding ability of cholestyramine. However, longer treatments $>10$ min, even at low pressures did not cause a significant increase of BS retention. Bean samples treated at 600MPa during 5min showed the highest BS-binding ability with 25.78% of BS retention, which was significantly different to the untreated samples. The effect of HHP conditions on viscosity followed a comparable trend (Figure
4.2 and 3A); in samples pressurized at 5 min, their BS-binding ability increased with the increase of viscosity, with 600MPa/5min samples showing the highest viscosity. No significant change in viscosity was observed in 10 and 15 min treated samples.-Therefore, the BS retention observed in HHP-treated bean flours could be partially correlated to a viscosity effect. Considering all processed samples, including HT-treated samples, a non-linear relationship with a correlation coefficient of $R^2=0.709$ was observed between BS-binding content and viscosity (Figure 4.3B).

Although SDF content is often described as an indicator for the viscosity effect caused by soluble fiber components, we could not establish a correlation between SDF content and viscosity of bean samples based on the obtained data (Figure 4.3). Hence, despite samples pressurized at 5 min had similar contents of SDF, their BS-binding ability increased linearly with the increase of viscosity. It has been reported that, depending on the surface area and porosity, IDF can contribute to viscosity acting as filler particles (Naumann et al., 2018). Thus, the porosity (Figure B1) together with the IDF and increasing RS content of samples pressurized 5 min at <600MPa (Figure 4.3) could explain their increasing viscosity, and their subsequent BS-binding efficiency.

Interestingly, while HHP 600MPa-5min and HT treatments induced a similar decrease in RS levels, these treatments led to the highest and lowest BS-binding ability of bean flours, respectively (Figure 4.3). Both treatments also led to reduced IDF levels, but the content of IDF in pressurized samples at 600MPa-5min is significantly larger than in heated samples. This suggests that the enhanced ability of 600MPa-treated samples to bind BS, is also, apart from increased viscosity, partly attributed to the contribution of IDF polymers different to RS found in beans.

Lignin is part of the IDF fraction, cell wall of common beans, and has long been considered to play a role in the mechanism of BS retention in high fiber-plant foods (Vahouny et al., 1980). The lignin content in bean samples was not significantly affected by any of the processing conditions used in this study (Table B1); however, microstructural characteristics could explain the enhanced BS sequestration. The partial disruption of the cell wall and more loosen-packed starch-protein-fiber complexes observed during HHP at 600MPa, may have resulted in a higher accessibility of lignin binding sites for BS. It is possible that this effect was assisted by the increased cell wall permeability and the porosity observed in 600MPa-treated samples. Previous studies have showed that modification of the cell wall of lupins could lead to an uncovering of lignin exposing binding sites for BS (Cornfine, Hasenkopf, Eisner, & Schweiggert, 2010), and that an increased surface area provides more binding sites for dietary fiber to bind BS (Huang, Du, & Xu, 2018). Hence, it would not be unreasonable to think that the partial cell wall disruption, loosened starch/protein/fiber complexes, and the porosity observed at this level and time of pressurization might cause optimal exposure of lignin, or other fiber components in bean flour, enhancing the sequestration of BS. Further studies considering other IDF fractions (i.e. cellulose, hemicellulose) and their implication in BS-binding capacity are currently ongoing. To date, relationships between BS-binding properties and microstructure of food matrices are limitedly described in the literature.
and no studies have been found about the effect of high hydrostatic pressure on BS retention. The findings from this study indicate that the improved BS-binding ability observed in HHP-treated samples at 5 min might be partially due to an increased viscosity, increased RS (in samples treated at pressures <600MPa) and to an optimal uncover of fiber components consequence of microstructural (cell wall and cytoplasmic matrix) modification (pressures = 600MPa), which altogether increase the accessibility of fiber to physically trap or adsorb BS and/or chemically bind BS. The limited BS retention observed in samples treated at HHP≤450MPa and at times>5min could be due to the varied cell surface porosity (number and size of these “openings”) and the tightly-packed arrangement of globular starch granules-protein-cell wall fiber formed at those pressures.

4.4 Conclusions

This study evaluated the effect of the composition and microstructure of bean flours subjected to traditional cooking (HT) and to a non-thermal technology (HHP), on the ability of beans to retain BS during in vitro digestion. The results demonstrated that HT and HHP processing had a different impact on BS-binding efficiency, consequence of diverse changes in bean microstructure, viscosity and dietary fiber content. We have showed for the first time that HHP processing can effectively preserve and significantly improve the ability of beans to retain BS. Our findings revealed that the BS-binding capacity of beans relies on a combination of compositional and structural factors. Viscosity and soluble fiber content alone are not sufficient to explain BS-binding. The degree of disintegration of the cell wall, protein matrix and starch gelatinization appeared to play an important role in BS-binding efficiency, possibly by promoting the formation of starch/protein/fiber networks of varied compactness and by providing binding sites and exposure of fiber components. Nevertheless, the contribution of insoluble fiber, including resistant starch, also seems crucial. Further studies on the structural modification of cell wall polysaccharides during bean processing would provide a deeper insight into the mechanisms of BS-binding of common beans. By understanding how food processing affects structural properties of beans, it would be possible to develop new strategies in the formulation of legume-based functional foods. This study highlights the importance of considering the structure of foods, and not just their nutrient content, when evaluating potential health impacts.

Acknowledgements

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Conflicts of interest
All the authors declare no conflict of interest.

References


Chapter 5: Retention of primary bile salts by dry beans (*Phaseolus vulgaris* L.) during *in vitro* digestion: Role of bean components and effect of food processing

*Summitted to Food Research International (Under Review)*

**Abstract**

The positive effect of common beans on reducing blood cholesterol levels has been linked to their ability to sequester bile salts (BS) and prevent their recycling. We have examined the preferences of major bean components (soluble/insoluble fiber, starch and proteins) to retain BS, and the role played by the bean matrix. Additionally, the kinetics of BS-release were evaluated in bean flours generated by a combination of hydrothermal or high-hydrostatic pressure (HHP), and mechanical treatments. An *in vitro* digestion model combined with dialysis was used to evaluate separately the retention of primary individual BS. Soluble fiber retained a significant proportion of BS mainly due to an increased digesta viscosity; however, the protein fraction exhibited the greatest BS retention without affecting viscosity. The thermal properties of proteins and starch were more significantly affected in presence of tauro-chenodeoxycholate, which correlated to the affinity of both fractions to retain more hydrophobic BS during digestion. Glycochenodeoxycholate and tauro-cholate were the most and least effectively retained BS by bean flours, respectively. Neither of the processing treatments had an impact on the binding preferences of bean flours to the primary BS; however, the largest BS retention was caused by HHP600 treatment. Bean materials preferentially delayed the release of chenodeoxycholate BS, which is probably related to BS micelle formation. These findings demonstrate that a combination of viscosity, molecular and compositional factors is triggering the BS-retention capacity of beans, and indicate the importance of evaluating contribution of individual bean components and as whole system.

**Keywords:** Common bean fiber, starch, protein; Processing; *In vitro* digestion; Bile salt-binding; Dialysis; Viscosity; Retention kinetics
5.1. Introduction

Bile salts (BS) are amphiphilic molecules primarily derived from cholesterol in the liver. They play a crucial role in regulating cholesterol metabolism an important factor for preventing cardiovascular disorders and related diseases (Mozaffarian et al., 2015). BS are conjugates of bile acids with the amino acids glycine and taurine. There are four types of primary BS in humans, including taurocholate, glycocholate, taurochenodeoxycholate, and glycochenodeoxycholate (Chiang, 2009). After food consumption, primary BS are secreted by the liver via the gall bladder and flow into the small intestine where they participate in lipid digestion and transportation (Maldonado-Valderrama et al., 2011). Almost 95% of BS are reabsorbed in the ileum, the last section of the small intestine, and returned to the liver in a process called enterohepatic circulation; the remaining 5% will continue to the colon, where they are transformed into secondary BS (Russell & Setchell, 1992). The loss of BS is compensated by de novo synthesis of BS from cholesterol, thus maintaining a constant BS pool in the human body (Chiang, 2009).

The recycling of BS between the liver and the intestine (i.e., enterohepatic circulation) is partially determined by the pattern of food intake (Kuipers et al., 2014). Several in vivo (Gunness et al., 2016) and in vitro studies (Naumann, Schweiggert-Weisz, Eglmeier, et al., 2019) have reported that fiber-rich foods, such as cereals or legumes, are able to bind or sequester BS, and prevent their reabsorption, modifying their pool size and composition. Therefore, fiber-rich foods can promote the formation of primary BS from cholesterol, thus leading to a reduction in plasma cholesterol levels (Gunness & Gidley, 2010). Dry beans, a complex food matrix with a high content of fiber, have shown ability to sequester BS (Kahlon et al., 2005; Lin et al., 2020) and therefore to lower blood cholesterol levels (Abeysekara et al., 2012; Amigo et al., 1992; Anderson & Gustafson, 1988). Despite that cholesterol-lowering activity is generally attributed to soluble dietary fibers (SDF), in a recent study, we showed that the BS-binding ability of dry beans was not proportional to the soluble fiber content, and it was more directly related to the amount of insoluble fibers (IDF) (including resistant starch, RS) (Lin et al., 2020). Other authors have also pointed out that other components, such as starch and protein, co-existing with dietary fibers may facilitate the binding of BS (Kim & White, 2012; Sayar et al., 2006). This has been demonstrated in oats; however, the extent to which major bean components (protein, starch, SDF, IDF) contribute individually to the BS-binding of beans still remains unknown. The main mechanism proposed for BS retention includes entrapment of BS by a viscous network (Dongowski, 2007; Gunness et al., 2010; Gunness & Gidley, 2010; Naumann et al., 2018). Nevertheless, in our previous study, we found a non-linear correlation between BS retention and viscosity, which suggested that factors other than viscosity may contribute to the BS-binding abilities of beans (Lin et al., 2020). It is now well-documented the existence of mechanisms distinctly independent of viscosity, involving molecular binding and/or adsorptive interactions with BS, which has been reported in fiber preparations from different grains and legumes including oats, lupins and peas (Naumann, Schweiggert-Weisz,
Haller, et al., 2019; Ngoh et al., 2017; Takahama & Hirota, 2011; Zhou et al., 2019). Adsorptive factors were shown to be different depending on the fiber preparation and type of primary BS. Binding to primary BS have also been studied in red lettuces and cruciferous vegetables (Yang et al., 2017). However, to the best of our knowledge, the binding preferences for individual primary BS has not been studied in common beans yet. Therefore, in the present study, the BS-binding ability of this complex food matrix has been extended to the analysis of the primary BS in human bile.

The bean matrix is characterized by a rigid cell wall and a cytoplasmic matrix enclosing starch granules and proteins (Rovalino-Córdova et al., 2019). This structural arrangement and related physico-chemical properties can be manipulated by thermal, high-pressure and mechanical treatments which in turn have an impact on the BS binding abilities of beans, as we have previously demonstrated (Lin et al., 2020; Lin & Fernández-Fraguas, under revision). However, it is not known yet if the role of the bean matrix on BS-binding changes as the sequence of those processing operations are changed; neither is it clear if the bean structural integrity is changed and delays the release of non-retained primary BS. Although we have tested the effect of processing on the in vitro BS-binding capacity of beans, individual BS were not quantified separately.

For these reasons, this study was undertaken to 1) investigate how major bean components contribute to the retention of primary BS, and the role played by the bean matrix, and to 2) evaluate the effect of bean processing on the kinetics of primary BS-release. Bean flours were generated by a combination of hydrothermal (HT) or high-hydrostatic pressure (HHP), and mechanical treatments. Furthermore, raw bean flours were fractionated into SDF, IDF, starch and protein. A standardized in vitro model mimicking conditions in the upper gastrointestinal tract, combined with dialysis and high-performance liquid chromatography (HPLC) was used to evaluate binding preferences of bean tissue materials to primary BS (sodium taurocholate, glycocholate, taurochenodeoxycholate, and glycochenodeoxycholate). The assessment of in vitro BS-binding ability was combined with viscosity measurements and thermal analysis to gain a better understanding on the interactions between bean tissue materials and primary BS.

5.2. Materials and Methods
5.2.1 Materials

Pinto beans were chosen as a representative variety of the market class of Phaseolus vulgaris since Pinto represents one of the most commonly consumed varieties worldwide (Gittlein, 2018). Dry pinto bean seeds (K1152-P) were kindly provided by ADM (Archer Daniels Midland Company, Decatur, IL, USA) and stored at room temperature. The plant material was grown in different regions of North Dakota and represented one harvest season, as reported by the manufacturer. Enzymes, including salivary α-amylase Type XIII-A (A1031) and pepsin (P-7125) as well as pancreatin (USPx8, P-7545) and bile salts (sodium taurocholate (NaTC, T4009), sodium
glycocholate (NaGC, G7132), sodium taurochenodeoxycholate (NaTCDC, T6260), and sodium glycochenodeoxycholate (NaGCDC, G0759)) were purchased from Sigma-Aldrich (St Louis, USA). Chemical and structural differences in the hydroxylation and conjugation of these primary bile salts are shown in Figure 5.1. Acetonitrile (HPLC grade), tetrabutylammonium phosphate and other chemicals were purchased from Fisher Scientific (Hampton, VA, USA). Servapor® dialysis tube with a molecular weight cut-off (MWCO) of 12–14 kDa was purchased from VWR International (Radnor, PA, USA).

<table>
<thead>
<tr>
<th>Conjugating group</th>
<th>Bile salt</th>
<th>Cholate (-C)</th>
<th>Chenodeoxycholate (-CDC)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Tauro- (T-)</strong></td>
<td>Sodium taurocholate (NaTC)</td>
<td>Sodium taurochenodeoxycholate (NaTCDC)</td>
<td></td>
</tr>
<tr>
<td>CMC: 3-18mM</td>
<td>CMC: 0.9-7mM</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Glyco- (G-)</strong></td>
<td>Sodium glycocholate (NaGC)</td>
<td>Sodium glycochenodeoxycholate (NaGCDC)</td>
<td></td>
</tr>
<tr>
<td>CMC: 4mM</td>
<td>CMC: 1-2mM</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

![Chemical structures](image)

**Figure 5.1** Chemical and structural differences of primary bile salts in human bile

5.2.2 Fractionation of bean flours

The fractionation processes applied to bean flours to obtain fiber fractions, starch and protein are shown in Figure 5.2. Insoluble (IDF) and soluble (SDF) fiber fractions were isolated
according to the methods of Feng et al. (2017) and Wang et al. (2016) with minor modifications, while taking into consideration the quantification method given by McCleary et al., 2012. This method, based on the AOAC Official Method of Analysis 2011.25, considers the resistant starch fraction as a part of the IDF fraction, as stated by the CODEX definition of IDF. Raw bean seeds were milled into flours (mean particle size D90 ≈ 500μm, as measured by a LS13320XR laser diffraction particle size analyzer (Beckman Coulter, Inc., USA)) by using a high-speed blender (51BL30, Waring commercial, Stamford, USA). Two grams of raw bean flour were dispersed in 80 mL maleate buffer (pH 6.0) containing a mixture of α- amylase (75U/mL) and amyloglucosidase (AMG) (0.3U/mL), and incubated in an orbital shaker water bath (360 Orbital Shaker Bath, Precision Scientific, Trichy, India) at 150 rpm and 37 °C. After 16 h of incubation, the pH was adjusted to 8.0 with the addition of 6 mL of 0.75 M Trizma base solution. Samples were then incubated in a water bath at 95-99 °C for 20 min, then cooled to 60 °C. Next, in order to hydrolyze proteins, 0.6 mL of the protease solution (350 tyrosine U/mL) was added and mixed for 30 min in a shaking water bath at 60 °C. The pH was then adjusted to 4.3 by adding 8 mL of 2 N acetic acid. The ultimate hydrolysate was centrifuged at 3200xg for 15min. The residue was washed twice with hot water (80 °C) and 95% (v/v) aq. ethanol, and centrifuged again (3200xg, 15min). The residue after centrifugation was collected as the insoluble dietary fiber (IDF) fraction and freeze dried. The supernatant collected from all the centrifugation steps was condensed into 1/10 volume by vacuum rotary evaporation at 55 °C and then mixed with 95% ethanol for 1 hour at 60 °C to precipitate the soluble fiber fraction. The residue was collected through centrifugation (3200xg, 15min) as the soluble dietary fiber (SDF) fraction and freeze-dried. The starch and protein fractions were isolated according to the methods of Sayar et al. (2006) and Sosulski & McCudry (1987) with minor modifications. First, 25g of flour were mixed with 200mL 0.02M NaOH at 25 ℃ for 30min, and this slurry was passed through a 105nm bolting cloth. The residue left on the cloth was transferred into 50mL centrifuge tubes, mixed with 40mL distilled water for 10min, neutralized with 1N HCl and then centrifuged at 3200xg for 15min at 25 ℃. The filtered slurry, which passed through the bolting cloth, was centrifuged at 3200xg for 15min at 25 ℃. The pellets were transferred into 50mL centrifuge tubes and mixed with 40mL DI water for 10min, neutralized with 1N HCl and then centrifuged (3200xg, 15min) again. The final residue was freeze-dried and used as the starch fraction. The supernatant after centrifugation was adjusted to pH 4.3 with 5N and 1N HCl and centrifuged (10,800xg, 15min). The pellet obtained was freeze dried and used as the protein fraction.
Figure 5.2 Fractionation scheme of bean flours.
SDF (Soluble Dietary Fiber) and IDF (Insoluble Dietary Fiber) isolation according to Feng et al., 2017; Wang et al., 2016; Protein and starch isolation according to Sayar et al., 2006 and Sosulski & McCudry, 1987.

5.2.3 Hydrothermal (HT) and High-Hydrostatic Pressure (HHP) treatments

Bean seeds were soaked in water at 1:2 (flour: water, w/v) ratio overnight at room temperature, before further processing treatment. High-Hydrostatic Pressure (HHP) treatments were conducted by using a hydrostatic pressurization unit (Quintus Food Press 35L-600, Avure Technologies, OH, USA). Soaked bean seeds were packed into 400mL plastic bags, vacuum-sealed, placed into the high-pressure vessel and treated at 150MPa, 450MPa, and 600MPa for 5min (samples named as HHP150, HHP300, HHP450, and HHP600) with extra pressure build-up (2-3 min) and release times (5-10s). The pressure-transmitting fluid was distilled water. Vessel temperature was controlled (25±3°C) and monitored by using a submerged thermocouple and circulating cool water. The pressure and temperature during treatment were recorded. Regarding hydrothermal treatment (HT), soaked bean seeds were mixed with water at a 3:1 (beans: water,
w/v) ratio and cooked at 90-95 °C for 2h, using a non-tight lid to avoid evaporation and increase of pressure. HT- and HHP-treated samples were frozen at -80 °C overnight, and then freeze-dried in a Labconco freeze dryer (Labconco, Kansas, MO, USA) for 2 days at a vacuum pressure of 28Pa and moisture collector temperature of -52 °C. Untreated soaked beans were freeze dried under the same conditions that HHP and HT-treated samples. Freeze-dried beans were milled into flours by using a high-speed blender (51BL30, Waring commercial, Stamford, USA), stored in vacuum-sealed plastic bags and airtight containers at room temperature. The mean particle size of flours was in the range of 450-550 µm as measured by a laser diffraction particle size analyzer (LS 13 320 XR, Beckman Coulter, Inc., Florida, USA).

5.2.4 In vitro determination of bile salt retention

The in vitro method for determining the ability of beans to retain BS, as illustrated in Figure 5.3, involved the following steps: 1) in vitro digestion of bean materials from oral to intestinal phase, 2) collection of permeated (unbound) BS through dialysis and 3) analysis of individual unbound BS by HPLC.

**Figure 5.3** Approach followed to determine the ability of bean materials to retain primary bile salts (BS), consisting of 1) three-phase in vitro digestion, 2) collection of non-retained, free BS using dialysis and 3) quantification of non-retained BS by HPLC analysis.

(SSF: simulating salivary fluid, SGF: simulating gastric fluid, SIF: simulating intestinal fluid, BS: bile salts)

5.2.4.1 In vitro digestion protocol

The standardized multistage static in vitro digestion protocol developed by the COST Infogest network (Brodkorb et al. 2019) was used. Electrolyte stock solutions simulating salivary...
(SSF), gastric (SGF) and intestinal fluid (SIF) were prepared and stored at 4°C. The fluids were pre-warmed to 37 °C before being used in the digestion. To simulate the oral phase, bean samples (flour or isolated fractions) were mixed with SSF (1:1, w/w) containing salivary α-amylase (to achieve 75U/mL) and shaken for 2 min by using a tube revolver (Thermo Fisher Scientific, Hampton, USA) placed in an incubator (Thermo Fisher Scientific, Hampton, USA) at 37 °C. For the gastric phase, the pH was adjusted to 3 with 1N HCl and the oral phase samples were mixed with SGF (1:1, w/w) containing pepsin to reach a final activity of 2000U/mL. Samples were shaken for 2 h in the tube revolver placed in the incubator at 37 °C. In the intestinal phase, the pH was adjusted to 7 by adding 1N NaOH and samples coming from gastric digestion were mixed with SIF (1:1, w/w), which contained pancreatin (to reach 100U/mL of trypsin activity) and a BS mixture (final concentration of 10 mM), and incubated for 2 h at 37 °C. The BS mixture consisted of primary BS: 35% NaTC, 35% NaGC, 15% NaTCDC and 15% NaGCDC, which is similar to the compositional ratio found in humans (Kim & White, 2012; Macierzanka et al., 2019). The final concentration of bean flours in the digestion mixture was 100mg/mL. The final concentration of isolated bean fractions in the chyme, considered the amount of each fraction in 100mg of bean flour determined according to Lin et al. (2020). Cholestyramine, a bile acid sequestrant and synthetic drug currently used for blood cholesterol reduction, was used as a positive control (Zacherl et al., 2011). The digestion of a substrate blank (no bean sample added) was also performed.

5.2.4.2 Bile salt release using dialysis

The dialysis method used to determine the amount of BS not retained (i.e. released) by bean materials was based on the method described by Naumann, Schweiggert-Weisz, Bader-Mittermaier, Haller, & Eisner (2018). Briefly, four grams of the digested bean samples were transferred into 12–14 kDa dialysis tubing with cut-off at 20 Å, since BS molecules have been reported to be approximately 20 Å long (Holm et al., 2013). The dialysis membrane was placed into a tube containing 36mL SIF, and shaken in an incubator (Innova 4230, New Brunswick Scientific, Edison, NJ, USA) at 160 rpm and 37 °C. Dialysate aliquots were sampled after 2, 4, 8, and 16 h of dialysis time and the permeated/released (i.e. non-retained) BS in the dialysate were quantified and analyzed by HPLC. In order to study the release kinetics of BS, the % of BS release (i.e. unbound BS) vs. time data were fitted to first-order kinetics (Eq.1) as reported by (Naumann et al., 2018) using Graphpad Prism8 (GraphPad Software Inc., San Diego, CA, USA):

\[ C_t = C_f \times (1 - e^{-kt}) \]  

Eq.5.1

Where \( C_f \) is the concentration of BS (% or 10^2 mM) after reaching equilibrium and \( k \) is the apparent release /permeability rate constant (h^-1). This equation describes the simplest model of diffusion under dialysis.
5.2.4.3 Quantification of Bile Salts by HPLC

The separation and quantification of the BS were performed by HPLC (Agilent 1100, Agilent Technologies, Santa Clara, CA, USA) using a Macherey-Nagel C18 Nucleodur reversed-phase column (250×4.6mm, i.d., 5 μm). The mobile phase, which has been used in medical research studies for the quantification of BS in human bile (Wildgrube et al., 1983), consisted of ultra-pure water, acetonitrile and 0.5M tetrabutylammonium phosphate (45:50:1, v/v/v). The mobile phase solutions were vacuum-filtered with WhatmanR filter paper (90 mm diameter, circles, ash less) (Maidstone, UK) and degassed using a ultrasonic cleaner (F320, Fisher Scientific, Hampton, VA, USA) before HPLC analysis. The mobile phase was eluted in isocratic mode at the flow rate of 1 mL/min with the column operating at 40 °C. A 5μL sample was injected for analysis with UV detection at 200nm. The HPLC Chem Station software (Agilent Technologies, Santa Clara, CA, USA) was applied to control and monitor the HPLC conditions and to collect the data. Bile salts were quantified using peak area analysis. A BS mixture including NaTC, NaGC, NaTCDC and NaGCDC at the same molar concentration (5mM) was used to verify the separation efficacy of the HPLC method. Complete separation was achieved for separate analysis of the four primary BS (Figure 5.4). Each individual BS standard (10mM) was injected to confirm their corresponding retention times and to obtain the area (Figure C1). The quantification of individual un-bound BS from samples was obtained according to the standard curves as prepared by the BS mixtures (35% NaGC, 35% NaTC, 15% NaTCDC and 15% NaGCDC) at a range of concentrations from 0.0625 to 10mM (Table C1). Linear regression of the standard curves showed good separation and quantification results (R≈1) in triplicate experiments. The amount of BS retained by bean samples was calculated as the difference between the initial amount of BS in the intestinal phase and the released (non-retained) BS in the dialysate quantified by HPLC. This value was expressed as the percentage (%) of bound BS.

5.2.5 Viscosity determination

Viscosity measurements were performed in a DHR3 rheometer (TA instruments, Waters Co., Ltd., Leatherhead, UK) using a parallel plate geometry (40mm diameter, 1mm gap) and a solvent trap to minimize moisture loss. The temperature was kept constant at 37 °C by using a circulating water system. An aliquot of digested samples was taken at the intestinal stage, loaded into the geometry and left to rest for 1 min before measurements. Viscosity was measured as a function of shear rate varying from 1 to 600 1/s. The value of viscosity at the shear rate of 15 s⁻¹ was recorded with the supplied software (Rheology Advantage, TA Instruments, Waters Co., Ltd.).
Figure 5.4 HPLC chromatogram showing the separation of primary bile salts (NaGC, NaTC, NaGCDC and NaTCDC)

NaGC: Sodium glycocholate
NaTC: Sodium taurocholate
NaGCDC: Sodium glycochenodeoxycholate
NaTCDC: Sodium taurochenodeoxycholate

5.2.6 Thermal analysis of bean materials-BS mixtures

The thermal profiles of bean materials in absence or presence of BS were obtained by using a Multi-Cell Micro- Differential Scanning Calorimeter (MC-DSC, TA Instruments, New Castle, DE, USA). Bean flours or isolated fractions were dispersed or dissolved in distilled water at room temperature to obtain a concentration of 2% (w/v). A 20mM bile salt stock solution was prepared in distilled water and stored at 4 °C. The final concentration of bean materials in the mixture was kept at 1% (w/v), whereas the BS concentration was either 0, 5mM or 10mM in the mixed systems. 800 ± 5mg of each sample mixture was placed in the DSC ampoules (made of Hastelloy) and sealed. Distilled water in the same range of weight was used as baseline of heat change in each ampoule. An empty ampoule was used as a reference. Sample and reference ampoules were placed in the DSC cells and equilibrated for 60min before starting scanning. Cells underwent a heating and cooling cycle from 10 °C to 150 °C at the rate of 1 °C/min. Thermograms were recorded and the enthalpy change (ΔH) of the transitions were calculated using the manufacturer supplied Nanoanalyze software (TA Instruments).

5.2.7 Data analysis

All measurements were performed in triplicate using freshly prepared samples, and values are reported as mean ± standard deviation. The data were evaluated by Analysis of Variance
(ANOVA), and the Tukey HSD comparison test was used to evaluate significant differences among experimental means (p < 0.05). All of the statistical analyses were conducted by JMP Pro13 (SAS Institute, Cary, NC, USA) and plotted by using Graphpad Prism8 (GraphPad Software Inc., San Diego, CA, USA).

5.3. Results and Discussion

5.3.1 Viscosity of *in vitro* digested bean flours

In order to understand how viscosity contributes to BS retention in the small intestine, the flow behavior of digested bean flours and their isolated fractions (i.e. protein, starch, soluble and insoluble fibre) at the intestinal stage was studied (Figure 5.5). In addition, the shear rate of 15s\(^{-1}\) was chosen to compare the viscosity of digested samples as this value is close to the small shear rates that occur in the gastrointestinal tract (Naumann, Schweiggert-Weisz, Eglmeier, et al., 2019). The flow curves of the digested starch and protein fractions showed a similar profile that the blank sample (i.e. digesta fluid) and tended to a plateau as the shear rate increased (Fig. 5A), displaying Newtonian-like behavior at high shear rates. This suggests a lack of polymer entanglement or crosslinking (Naumann et al., 2018), which is probably result of the dismantle of protein chains and starch polymers into smaller fragments by the action of pepsin in the gastric phase, and of salivary \(\alpha\)-amylase in the oral compartment, respectively. Both fiber fractions (IDF and SDF) showed a similar pattern of shear-thinning behavior (Figure 5.5A), indicating entanglement of undigested fiber polymers. When the shear rate increased, the entangled polymers partially disentangled and the flow resistance (i.e. viscosity) decreased. Compared to fiber samples, the digesta containing bean flour showed a weaker shear-thinning profile with tendency to a plateau at high shear rates, suggesting less degree of entanglement of bean fibers in the bean dispersion.

Complementing flow behavior, Figure 5.5B shows that the starch and protein fractions did not significantly increase the viscosity of digesta. These fractions were significantly less viscous than the chymes containing both, bean flour or fiber fractions, which could be partially explained by the fact that the fiber fractions consist of undigested longer molecules, able to form more entanglements that contribute to an increased digesta viscosity. The particularly viscous network formed by IDF, which was significantly more viscous than the blank digestion, could be also due to the presence of resistant starch in the IDF fraction. This hypothesis is supported by the work of Kim & White (2012), who reported that the non-hydrolyzed amylose and amylopectin polymers present in resistant starch increased the viscosity of digesta fluids. The IDF fraction could also contribute to the viscosity of the chyme by acting as filler particles, as reported by (Naumann et al., 2018) who partially explained the high viscous effect of citrus fiber by its high IDF content. The significantly high viscosity of the bean flour compared to the isolated fractions, suggest that apart from DF fractions, proteins and starch are contributing to viscosity in
a higher degree than when they are in their isolated form. This is probably related to the barrier effect exerted by the bean cell wall, that prevent a high extent of protein and starch enzymatic hydrolysis during in vitro digestion (Rovalino-Córdova et al., 2018, 2019).

Figure 5. Flow behavior (viscosity as a function of shear rate) and viscosity values at shear rate of 15 s\(^{-1}\) of in vitro digested isolated bean fractions (A&B) and in vitro digested beans processed by hydrothermal and high-hydrostatic pressure treatments (C&D).

(Data are expressed as the mean values (n=3) ± standard deviation (SD) bar. Mean values with different letters are significantly different (P < 0.05)).

The flow curves of digesta samples containing untreated and processed bean flours were similar (Figure 5.5C), which indicates that HT and HHP, when applied to whole beans previously to mechanical treatment, did not significantly affect the network integrity when bean flours are digested. By comparing the digesta viscosity values (Figure 5.5D), HT-treated beans showed the highest viscosity, which could be due to the presence of gelatinized/retrograded starch in these samples.
5.3.2 Ability of bean flour and its isolated fractions to retain individual primary BS in vitro

The ability of bean materials to retain BS was studied by using dialysis and quantifying released or permeated (i.e. non-retained) BS after 2h of dialysis. The % BS retained by beans was calculated as the difference between the initial amount of BS and the amount of permeated BS in the dialysate, taking into consideration the % BS retained in the blanks. In order to understand the contribution of bean components to the ability of bean flours to retain BS and how that contribution relates to the viscosity generated during digestion, the amount of isolated fractions used in this study was equivalent to their partial ratio in the whole flour (Lin et al., 2020). Table 5.1 shows that bean flour and isolated bean fractions showed different levels of BS retention to the BS mixture. The BS-binding ability of cholestyramine, the positive control, was 100%. The protein fraction showed the greatest BS retention (38.8%) among all bean fractions, retaining even more BS than the flour (30.81%). The starch and SDF fractions also retained some BS (29.71 and 22.02 %, respectively), and IDF retained less BS (15.33%) than the rest of samples. Specifically, the contribution of the individual fractions to retain the mixture of BS was, in a decreasing order, Protein >Starch> SDF> IDF.

Considering the low content of SDF (~7%) in bean flours in comparison to IDF, starch and protein content, the capacity of SDF to retain BS is, together with the protein fraction, the highest among the samples when the binding capacity is expressed on an equal dry weight basis (Figure C2). This high capacity can be related to the viscous matrix generated by soluble fiber polymers in the digesta (Figure 5.5A&B), which allowed entrapment of BS. Viscosity has been broadly considered a major mechanism to explain retention of BS by soluble dietary fiber, and it has been demonstrated for a number of high-fiber preparations and purified soluble fiber, including β-glucans (Kim & White, 2012) and arabinoxylans (Dongowski, 2007). Retention of BS in cereals and legumes has also been related to insoluble fiber structures (Kahlon & Woodruff, 2003) which is consistent with our current results. However, although IDF and SDF increased viscosity of the digesta to similar levels (Figure 5.5A&B), the IDF fraction had the lowest impact on BS retention among the bean fractions. This suggests that the superior ability of SDF to retain BS was also due to mechanisms distinctly independent of viscosity, probably the so-called adsorptive effect which has been recently documented (Gunness et al., 2010; Naumann et al., 2018). Therefore, a direct molecular interaction between bean SDF and BS could constitute another means to retain BS by bean SDF. The comparatively moderate BS retention observed by IDF (15.33%) can be associated to a viscosity effect (Figure 5.5A&B). As we showed in our previous study, the BS-binding ability of beans was non-linearly correlated with IDF and resistant starch (RS) content (Lin et al., 2020). We also suggested that IDF polymers different to RS, such as lignin, were partly responsible for the BS retention of beans. Similarly, other authors have proposed that lignin contributes to the ability of lupins to retain BS mainly through adsorptive
interactions (Naumann, Schweiggert-Weisz, Eglmeier, et al., 2019). Therefore, in the present study, an adsorptive factor from insoluble fiber components, such as lignin, cannot be ignored.

**Table 5.1 Retention of primary Bile Salts in vitro (%BS_retained) by bean flour and its fractions**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>BS mixture</th>
<th>NaTC</th>
<th>NaGC</th>
<th>NaTCDC</th>
<th>NaGCDC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bean flour</td>
<td>30.81±1.33ab</td>
<td>22.87±1.27abB</td>
<td>24.53±2.45abBB</td>
<td>45.07±0.33aA</td>
<td>49.69±3.91aA</td>
</tr>
<tr>
<td>IDF</td>
<td>15.33±2.28c</td>
<td>6.37±1.15bbB</td>
<td>17.61±1.96bA</td>
<td>15.10±6.87bA</td>
<td>25.36±6.99bA</td>
</tr>
<tr>
<td>SDF</td>
<td>22.02±2.75bc</td>
<td>24.39±1.67aAB</td>
<td>20.50±1.75bB</td>
<td>23.83±1.61bAB</td>
<td>34.07±2.51aA</td>
</tr>
<tr>
<td>Starch</td>
<td>29.71±3.85ab</td>
<td>26.47±2.81aA</td>
<td>30.95±2.95abA</td>
<td>30.07±2.90abA</td>
<td>35.39±2.71aA</td>
</tr>
<tr>
<td>Protein</td>
<td>38.85±0.46a</td>
<td>29.60±2.57abB</td>
<td>36.75±2.71aB</td>
<td>45.26±2.03aA</td>
<td>45.26±2.91aA</td>
</tr>
<tr>
<td>Blank</td>
<td>4.01±0.61d</td>
<td>2.19±0.36cB</td>
<td>2.66±0.72cB</td>
<td>4.01±0.37cAB</td>
<td>6.10±0.62cA</td>
</tr>
</tbody>
</table>

The amount of bean fractions used were equivalent to their ratio in 100mg/mL bean flours. The % BS_Bound by cholesteryamine is 99%. Data are expressed as the mean values (n=3) ± standard deviation (SD). Comparisons for all pairs were made using Tukey-Kramer HSD. *Within a column, mean values with different letters are significantly different between different bean fractions (p < 0.05). A-BWithin a row, mean values with different capitals are significantly different between different primary BS (NaTC, NaGC, NaTCDC and NaGCDC) (p < 0.05).

Regarding the protein and starch fractions, when the retention capacity is expressed on an equal dry weight basis, the %BS retained by the protein fraction remains the highest, whereas for the starch fraction, it decreases to levels below the corresponding to SDF (Figure C2). Despite that the fractionated protein showed the greatest ability to retain BS, it did not significantly increase the viscosity after in vitro digestion (Figure 5.5A&B), which suggests that the ability of the protein fraction to retain BS during digestion was due to factors other than viscosity and could be explained by its adsorptive properties. Other studies have also described the potential contribution of proteins from black and pinto beans to BS-binding (Kahlon et al., 2002). Sayar et al. (2006) also reported that proteins from oats considerably contributed to the BS-binding capacity of oat flour; Kim & White (2012) found that the hydrolysis of protein into peptides increased BS-binding by oat slurries. Hence, it would not be unreasonable to suggest that the observed high retention of BS by the protein fraction could be due to the existence of molecular interactions between BS and protein hydrolysates and peptides formed during digestion of bean proteins. The BS retention observed for the starch fraction is in line with the study of (Simsek & El, 2012), who reported the in vitro binding of BS by taro starch, which is also consistent with in vivo data showing reduced levels of free fecal bile acids after feeding a starch-rich diet to mice (Caderni et al., 1993). Since the starch fraction neither show a significant increase in digesta viscosity (Fig.5A&B), the observed BS retention could be attributed to a complexation of BS with starch helical structures, mainly of
amylose (Takahama & Hirota, 2011). The results also show that bean components in their isolated form seem to be more effective at retaining BS during in vitro digestion than when they are part of the bean matrix. This would suggest non-existence of a synergistic effect of all bean flour fractions on BS retention, in contradiction to what has been reported in oats (Sayar et al., 2006). This fact could be explained by the rigid and thick cell wall structure of legumes in comparison to cereals. In beans, the cell wall acts as barrier encapsulating starch granules and proteins, thus hindering the access of digestive molecules to bean components (Rovalino-Córdova et al., 2018, 2019). Since the cotyledon cell wall structure still prevails after thermal processing and mechanical treatment (ref Pallares, 2018), the lack of synergy observed in bean flours is probably due to a limited accessibility of BS to the enclosed protein and starch.

In order to investigate the binding preferences of beans for primary BS and gain more insight into the type of binding, individual non-retained BS (NaGCDC, NaTCDC, NaGC and NaTC) were quantified separately. Table 5.1 shows that BS retention was dependent on the structure of the primary BS. NaGCDC showed the largest retention levels (of up to 49%), which were considerably high in the protein fraction and the flour. NaTC was the less effectively retained BS (excepting for SDF), and was adsorbed only to a minor extent by IDF. There was a trend for bean samples to retain a higher proportion of di-hydroxy BS (chenodeoxycholates, -CDC) than tri-hydroxy BS (cholates, -C), as well as to bind more effectively to glyco-conjugated (-G) than with taurine-conjugated BS (-T). The greater preference for dihydroxy-bile acids than for trihydroxy-bile acids has already been proposed by several authors (Eastwood & Hamilton, 1968; Dongowski, 2007). The BS hydroxylation degree considerably influenced BS binding by the protein fraction and the flour (the two samples showing the highest adsorption). However, the conjugation had no significant influence on BS retention, excepting for the binding of cholates by IDF. Recent studies have reported that while a minor difference in the main part of the BS structure (i.e. number and position of hydroxyl groups on the sterol ring) has a major effect on BS adsorption behavior to hydrophobic surfaces (Parker et al., 2014; Zornajk et al., under review), major structural changes (i.e. conjugation with either taurine or glycine) has much less little impact (Parker et al., 2014). Since the removal of one of the ring hydroxyl groups (from cholates -C to chenodeoxycholates -CDC) in the BS, leads to an increase in BS hydrophobicity and an increased binding to bean fractions, the hypothesis of a hydrophobic interaction between BS and bean components is considered. In the case of the protein fraction, this hypothesis is supported by the study of (Ngoh et al., 2017) who found that peptides from pinto beans bound to bile acids through hydrophobic interactions. Other studies have also reported the involvement of hydrophobic peptides resulting from protein digestion in the BS-binding shown by plant proteins such as buckwheat, corn and soybean proteins (Kongo-Dia-Moukala et al., 2011; Ma & Xiong, 2009; Nagaoka et al., 2010). Furthermore, the highest binding affinity displayed by bean flour and isolated proteins to more
hydrophobic BS during *in vitro* digestion, suggests that hydrolyzed proteins are major contributors to the BS binding of flours by means of an adsorptive mechanism.

### 5.3.3 Effect of BS on the thermal properties of bean flours and their fractions

To gain more insight into the mechanism of binding between beans and BS, and into the contribution of bean components to this binding, the thermal properties of bean flours and their isolated fractions were determined in the absence and presence of BS by means of DSC analysis. Since the BS conjugation group had less impact on BS-binding, only taurine-conjugates, NaTC and NaTDCD, were selected to represent BS with different degree of hydrophobicity. Micro-DSC thermograms displaying the peaks on heating for bean flour, and starch and protein fractions at a fixed concentration (10mg/mL), as well as for their mixtures with BS at two BS concentrations (5 and 10 mM) are shown in Figure 5.6. The corresponding enthalpy changes (ΔH) are shown in Table C2. In the absence of BS, the thermograms corresponding to bean flours show two endothermic transition peaks, at 78 °C (Tp1) and 95 °C (Tp2) (Figures 5.6A&D), which are associated with starch gelatinization and protein denaturation (Lin & Fernández-Fraguas, under review). Similar individual transition peaks, at 78 °C or 95 °C, were also observed in the isolated starch (Figures 5.6B&E) or protein fraction (Figures 5.6C&F), respectively. Fiber fractions (IDF and SDF) did not show any peak probably due to the fact that the thermal transition temperature of fiber components was out of range of the instrument maximum temperature (150 °C). The thermal degradation temperature of cellulose and lignin, insoluble fiber polymers, has been reported to be above 250 °C (Brebu & Vasile, 2010) and that of pectin, soluble fiber polymer, is in the range of 200-250°C (Dranca & Oroian, 2019).

When presented with BS, shifts in transition temperatures and/or change of ΔH values were observed in all the bean materials (Figure 5.6 and Table C2), suggesting some type of association or adsorptive interaction between BS and bean components. However, differences were observed between bean samples and BS. In NaTC mixtures, the thermal profile of bean flours did not show significant change in terms of temperature or ΔH associated to starch gelatinization, while a significant decrease of peak (Tp2) and ΔH associated to protein denaturation was observed with the increasing of NaTC concentration. The incorporation of increasing concentrations of NaTC in the isolated starch fraction decreased the enthalpy of starch gelatinization, an effect that was clearly perceptible even at 5mM NaTC, without affecting the starch gelatinization temperature (Figure 5.6B). In the case of the isolated protein fraction, addition of NaTC slightly shifted the temperature of protein denaturation towards lower values and reduced ΔH values as the BS concentration increased, this effect being more significant at higher NaTCDC concentrations (Figure 5.6C). Regarding mixtures with NaTCDC, the addition of the BS to bean flours slightly shifted Tp1 and Tp2 to lower temperatures and decreased the ΔH of both transitions, with higher NaTCDC concentrations showing a larger effect (Figure 5.6D). NaTCDC had a similar effect on the isolated
starch fraction than NaTC but slightly decreased gelatinization temperature (Fig. 6E). A clear effect of NaTCDC on the enthalpy associated to protein denaturation was observed in the isolated protein fraction as the BS concentration increased; yet, only slight differences between NaTC concentrations were observed (Figure 5.6C&F).

**Figure 5.6** Thermal analysis of 1% (w/v) bean flour (A&D), starch (B&E) and protein (C&F) fractions in the absence and presence of 5 or 10mM bile salts (NaTC or NaTCDC).

(Each sample was analyzed in triplicate and the graphs show a representative profile for each sample)

These results show that the effect of both BS on starch gelatinization and protein denaturation was very limited in bean flours (Figure 5.6A&D), compared to the isolated starch and protein fractions (Figure 5.6B&E). This could be explained by the barrier effect characteristic of the legume cell wall limiting the accessibility of BS to proteins or starch, which is in accordance
with the lack of synergistic effect of flour fractions on BS retention. It has been also reported that the cytoplasmic proteins in beans could act as a second encapsulation system preventing access to starch (Rovalino-Córdova et al., 2019). This could explain the limited effect that BS have on starch gelatinization in bean flours, compared to the effect on protein denaturation (Figure 5.6A&D). Fig. 6A&D also shows that interactions between bean components and BS were dependent on the BS structure, mainly for the protein fraction. The more hydrophobic BS, NaTCDC, had a more significant effect on the change of thermal properties related to protein denaturation than starch gelatinization of bean flour compared to NaTC (Figure 5.6A&D and Table C2), in line with the greater ability of bean flour to retain more hydrophobic BS during in vitro digestion (Table 5.1).

The greatest effect on protein denaturation in the isolated fraction was also observed with NaTCDC, the di-hydroxy BS, at both concentrations, which further supports a more efficient association, likely of hydrophobic character, between NaTCDC and bean proteins. We propose that the different effects observed with cholates and chenodeoxycholates are related with the micellar structure of the BS in solution, which reflects the hydrophobicity of the BS. The BS concentrations tested, 5mM and 10mM, were both below the CMC (~13mM) of NaTC and within or above the CMC values (0.9–7mM) reported for NaTCDC, respectively (Madenci & Egelhaaf, 2010). Therefore, the taurochenodeoxycholate, which is forming micelles at 10 mM (and probably at 5mM, which could be assumed by the thermogram profile) is more susceptible to interact with bean proteins than NaTC, which in form of monomers at both concentrations. Similarly, in a recent DSC study looking at the effect of BS structure on thermal transition of cellulose derivatives, we observed that NaTDC (taurodeoxycholate), also a di-hydroxy BS, bound more efficiently to HPC than NaTC (Zornjak et al., under review) when it was in micellar state.

5.3.4 Effect of HT or HHP processing on the ability of beans to retain primary BS

Whole beans were treated either by HT or HHP before mechanical disintegration to investigate how processing affects beans capacity to retain BS. As shown in Table 5.2, and compared to cholestyramine, which bound 99% BS, untreated bean flours bound 30.81% of BS mixture. This value was considerable higher than obtained for bean flours in our previous study (19%) (Lin et al., 2020), which is likely due to the different methods used to determine BS-binding ability and/or to quantify unbound BS. The centrifugation method used in the previous study to separate bound from unbound BS, combined with a colorimetric-enzymatic BS assay, is the most common method used to evaluate BS-binding. However, this method is based on the hypothesis that fiber, or another BS sequestrant, binds BS forming insoluble complexes that precipitate with centrifugation. While the BS retained by IDF could be probably separated from the unbound BS (Zacherl et al. 2011), it is not clear whether BS retained by SDF or other soluble molecules, or by a viscous matrix, will be fully precipitated after centrifugation. The dialysis-based method used in the present study includes a membrane with a cut off (12–14 kDa), close to the molecular size of
BS (Holm et al., 2013) to prevent the diffusion of BS retained by bean components, while considering the viscosity of the media. Therefore, the centrifugation method underestimates the BS-binding ability of samples which explains the higher % of retained BS obtained in the present study.

Table 5.2 Retention of primary Bile Salts in vitro (%BS_retained) by beans processed by hydrothermal (HT) or high hydrostatic pressure (HHP) treatments

<table>
<thead>
<tr>
<th>Treatment</th>
<th>BS mixture</th>
<th>NaTC</th>
<th>NaGC</th>
<th>NaTCDC</th>
<th>NaGCDC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>30.81±1.33^b</td>
<td>22.87±1.27^ab</td>
<td>24.52±2.45^a</td>
<td>45.05±0.33^b</td>
<td>49.69±3.91^a</td>
</tr>
<tr>
<td>HT</td>
<td>33.02±0.66^ab</td>
<td>24.98±0.47^ab</td>
<td>26.56±0.97^a</td>
<td>50.65±1.02^a</td>
<td>51.45±0.15^a</td>
</tr>
<tr>
<td>HHP150</td>
<td>29.15±0.94^b</td>
<td>21.40±0.79^b</td>
<td>22.43±1.13^a</td>
<td>45.01±0.46^b</td>
<td>46.96±1.42^a</td>
</tr>
<tr>
<td>HHP450</td>
<td>32.49±1.17^ab</td>
<td>25.21±1.68^ab</td>
<td>26.63±0.90^a</td>
<td>47.20±0.61^b</td>
<td>48.35±1.04^a</td>
</tr>
<tr>
<td>HHP600</td>
<td>34.37±0.52^a</td>
<td>26.56±0.25^a</td>
<td>27.16±0.76^a</td>
<td>51.10±1.25^a</td>
<td>52.25±1.14^a</td>
</tr>
</tbody>
</table>

The % BSBound by cholestyramine is 99%. Data are expressed as the mean values (n=3) ± standard deviation (SD). Comparisons for all pairs were made using Tukey-Kramer HSD; ^a-cWithin a column, mean values with different letters are significantly different between different bean samples (p < 0.05). ^A-BWithin a row, mean values with different capitals are significantly different between different primary BS (NaTC, NaGC, NaTCDC and NaGCDC) (p < 0.05).

Although HT treatment of beans slightly increased BS retention (33.02%), probably due to the increased digesta viscosity (Figures 5.5C&D), processing treatments did not have a significant effect on the retention of the BS mixture. These results are not in agreement with those obtained in our previous study, probably because of the different sequence of processing operations performed in both studies. In the previous study, whole seeds were mechanically milled into flours before being subjected to HT or HHP treatments (Lin et al., 2020), whereas in the current study, HT or HHP are applied to whole beans before undergoing mechanical disintegration. The partial damage caused in the cell wall integrity and cytoplasmic matrix (i.e. proteins bodies attached to starch granules) of beans by an initial mechanical treatment, can be further compromised by the application of HT or HHP treatment (Lin et al., 2020). However, since in whole beans, the starch granules are embedded in a protein matrix and both components are enclosed in the rigid cell wall, a preceding thermal or pressure treatment followed by mechanical disintegration would probably have less impact on the structural integrity and associated factors. This ultimately would translate in minor differences among BS-binding abilities.

Regarding the effect of processing on retention of individual BS, only the most severe treatments (HT and HHP 600) significantly increased the ability of bean flour to retain NaTCDC. However, neither of the HT or HHP treatments had an impact on the binding preferences of bean flours to the primary BS (Table 5.2). Similar to the trend observed in untreated bean flours and
their isolated fractions, processed bean samples showed a significative greater binding preference to chenodeoxycholates, -CDC than to cholates, -C, with no detectable influence resulting from conjugation. A positive but low correlation ($R^2=0.59$, $p=0.036$) was observed between BS retention and digesta viscosity (Figure C3), which suggest that the capacity of processed bean samples to retain BS cannot be explained solely by the viscosity generated during digestion.

### 5.3.5 In vitro Bile salt-binding kinetics of beans

In order to better differentiate viscosity and adsorptive factors, and gain a deeper insight into the possible mechanisms of BS retention, the effect of processed bean matrices on the kinetics of BS release was investigated. Kinetics were determined by quantifying released (i.e. non-retained) BS at regular dialysis time intervals for a total of 16 h. The release kinetics of individual primary BS are displayed in Figure 5.7. The fitting parameters ($C_f$, equilibrium concentration of released BS (%), and $k$, BS release rate constant) obtained by nonlinear regression (Eq. 5.1) are shown in Table 5.3. Correlation coefficients ($R^2$) were higher than 99% (data not shown), indicating that this model is appropriate to describe the release of BS as a function of time. The %BS release by cholestyramine was ~0% and, as expected, it did not significantly change with time (Figure 5.7A), as cholestyramine could strongly bind BS to form insoluble complexes that precipitate and do not permeate (Oakenfull & Fenwick, 1978). The substrate blank curves show a rapid release of BS with time and reached almost 100% release after 8 h of dialysis, indicating that, in absence of bean flours, BS were fully permeated through the dialysis membrane after 8 h (Figure 5.7A).

Release of both, the BS mixture and individual BS, by in vitro digested untreated bean flours increased with dialysis time (i.e. the amount of bound BS by beans decreased), tending towards a plateau after 8 h (Figure 5.7A), when the release equilibrium, $C_f$ (89.86%) was reached (Table 5.3). This sample also showed a delayed release (i.e. lower $k$) of the BS mixture and each individual BS in comparison to the blank substrate. None of the processing treatments had a significant effect on the release kinetics of BS mixture (Figure 5.7A), resulting in no significative different $C_f$ (90-91%) and $k$ (0.62-0.75) values (Table 3). The viscosity of digested bean flours was negatively correlated with releasing rate $k$ and BS release equilibrium concentration ($C_f$) (Figure C4), which suggests that the viscosity generated by bean flours is partially responsible for the delayed and decreased amount of BS mixture released, in line with the findings of Naumann et al. (2018) on fiber preparations. Regarding the effect of processing on the release of individual BS, both HT and HHP (especially higher pressure at 450MPa and 600MPa) treatments seemed to slightly decrease the release extent of NaTC and NaGCDC (i.e. lower $C_f$ values), while increasing that of NaGC with no significant effect on NaTCDC (Table 3). The largest effect was caused by HHP600 treatment which led to the lowest release of NaGCDC among all samples. The effect of processing on the BS release rate ($k$) did not show an identifiable trend, however and even if the effect was not significant, in comparison to untreated beans, decreased $k$ values were observed for
Figure 5.7 Concentration of BS (%) released as a function of dialysis time during in vitro intestinal digestion of beans (treated by HT and HHP processing) for (A) the BS mixture, (B) NaTC, (C) NaGC, (D) NaTCDC and (E) NaGCDC. (A blank sample (digesta without beans) and reference (cholestyramine) have been included. Data are expressed as the mean values (n=3) ± standard deviation (SD) bar. The solid line represents the best fit between the experimental data and the mathematical model (Eq. 5.1))
each primary BS in HHP-treated beans except for NaTC in HHP600-treated beans (Table 5.3). In particular, pressurization at 600MPa increased NaTC and NaGDC retention while accelerating NaTC release and delaying NaGDC release during dialysis. As observed in Figure 5.7, NaGDC was the BS whose retention by beans was mostly affected by HHP processing.

As for the preferences of processed beans to retain a particular BS, the BS release kinetics from untreated and processed bean flours differed depending on the BS structure (Figure 5.7B,C,D,E & Table 5.3). We can observe that BS hydroxylation affected % and rate of BS release to a considerable extent: All bean samples, regardless of processing treatment, released a higher proportion of cholates (tri-hydroxy BS) compared to chenodeoxycholates (di-hydroxy BS), with NaTC being the most released BS and NAGDC least released. The release rate was also higher in cholates than in chenodeoxycholates BS, which indicates that cholates were the least preferred and more quickly released BS by bean samples during dialysis, probably because of a lower affinity between bean materials and less hydrophobic BS. Similarly, (Naumann, Schweiggert-Weisz, Eglmeier, et al., 2019; Naumann, Schweiggert-Weisz, Haller, et al., 2019) reported a lower diffusion rate and a higher retention of chenodeoxycholates compared to cholate BS by a range of fiber-rich ingredients. The conjugation of the BS only had an effect on the amount of BS retained, and mainly on chenodeoxycholates, with a significant lower amount of glyco-conjugated released in comparison to tauro-conjugated BS from all samples. These findings are probably related to the formation of BS micelles. It is known that BS aggregate and form micelles when their concentration in solution is above their critical micelle concentration (CMC). Since micelle formation is mainly driven by hydrophobic associations, the CMC reflects the hydrophobicity of the BS (Hofmann & Hagey, 2008). Thus, as illustrated in Figure 5.1, the micellization of chenodeoxycholates (-CDC), the more hydrophobic BS, occurs at lower CMC than the cholate BS (-C). In this study, the initial BS concentration was 10mM, which decreased to ~1mM (final dilution concentration) as the dialysis continued. The dialysis membrane (cut-off,12-14KDa) might hinder BS micelles from diffusing as reflected in the profiles and fitting parameters of blank substrates (Figure 5.7&Table 5.3). Blank data show that, in absence of bean materials, permeation of BS micelles increases with time and chenodeoxycholates are released more slowly than cholates, likely because of an earlier onset of micellization. Therefore, the lower release rate and equilibrium concentration (C_t) observed for beans-chenodeoxycholates mixed systems during dialysis, is partially due to BS micelles slowly diffusing through the membrane. In any case, in the presence of bean materials, the diffusion of -CDC is almost half of that of -C, indicating that the formation of micelles plays a significant role on the ability of beans to retain BS.
Table 5.3 Correlation coefficients ($R^2$), %BS bound after reaching equilibrium ($B_p$), and decaying rate constant ($k$) determined by exponential one phase decay kinetics fitting of *in vitro* primary bile salts-binding (%BS_Bound) of beans treated by hydrothermal (HT) and high hydrostatic pressure (HHP) treatment during dialysis.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>BS mixtures</th>
<th>NaTC</th>
<th></th>
<th>NaGC</th>
<th></th>
<th>NaTCDC</th>
<th></th>
<th>NaGCDC</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$B_p$</td>
<td>$k$</td>
<td>$B_p$</td>
<td>$k$</td>
<td>$B_p$</td>
<td>$k$</td>
<td>$B_p$</td>
<td>$k$</td>
<td>$B_p$</td>
</tr>
<tr>
<td>Untreated</td>
<td>8.38±0.17$^a$</td>
<td>0.50±0.01$^a$</td>
<td>5.60±0.11$^b$</td>
<td>0.68±0.01$^a$</td>
<td>8.91±0.18$^a$</td>
<td>0.44±0.01$^b$</td>
<td>8.41±0.17$^b$</td>
<td>0.38±0.01$^b$</td>
<td>10.18±0.20$^b$</td>
</tr>
<tr>
<td>90°C/2h</td>
<td>8.34±0.25$^a$</td>
<td>0.45±0.01$^a$</td>
<td>7.73±0.23$^a$</td>
<td>0.61±0.02$^b$</td>
<td>7.75±0.23$^b$</td>
<td>0.47±0.01$^b$</td>
<td>8.10±0.24$^b$</td>
<td>0.41±0.01$^{ab}$</td>
<td>10.69±0.32$^b$</td>
</tr>
<tr>
<td>150MPa/5min</td>
<td>7.75±0.38$^a$</td>
<td>0.54±0.03$^a$</td>
<td>5.20±0.25$^{bc}$</td>
<td>0.66±0.03$^{ab}$</td>
<td>5.53±0.27$^c$</td>
<td>0.60±0.04$^a$</td>
<td>10.10±0.49$^{ab}$</td>
<td>0.36±0.02$^b$</td>
<td>13.18±0.54$^b$</td>
</tr>
<tr>
<td>450MPa/5min</td>
<td>8.11±0.19$^a$</td>
<td>0.52±0.01$^a$</td>
<td>4.68±0.11$^c$</td>
<td>0.31±0.001$^c$</td>
<td>5.17±0.12$^c$</td>
<td>0.28±0.01$^c$</td>
<td>9.24±0.22$^b$</td>
<td>0.37±0.01$^b$</td>
<td>11.60±0.28$^b$</td>
</tr>
<tr>
<td>600MPa/5min</td>
<td>8.77±1.38$^a$</td>
<td>0.40±0.08$^a$</td>
<td>2.98±0.47$^d$</td>
<td>0.19±0.03$^d$</td>
<td>5.34±0.84$^c$</td>
<td>0.49±0.08$^b$</td>
<td>11.74±1.85$^a$</td>
<td>0.47±0.07$^a$</td>
<td>18.89±1.98$^a$</td>
</tr>
<tr>
<td>Blank</td>
<td>-1.19±0.02$^b$</td>
<td>0.24±001$^b$</td>
<td>-1.44±0.03$^e$</td>
<td>0.23±0.01$^e$</td>
<td>-1.83±0.04$^d$</td>
<td>0.35±0.01$^c$</td>
<td>0.01±0.00$^e$</td>
<td>0.14±0.00$^e$</td>
<td>-1.07±0.02$^e$</td>
</tr>
</tbody>
</table>

Data are expressed as the nonlinear regression of the mean values (n=3) ± standard deviation (SD). Comparisons for all pairs were made using Tukey-Kramer HSD. Along the column, mean values with different letters are significantly different between different bean fractions (P < 0.05).
5.4. Conclusions

In this study, the role of bean components and matrix in the ability of beans to sequester individual primary BS was investigated under in vitro conditions simulating the upper gastrointestinal tract using a dialysis-based method. The effect of processed beans on the kinetics of primary BS release was also addressed. We have demonstrated that the barrier role of bean cell walls impacts the ability of bean components to retain BS during in vitro digestion. Our results indicate that the considerable BS retention observed for the SDF fraction was partially due to the increased viscosity generated in the digesta. In addition to SDF, bean proteins are major contributors to the ability of beans to retain BS under intestinal conditions, and factors involving adsorption or molecular interactions between protein hydrolysates and BS are proposed. Furthermore, the role that resistant starch and IDF might play in BS retention should not be ignored either, and a combination of viscous and adsorptive effects are likely to be involved. Nevertheless, a detailed knowledge of the specific molecular structures responsible of BS retention is still required. The preference of digested bean materials to retain glycine-conjugated and more hydrophobic di-hydroxy-BS (i.e. chenodeoxycholates), and the greater impact of taurochenodeoxycholate on the thermal properties of the protein fraction, support the hypothesis of hydrophobic interactions between bean proteins and BS. We have also shown that HT or HHP processing followed by a mechanical treatment, did not significantly influence the preference of beans to retain more hydrophobic BS and delay their release. The formation of BS micelles was probably responsible for the different release of unbound BS from bean flours observed for cholates and chenodeoxycholates, which emphasizes that BS hydroxylation and BS micellar structure play essential roles on the ability of beans to retain BS. A different sequence of the same processing operations has an impact on BS retention possibly because of a distinct structural damage of the bean matrix and accessibility of BS to bean components. Hence, the impact of, not only the types of processing methods, but also their order should be considered more critically when applied in combination to complex food matrices. These findings, therefore, provide a deeper insight into the factors responsible for the cholesterol-lowering effect of beans, which could advance the development of new strategies for the formulation of legume-based functional ingredients.

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**Conflicts of interest**
All the authors declare no conflict of interest.

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Chapter 6: Dry bean (*Phaseolus vulgaris* L.) modulate the kinetics of lipid digestion *in vitro*

**Abstract**

Common beans have been shown to play a positive role in reducing blood cholesterol and lipid levels. The mechanisms behind these health benefits are mainly attributed to the ability of dietary fiber (DF) in common beans to modulate lipid digestion in the duodenum. However, there is currently a poor understanding of the precise physicochemical mechanisms by which DF fractions could delay lipid digestion, and of the role of the bean matrix and bean processing. Therefore, this study aims to investigate possible mechanisms behind the lipid-lowering effect of beans and how different processing treatments affect the ability of beans to modulate lipolysis *in vitro*. Both SDF and IDF fractions from beans were isolated. Beans were either milled into different particle sizes and/or processed by hydrothermal (HT) or hydrostatic-pressure (HHP) operations. The effect of bean tissue materials on the particle size distribution, microstructure and viscosity of emulsions during *in vitro* gastrointestinal digestion was analyzed to understand their impact on lipolysis rate and extent of emulsions, as well as on the profile of free fatty acid (FFA) released. A decrease of lipolysis was achieved by both SDF and IDF fractions, with SDF showing a larger reduction, by increasing the digesta viscosity, entrapping lipid droplets, and causing depletion-flocculation and/or coalescence of lipid droplets. However, bean flours led to a more significant effect on reducing lipolysis than isolated fibers, possibly due to the presence of more complex network. Medium bean particles showed a larger reduction in lipolysis (rate and extent) than fine and coarse particles. HT treatment, which caused more damage of bean matrix, significantly reduced the ability of beans to modulate lipid digestion while HHP-600MPa/5min showed an enhanced effect. These findings show that the lipid-lowering effect of dry beans is not only related to bean fibers but also to the whole bean matrix, which could be manipulated by the physical state (i.e. particle size) and processing of beans.

**Keywords:** Dry beans, Dietary fiber, Processing, lipolysis; Microscopy; GC-MS
6.1 Introduction

Dry beans (*Phaseolus vulgaris* L.) have a complex matrix comprised of two pieces of cotyledons and a seed coat, the outer layer of beans. They are rich in minerals, phenolic compounds, and especially dietary fiber (Los et al., 2018). Consumption of dry beans has been shown to lower blood cholesterol and lipid levels and therefore to reduce the risk of cardiovascular disease (Abeysekara et al., 2012a; Anderson & Gustafson, 1988; Padhi & Ramdath, 2017). The USDA has ranked dry beans as the top source of dietary fiber for increasing the level of dietary fiber in the human diet (B. W. Li et al., 2002). The most recent U.S. dietary guidelines, *MyPlate*, also recommends the public to have a healthy diet rich in vegetables, fruits, grains, and legumes. Such an eating pattern is high in fiber and low in energy density, which is claimed to be particularly crucial for controlling lipid and cholesterol levels (CDC, 2011).

Dietary lipids usually include triacylglycerols (TAG), cholesterol, cholesterol esters, and phospholipids (Gropper et al., 2009). Generally, lipid digestion mainly occurs in the small intestine (70-90%) by the hydrolysis of pancreatic lipase, with a small part in the stomach by the hydrolysis of gastric lipase (Maldonado-Valderrama et al., 2011). The duodenum, as the first part of the small intestine, is the major site for lipid digestion. When chime enters the duodenum, this stimulates the pancreas to secrete pancreatic lipase, a mixture of enzymes including α-amylase, proteases, and lipase, to hydrolyze starch, proteins, and fat, respectively (Bauer et al., 2005). Most TAGs are hydrolyzed into free fatty acids (FFAs) (at the sn-1 and sn-3 positions) and sn-2-monoglycerides (MAGs) by pancreatic lipase in the duodenum (Mu & Høy, 2004). The key player help to achieve lipolysis (the digestion of lipids by the enzymatic hydrolysis) are bile salts (BS), the amphipathic molecules which could convert lipids into smaller droplets and emulsify lipid droplets thus facilitating the efficient attachment of pancreatic lipase to the droplets surface (Gropper et al., 2009).

The specific mechanisms of dry beans on cholesterol- and lipid-lowering effects are not fully understood; nevertheless most studies point out to factors mainly related to the physicochemical and physiological properties of dietary fiber in dry beans (Abeysekara et al., 2012b). Specifically, soluble fibers may generate viscosity in the upper gastrointestinal tract (GIT) that delay the transport and digestion of lipids. Also, dietary fiber is reported to sequester BS thus modulating lipid digestion (Capuano, 2017). Besides, other bioactive compounds like polyphenols, saponins, and proteins from dry beans are also reported to be responsible for reducing hyperlipidemia and hypercholesteremia (Amigo et al., 1992; Macarulla et al., 2001; Ramírez-Jiménez et al., 2015). The functionality of food components is controlled by major factors related to the complexity and structure of the food matrix and/or the interactions between different components. Therefore, whole seeds, which contain complex matrix structures and important bioactive substances, are more informative, effective and important for long-term health than isolated fiber (Jacobs Jr, 2015), which is worth studying further. While the effect of purified or
extracted dietary fibers from fruits or cereals (Abdul-Hamid & Fennema, 1995; Espinal-Ruiz et al., 2014, 2016) on lipid digestion has been investigated, the impact of whole beans and their dietary fiber fractions on the digestibility of lipids has received little attention.

Processing can influence the physicochemical, macro- and microstructural properties of beans (Lin et al., 2020; Lin & Fernández-Fraguas, under revision) which might have a further impact on digestion. Our previous study has shown that hydrothermal treatment (HT) and high hydrostatic pressure (HHP) could affect the rheological, thermal, pasting and functional properties of dry beans to a different extent. Moreover, HT and HHP treatment of bean slurries could manipulate the dietary fiber profile, resistant starch content, and microstructure of bean matrix to further impact the in vitro BS binding ability of beans (Lin & Fernández-Fraguas, under revision). Therefore, it would be necessary to explore how these structurally modified bean matrix produced by HT and HHP treatment impact the digestion of lipids, which have not been investigated yet. Moreover, the integrity of beans’ structures (i.e. physical state and particle size) may also affect the digestibility of bean components and further affect their physiological functions (Dhital et al., 2016; Scanlon et al., 2018). Different degrees of beans’ structural integrity can be generated by mechanical treatment such as milling and blending. Large particles created by gentle mechanical process usually have more clusters of intact cells of higher physical strength that may form stronger barriers for enzyme diffusion thus increasing the survival of cellular structure of food matrix during the GIT digestion (Dhital et al., 2016). Studies have shown decreased blood glucose response in whole beans compared to milled beans (Golay et al., 1986), and an improved in vitro starch digestibility with the increase of mechanical force on legumes, including chickpea, mung and kidney beans (Dhital et al., 2016). There are studies reporting that milling and processing of cereals (i.e. wheat and oat) could affect their ability to reduce the lipolysis rate and extent in vitro (Cara et al., 1992; Grundy et al., 2017). However, the effect of the structural integrity of the dry bean matrix on the lipid digestion has not been studied.

Therefore, the present work aims to investigate the role of dry bean matrix and their fiber fractions and the structural integrity of dry bean matrix on the in vitro lipid digestion of extrinsic oil-in-water emulsions. Specifically, we isolated bean fibers (soluble and insoluble fiber) to study their impact on in vitro lipid digestion, as compared with whole bean flours. We also determined the effect of bean tissue materials varying in particle size and the type of processing on lipolysis. We studied the stability of lipid droplets as they passed through the GIT and observed the digestibility of bean particles by confocal and optical microscopy, to provide a mechanistic understanding of the influence of dry bean matrix on their lipid-lowering effects. Finally, by using Gas chromatography-mass spectrometry (GC-MS), we also determined how the profiles of free fatty acids (FFA) released during lipolysis were affected by bean fiber and the different bean tissue materials.
6.2 Materials and Methods

6.2.1 Materials

Pinto beans were chosen as a representative variety of the market class of *Phaseolus vulgaris* since Pinto represents one of the most commonly consumed varieties worldwide (Gittlein, 2018). Dry pinto bean seeds (K1152-P) were kindly provided by ADM (Archer Daniels Midland Company, Decatur, IL, USA) and stored at room temperature. The plant material was grown in different regions of North Dakota and represented one harvesting season as reported by the manufacturer. Sunflower oil was purchased in a local grocery store (Kroger, Blacksburg, VA). The sunflower oil used in our study is a medium oleic acid oil, or called as NuSun oil. Enzymes, including salivary α-amylase Type XIII-A (A1031), porcine pepsin (P-7012) and porcine pancreatin (USPx8, P-7545) as well as bovine bile extract B3883 and lauric acid C12:0 were purchased from Sigma-Aldrich (St Louis., USA). According to (Naso et al., 2019) the composition of the bile extract B3883 is 30–40% taurocholic acid, 10–20% glycocholic acid, 5–10% glycodeoxycholic acid and 5–10% taurodeoxycholic acid, accounting for 60% cholic acid by mass of the total BS; the average molecular mass is ~442 g/mol. Lipase activity in porcine pancreatin was determined according to the protocol reported by (Brodkorb et al., 2019). All other chemicals such as Sulphuric acid, tributyrin, diethyl ether (HPLC grade), heptane (HPLC grade), and 12% boron trifluoride (BF₃) in Methanol were purchased from Fisher Scientific (Hampton, USA) and VWR International (Radnor, USA).

6.2.2 Mechanical and processing treatments of beans

Figure 6.1 Flow chart of the mechanical and processing treatment of beans

$d_{50}$: median particle size ($\mu$m)

Sample denotation, applied here and elsewhere:

CP, coarse particles of raw bean flour; MP, medium particles of raw bean flour; FP, fine particles of raw bean flour
FP-HT, fine particles of hydrothermal treated bean flour
FP-HHP600/5, fine particles of High hydrostatic pressure (600MPa/5min) treated bean flour
FP-HHP300/15, fine particles of High hydrostatic pressure (300MPa/15min) treated bean flour

A flow chart showing the processing treatments applied to beans is shown in Figure 6.1. Whole dry beans were soaked in water at the ratio of 1:2 (weight/volume, flour: water) overnight at room temperature. Soaked beans were packed into 400mL plastic bags, vacuum-sealed and then processed using high hydrostatic pressure (HHP). HHP treatments were conducted by using a hydrostatic pressurization unit (Quintus Food Press 35L-600, Avure Technologies, OH, USA). Bean samples were put in the high-pressure vessel and treated at room temperature (~25 °C) at 600MPa for 5min and 300MPa for 15min with extra pressure build-up (2-3 min) and release times (5-10s). The pressure-transmitting fluid was distilled water. Vessel temperature was monitored by using a submerged thermocouple and controlled (25 ± 3 °C) by using circulating cool water. Pressure and temperature during treatment were recorded.

One part of soaked beans was cooked by three times its weight of tap water at 90-95 °C for 2h (HT treatment), packed in plastic bags then vacuum-sealed (HT-treated samples). Another part of soaked beans was directly packed into 400mL plastic bags then vacuum-sealed (untreated sample). The untreated, HT- and HHP-treated samples were frozen at -20 °C overnight, and then freeze-dried by using a Labcono freeze dryer (Labcono, Kansas, MO, USA) for 2 days at a vacuum pressure of 28Pa and moisture collector temperature of -52 °C, and then milled into flours by using a high-speed blender (51BL30, Waring commercial, Stamford, USA).

Untreated bean samples were also milled and separated into different size fractions using a series of sieves. Three distinct fractions representing particle sizes that occur during food processing and in vivo mastication were selected and denoted as coarse (CP), medium (MP) and fine particles (FP) according to their mean particle size d50, which was 1500 μm, 900 μm, and 200 μm, respectively. HT and HHP-treated samples, which were produced from FP bean flours, were identified as FP-HT and FP-HHP600/5 and FP-HHP300/15. All the samples were stored in vacuum-sealed plastic bags and airtight containers at room temperature until further analysis.

### 6.2.3 Extraction of insoluble and soluble dietary fiber

Insoluble (IDF) and soluble (SDF) fiber fractions were isolated from beans according to methods in the literature (Feng et al., 2017; Wang, Huang, Tu, Ruan, & Lin, 2016) with some modifications. Two grams of raw bean flours were dispersed in 80 mL maleate buffer (pH 6.0) with an α-amylase/amyloglucosidase (AMG) mixture and then incubated for 16 h at 37 °C at 150 rpm in an orbital shaker (360 Orbital Shaker Bath, Precision Scientific, Trichy, India). After 16 h, an iodine test was used to check for starch hydrolysis and then the pH was adjusted to approximately 8.0 with the addition of 6 mL of 0.75 M Trizma base solution. Samples were then
incubated in a water bath at 95-99 °C for 20 min, then cooled to 60 °C. Next, 0.6 mL of the protease solution (350 tyrosine U/mL) was added to hydrolyze protein for 30 min in a shaking water bath. After this, the pH was adjusted to approximately 4.3 via the addition of 8 mL of 2 N acetic acid. The ultimate hydrolysate was centrifuged at 3200xg for 15min. The residue was washed twice with hot water (80°C) and 95% ethanol and then centrifuged at 3200xg for 15min. The residue after centrifugation was collected and freeze-dried as insoluble dietary fiber (IDF) fraction. And the supernatant from the hydrolysate was collected and condensed into 1/10 volume by vacuum rotary evaporation and then mixed with 95% (v/v) aq. ethanol for 1 hour at 60°C to precipitate soluble dietary fiber (SDF). The residue was collected through centrifugation (3200xg for 15min) and freeze-dried to obtain SDF.

6.2.4 Sample preparation

First, the source of lipids (i.e. oil-in-water emulsions) was prepared using Tween 20 as emulsifier as it is a common food-grade non-ionic surfactant used in the food industry. An emulsion premix was prepared by mixing 20% (w/w) of sunflower oil and 80% (w/w) emulsifier solution (2.5% Tween 20 solution in 5mM phosphate buffer pH7.0) and pre-homogenized using vortex for 1 min. The coarse emulsion was then passed through a high-shear processor (Microfluidizer LV.1, Microfluidics Inc., Newton, MA) at 5000psi 5 passes and stored for at least one hour in the dark before carrying out any further treatment and/or analysis.

Second, bean samples (~7g) were prepared by mixing 2.24g stock emulsion (containing 20% (w/w) sunflower oil and 2% (w/w) Tween 20), 0.7 g beans flour and 3.84g buffer solutions with a final concentration of 6.4% oil and 10% beans. Fiber-emulsion mixtures were prepared by adding SDF (0.7%) or IDF (2.5%) equivalent to their amount in the 10% of beans in the bean-emulsion mixtures. Bean fibers were previously pre-hydrated in buffer for 30min before mixing with stock emulsions. Bean and fiber-emulsion mixtures were further mixed by vortex for 1min. Blank samples were performed by mixing fiber or beans with buffer without adding emulsions (replace emulsion with buffer).

6.2.5 In vitro digestion

A standardized multistage static in vitro digestion protocol developed by the COST INFOGEST network -INFOGEST 2.0- that simulates the mouth, stomach, and small intestine was used (Brodkorb et al., 2019). The electrolyte stock solutions (simulated salivary fluid (SSF) pH 7.0, simulated gastric fluid (SGF) pH 3.0, and simulated intestinal fluid (SIF) pH 7.0) were prepared as described previously by (Brodkorb et al., 2019) and stored at 4 ℃. Fluids were pre-warmed to 37°C before being used for digestion. NaHCO₃ was replaced with NaCl at the same molarity in all of the simulated fluids since the pH-stat method and digestion were used in open-vessels instead of sealed containers (to prevent the pH increasing due to the release of CO₂)
(Brodkorb et al., 2019). Each assay will be performed in mechanically stirred reaction vessels of a pH-stat automatic titration device equipped with a double unit (Titrino 907, Methrohm USA) to have duplicate samples tested. All testing samples taken from each stage of digestion were immediately put into an ice bath to inhibit enzyme activity.

6.2.5.1 Oral phase

Five grams of bean/fiber-emulsion mixture or blank/control samples (initial phase) were mixed with 4mL SSF (ratio 1:1 w/w), 0.5 mL α-amylase (final activity 75U/mL), 0.475mL distilled water and 25uL CaCl₂ (2mM) and the resulting mixture containing 3.2% (w/w) oil and 5% (w/w) beans sample. During the oral stage, the mixture was incubated at 37 °C with continuous stirring in the vessel for 5min, with a temperature-controlled water circulating bath (Rte100, Neslab Instruments, Inc., Newington, NH, U.S.A.).

6.2.5.2 Gastric phase

Samples taken from the oral phase were mixed with 7.2mL SGF (ratio 1:1 w/w) with 4.5uL CaCl₂ (2mM) and adjusted pH to 3 by adding 1N HCl. Pepsin (0.45mL, 32mg/mL) was added to reach the final activity as 2000U/mL. The final mixture contained 1.6% (w/w) oil and 2.5% bean samples and was incubated at 37 °C with continuous stirring for 2h.

6.2.5.3 Intestinal phase

Samples taken from the gastric phase were mixed with 17mL SIF (ratio 1:1 w/w) with 34uL CaCl₂ (0.3M) and adjusted pH to 3 by adding 1N NaOH. Bile salts extract solution (2.125 mL, 160mM) was added to reach the final concentration at 10mM in the mixture. The final composition in the intestinal phase was 0.8% (w/w) oil and 1.25% (w/w) sample. Pancreatin (400 U/mg lipase activity) was added in 4.25mL to reach the final activity of 100U/mL. The triacylglycerols (TAGs) are hydrolyzed into two free fatty acids (FFAs) (at the sn-1 and sn-3 positions) and sn-2-monoglycerides (MAGs) by pancreatic lipase (Mu & Høy, 2004).

6.2.6 Kinetics of FFA release determined by the pH-stat method

Lipolysis kinetics in the intestinal phase were determined by in situ titration of the digestion mixture as the lipolysis progresses using the pH-stat method. The FFAs released during intestinal lipolysis were continuously neutralized with 0.1M NaOH automatically added by the pH-stat titration device. The double unit allowed the simultaneous performance of two assays avoiding problems of reproducibility. The volume of NaOH required to maintain the pH at 7 overtime was used to calculate the concentration of FFAs released during lipolysis. Considering two FFAs being released during lipolysis, the amount of FFA (% w/w) was calculated according to (Y. Li & McClements, 2010) using Eq. 6.1:
\%
\text{FFA} = 100 \times \left( \frac{V_{\text{NaOH}} \times m_{\text{NaOH}} \times M_{\text{lipid}}}{w_{\text{lipid}} \times 2} \right) \quad \text{Eq. (6.1)}

$V_{\text{NaOH}}$ is the volume of NaOH used to neutralize FFAs produced; $M_{\text{NaOH}}$ is the concentration of NaOH solution (0.1M), $w_{\text{lipid}}$ is the total mass of TAG initially present in the reaction vessel (0.272g in intestinal phase), and $M_{\text{lipid}}$ is the molecular weight of oil (876 g/mol for sunflower oil). The following first-order kinetics model (Ye et al., 2013) was applied to analyze the percentage of FFAs released ($Y_m$) over time ($t$):

$$Y = Y_m \times (1 - e^{-kt}) \quad \text{Eq. (6.2)}$$

Where $Y_m$ is the maximum percentage of FFA released at the end of the reaction (extent of lipolysis), $k$ is the FFA release rate constant (lipolysis rate), and $t$ is the lipid digestion time (s).

Titration blanks will be performed in absence of lipase for each sample to ensure the accurate quantification of newly formed FFAs derived from the test samples. In addition, control experiments will be performed on the digestive fluids alone, without any added substrate sample, to determine whether the hydrolysis (if any) is coming from the auto-digestion of the digestive fluids. In order to account for the lipolysis of lipids contained in beans, baseline control experiments were performed for control samples containing bean materials without the emulsion. Also, digestions of emulsions alone were performed.

6.2.7 FFA analysis and FFA release kinetics determined by Gas chromatography-mass spectrometry (GC-MS)

Duplicate samples (0.5 mL) at the intestinal phase were taken for GC-MS analysis at different times: 0 (no pancreatin), 5, 10, 15, 30, 60 and 120 min, following initiation of the lipolysis (i.e. after addition of pancreatin), and put into an ice bath. FFAs were immediately extracted from digested samples using ethanol and diethyl ether/ heptane (1:1 v/v) and protonated by 2.5M sulphuric acid, according to the protocol reported by (Helbig et al., 2012) and stored at -80 °C before being analyzed by GC-MS. Lauric acid C12:0, used as internal standard, was added to the extracted FFA samples to obtain a final concentration of 0.1mg/mL. GC-MS analysis was performed using a Shimadzu GC2010 coupled with a TQ8030 triple quad mass spectrometer in the Q3 single quad mode (Shimadzu, Kyoto, Japan). Samples containing internal standard (1uL) were injected into a liner with a split ratio of 50:1 by an auto-injector (AOC-20I, Shimadzu, Kyoto, Japan) and analyzed by flame ionization detector. A Carbowax column (60 m × 0.25 mm i.d. × 0.25 μm film thickness, Phenomenex ZB-Wax Plus, Phenomenex, Torrance, CA, USA) was used. Helium was used as carrier gas at the linear velocity of 30 cm/sec. The program started an initial temperature of 175 °C with an increasing rate of 5 °C /min to 250 °C, and then hold at 250 °C for 15 min. The total oven run time was 30 min. The fatty acid composition of sunflower oil was determined according to the AOCS official method (AOCS, 1998) by using GC-MS to analyze the
fatty acid methyl ester (FAME) derived from the transesterification of fats by boron trifluoride (BF$_3$) and methanol.

6.2.8 Characterization of fiber/beans-emulsion mixture

6.2.8.1 Particle size distribution analysis

The particle size and distribution (PDS) of emulsions, either alone or in the presence of bean materials was determined at each stage of digestion by using a laser diffraction instrument (LS 13 320 XR particle size analyzer, Beckman Coulter, Inc., Florida, USA). Prior to measurements, the emulsions were diluted to appropriate droplet concentration using distilled (DI) water to avoid multiple scattering effects. A refractive index of 1.47 (sunflower oil) and of 1.333 for carrier fluid (DI water) were used. Particle size data were reported as distribution profiles and as the surface-weighted mean diameter $d_{3,2}$ (software LS 13 320XR). All the tests were performed in triplicate for each lipolysis sample.

6.2.8.2 Microstructure analysis

The microstructure of the emulsions, with or without bean materials, was visualized at each stage of digestion by confocal microscopy (Zeiss LSM 880 confocal laser scanning microscope) according to the method reported by (Espinal-Ruiz et al., 2014). A small aliquot of the emulsion was transferred to a glass microscope slip and stained with the fat-soluble fluorescent dye, Nile Red (0.1% (w/w) dissolved in 100% (w/w) ethanol), to identify the location of lipid droplets. The stained samples were covered with a glass coverslip, and nail polish was used to fix the coverslip and to prevent evaporation. The excitation argon laser (543 nm) and emitted light between 555-620nm were used to take the confocal images. Regular optical images were taken by turning off the laser and turning on the regular white light. The images were analyzed by the instrument software (EZ CS1 version 3.8, Niko, Melville, NY). Red (confocal) and/or white/black (optical) images allowed to visualize lipid droplets and bean tissue materials, respectively. Mixed color (red + white/black) images using transmitted light (both laser and bright light) displayed both lipid droplets and bean tissue materials together in the images.

6.2.8.3 Apparent viscosity measurements

The apparent viscosity of the emulsions, either alone or in the presence of bean materials, was immediately determined at each phase of digestion by using a DHR3 rheometer (TA instruments, Waters Co., Ltd., Leatherhead, UK) equipped with a parallel plate geometry (40mm diameter and 1mm gap) and a solvent trap to minimize moisture loss. The test temperature was set at 37 °C and controlled by a circulating water system. Samples were allowed to equilibrate and rest for 1 min. Flow curves were obtained at a shear rate varying from 0.1 to 600 1/s. The shear rate of 15s$^{-1}$ was chosen to compare the viscosity of digested samples as this value is close to the small shear rates that occur in the gastrointestinal tract (Naumann et al., 2019).
6.2.9 Data analysis

The obtained data were analyzed using Analysis of Variance (ANOVA). Values are reported as means (n=2) and standard deviation. The Tukey HSD comparison test was conducted to evaluate significant differences among experimental mean values (P < 0.05). All the statistical analyses were conducted by using JMP Pro13 (SAS Institute, Cary, NC, USA) and plotted by using Graphpad Prism8 (GraphPad Software Inc., San Diego, CA, USA).

6.3 Results and discussion

6.3.1. Microstructure and particle size analysis of emulsions in presence of bean fibers

Particle size analysis and transmitted images (obtained by combined confocal and light microscopy) enable us to investigate the effect of bean fibers on the stability and microstructural changes of emulsions together during in vitro digestion. Therefore, combined microscopy images as well as PSD for each emulsion system (with or without bean flour/fibers), at each stage of digestion are shown in Figure 6.2; d$_{3,2}$ values are summarized in Table D1. The control emulsion, (i.e., no bean materials added), initially showed a monomodal distribution (Figure 6.2A1) characterized by a d$_{3,2}$ of 0.62μm (Table D1). The emulsion remained stable in the oral phase and the monomodal distribution and mean particle size were retained (Figure 6.2A2).

The emulsion still maintained a single peak of similar size during the gastric phase (Figure 6.2A3). Despite the acidic environment of the stomach change the electrostatic balance of lipid droplets and cause flocculation, the emulsifier used in our study, Tween 20, is non-ionic and hence, less likely affected by the change in pH and the presence of pepsin in gastric fluids, in comparison to ionic emulsifiers (like protein and methylcellulose) (Espinal-Ruiz et al., 2014). In particular, the high stability of Tween 20-stabilized emulsions at low pH is probably due to steric repulsion between lipid droplets, consequence of the bulky polyethylene head group of Tween 20 (Gasa-Falcon et al., 2019). A similar insignificant change of droplet size after gastric digestion was also reported in other studies in Tween 80-stabilized emulsions (Golding et al., 2011). After intestinal digestion, no lipid droplets were found in the images, indicating a large extent of digestion in the control emulsions. The PSD showed multimodal distribution and the d$_{3,2}$ value was increased to 1.47 μm (Figure 6.2-A4 and Table D1). The peak at the smallest particle size (0.6-1 μm) was at a similar particle size range of the sample at initial, oral and gastric phase, therefore they could be the lipid droplets that were not fully hydrolyzed and remained in the fluids. Many small peaks were shown at the particle size from 1 to 1000 μm (Figure 6.2A4.). Presumably, these small peaks would be mainly BS micelles and/or mixed micelles of BS and lipolysis products that cannot be stained or shown in the images (Chang & McClements, 2016; Espinal-Ruiz et al., 2016; Liu et al., 2019). Also, bile salts, which are negatively charged, could replace the surfactant from the droplet surface and then the cationic sodium (Na$^+$) and calcium (Ca$^{2+}$) may promote droplet flocculation through charge neutralization and bridging effects (Y. Li & McClements, 2010). Finally, the
lipolysis products such as FFAs and MAGs were shown to partition into the oil-water interface instead of dispersing within the aqueous phase, leading to coalescence. (Liu et al., 2019; Pafumi et al., 2002). An increase in the size of Tween 20 stabilized emulsions after intestinal digestion was also observed by (Gasa-Falcon et al., 2019; Koukoura et al., 2019).

Figure 6.2 Particle size distribution and microstructure (transmitted images, a combination of confocal and optical images) of control emulsions and emulsions added with SDF, IDF and FP bean flours as they passed through the stimulated in vitro gastrointestinal tract.

(Lipid droplets (Nile red), lipid droplets depletion flocculation (green arrows), lipid droplets coalescence (blue arrows), and entrapment of lipid droplets by fiber or bean particles (green square))
The incorporation of bean SDF to the emulsion considerably affected the state of lipid droplets. As shown in Figure 6.2B1, the volume % of the first peak, which corresponds to the emulsion droplets, significantly decreased. At the same time, a second peak appeared resulting in an increase of d$_{3,2}$ to 2.25 µm (Table D1). This second peak corresponds to the SDF network (clearly visible in the image) as the PSD of SDF-only system (no emulsion) shows the similar location of this peak (Figure D1). In addition, flocculation and/or coalescence of lipid droplets was considerably significant as observed in Figure 6.2B1. In particular, flocculated and coalesced droplets were mainly located close to the fibers or entrapped inside the polymer network, while the droplets distant from SDF appeared to keep their identity and to be still randomly dispersed in the aqueous phase. The entrapment of lipid droplets by SDF, could decrease the distance between each droplet, leading to the Van Der Waals attractive forces overcome the electrostatic repulsive force between droplets, thus causing depletion flocculation and coalescence (McClements & Weiss, 2005). On the other hand, bean’s SDF mainly includes arabinose rich-pectin (Shiga & Lajolo, 2006), which possesses anions that may alter the electrostatic equilibrium and reduce the electrostatic repulsion between droplets, thus causing flocculation and coalescence (McClements & Weiss, 2005). At the oral and gastric phase, the coalesced lipid droplets disappeared but the flocculated droplets remained (Figure 6.2B2 and B3). Under gastric conditions, the volume % of the first peak (emulsion droplets) decreased and the second peak increased (fiber particles), which is in line with the aggregation of fiber particles and lipid droplets observed (Figure 6.2B3). (Espinal-Ruiz et al., 2014) reported similar phenomena in emulsions added with soluble fibers. These authors proposed the aggregation to be caused by the reduction of negative charges in the acidic environment. After the intestinal phase, most lipid droplets disappeared, and the ones that remained are embedded in a clearly visible SDF network (Figure 6.2B4). These results agree with the PSD showing no first peak and a broader second peak, which results in a significantly increased d$_{3,2}$ (Table D1). Moreover, the larger d$_{3,2}$ value of the SDF-emulsion mixture compared to the corresponding only-SDF sample (Figure D2), further indicates that the second peak, is the result of SDF entrapping not hydrolyzed lipid droplets and/or mixed BS-lipolysis products micelles. Comparing to the disappearance of first peak, the remaining of second peak (i.e., entrapped droplets by SDF) indicated the less accessibility of bile salts or enzymes to the surface of those entrapped lipid droplets, therefore decreasing their lipid digestion.

The addition of IDF to emulsions also had an effect on the state and size of lipid droplets. Similar to the SDF system, a bimodal distribution was observed (Figure 6.2C1). The population of small particles had equivalent size but a lower volume % than both control emulsions and SDF-emulsion mixtures, indicating that this peak corresponds to emulsion droplets. The second population of larger particles, which shift to a larger size (~1000 µm) corresponds to IDF particles as the PSD of IDF-only system shows the similar location (Figure D1). The initial lipid flocculation and a more noticeable entangled network of insoluble fibers and lipid droplets was
observed after the oral phase (Figure 6.2C2). Possibly due to the further dilution effect by digestion fluids, a lower extent of flocculation was observed in the gastric phase (Figure 6.2C3) and seemed to be weaker than the one in the SDF-emulsion mixture. Similar to SDF-emulsion mixtures, gastric fluids led to a decrease in the volume% of emulsion droplets and to an increase of IDF particles. As shown in Figure 6.2C4, after the intestinal phase, coalesced droplets were entrapped by even more notable IDF structures, which is in accordance with the disappearance of smaller lipid droplets that shifting towards larger particles and with a significantly increased particle size (Table D1). Even though both, insoluble and soluble bean fibers, led to depletion-flocculation phenomena and formed structures that entrapped lipid droplets during in vitro digestion, this process and arrangements seem to be different. Specifically, emulsion droplets were clearly distinguishable with no extensive interpenetration occurring in the compact network of insoluble fibers (Figure 6.2-C4) whereas lipid droplets seemed to be interlinked with a less dense gel network of soluble polymers (Figure 6.2B4) that allowed free diffusion of lipids through the solution.

The emulsion added with bean flours of fine particles (FP) initially showed a large number of clearly distinguishable starch granules (Figure 6.2D1), which showed that the rigid bean cell wall protects starch, even when a severe mechanical disintegration of beans is done. The emulsion droplets were largely entrapped by bean particles (mostly starch granules) (Figure 6.2D1). The complete disappearance of the small peak (~1 μm) of FP-emulsion mixtures also indicated a larger entrapment of lipid droplets, compared to the SDF and IDF emulsions. The FP-emulsion mixtures showed peaks only in larger size range, with one around 50 μm and the other around 500 μm (Figure 6.2D1), resulting in an increase of d₃,₂ value (Table D1). This result was analogous to the PSD of FP blank samples (only FP, no emulsions) (Figure D2). However, the d₃,₂ value of bean added emulsion was significantly larger than the d₃,₂ value of bean blank samples (Table D1). It means the peak at larger particle size (500 μm) may correspond to not only the bean particles but also the bean particles with entrapped lipid droplets. The peak at smaller particle size (50 μm) may partially come from the flocculation/entrapment of lipid droplets by SDF in beans, since this particle size range is within the particle size of the second peak of SDF-added emulsions (Figure 6.2-B1). After the oral phase, FP-emulsion mixtures showed fewer bean particles (and starch granules), as well as fewer lipid droplets than initial samples (Figure 6.2D2). On the other hand, the salivary α-amylase hydrolyze a certain amount of starch, therefore, causing the decreased volume% of the peak corresponding to bean particles. This is supported by the corresponding PSD of FP blank samples (Figure D2) which also showed an increase in the volume% of the first peak and a decrease in the second peak after oral digestion. After the gastric phase, much fewer bean particles and lipid droplets showed (Figure 6.2-D3), compared to the oral phase. Comparing to the FP blanks, which showed insignificant change in the PSD in the gastric phase, we may suspect that the increased volume% of the first peak of smaller size is mainly due to the lipid droplets released from the entrapment by bean particles. After the intestinal phase, most of starch granules
and lipid droplets disappeared possibly due to their hydrolysis by pancreatic α-amylase and lipase, respectively (Figure 6.2D4), leaving remnants of the cell wall. Some resistant starch also remained and entrapped the lipid droplets, mainly due to the rigid cell wall of beans and the relative resistant structure of bean starch. Dry beans were known to have a low starch digestibility since it consists of high ratio of amylose (30-40%) and a more ordered crystalline structures of starch (B-type and C-type), compared to cereals starch (A type) (Madhusudhan & Tharanathan, 1995; Rebello et al., 2014).

6.3.2. Microstructure and particle size analysis of emulsions in presence of bean tissue materials of varied microstructural integrity

The confocal microscopy and optical microscopy of bean-emulsions mixtures are shown in Figure 6.3 and Figure 6.4. The corresponding PSD results are shown in Figure 6.5. The confocal microscopy focus on the change of physical stability of the lipid droplets; while the optical microscopy, where the bean starch and cell wall structure can be seen clearly, demonstrates the different structures of these bean flour samples and their different digestibility during each digestive stage. The microscopy and PSD of control emulsions and FP beans-added emulsions were also shown in Figure 6.3, 6.4 & 6.5 as comparisons for other bean-emulsion mixture samples.

As shown in Figure 3C, the addition of bean flours of medium particle size (MP) to emulsions seemed to cause more significant droplet flocculation initially, and at the oral and gastric phases than emulsions added with fine particles flours (FP) (Figure 6.3B), which is in accordance with the higher volume% of larger particles (>1000 μm) (Figure 6.5C) and larger $d_{3,2}$ values (Table D1). After the intestinal phase, the undigested lipid droplets (Figure 6.3C4) in MP-emulsion mixtures seemed to be more than the lipid droplets in FP-emulsion mixtures (Figure 6.3B4) and CP-emulsion mixtures (Figure 6.3D4) . In the optical microscopy, the starch structure of MP-emulsion samples after oral and gastric phase were different from the ones in FP-emulsions. The former one, generally, still showed the polarized structure, indicating native starch structures, while a lot of starch in the latter one had lost the polarized structure, probably due to the hydrolysis by oral and gastric enzymes. This was caused by the higher cell wall or structural integrity in former samples (MP-samples) which were processed by moderate mechanical treatment and could decrease the accessibility of enzymes to bean starch granules. Even after intestinal phase, there were still intact resistant starch - enzymatic inaccessible starch granules shown in MP-emulsion mixtures (Figure 6.4C4). In comparison, in the FP-emulsions, all the starch lost their native structures even though some of them remain undigested (Figure 6.4B4). It seems not only cell wall fiber, but also these undigested starch and more intergraded bean matrix in MP-emulsion mixtures also interfered with lipid droplets as shown in Figure 6.3C2-4 and Figure 6.4C2-4.

In the emulsions added with coarse flours (CP), flocculation can also be observed in the initial, oral and gastric phase (Figure 6.3D1-3). However, different from FP and MP samples, the
PSD of CP-emulsion showed a large volume % of the first peak (at ~1 µm) from initial to gastric phase (Figure 6.5D). This may because more emulsion were sampled than bean particles as the consequence of the non-homogenous system in CP-emulsions samples. The coarse particles were too large to mix well with emulsions. It can also be explained by the optical microscopy which showed less amount of bean or starch particles in these sample (Figure 6.4D1-3) than FP and MP samples. Due to the non-homogenous situation, when sampling the mixture samples by the pipettes, emulsions were more easily to be sampled and retained in the microscopy slip than the coarse particles, so that less bean particles were presented and shown in the microscopy (Figure 6.4D1-3). As a consequence, there were a higher ratio of the flocculated lipid droplets in the microscopy (Figure 6.3D1-3) than FP samples, even though in the whole CP-emulsion mixture system, they may not have more flocculation than FP samples. Similar to MP samples, due to the more integrated bean structure in coarse particles than FP bean samples, the native, polarized structure of bean starch were also observed in these CP samples after oral and gastric phase (Figure 6.4-D2-3). Moreover, after intestinal phase, a lot more undigested starch granules were shown in CP-emulsion mixtures than FP and MP samples, further indicating the highest integrated bean matrix in CP samples reduced the accessibility of enzymes to hydrolyze starch granules. This were in accordance with the unneglectable volume% of large particles in the PSD results (Figure 6.5D). However, less free lipid droplets were remined in CP-emulsions mixtures than FP and MP samples, indicating larger extent of lipolysis in former samples.

HT-treated beans/emulsion mixtures (FP-HT) also induced flocculation and some coalescence of lipid droplets at the initial stage (Figure 6.3E1), and showed a high extent of starch gelatinization (Figure 6.4-E1). The difference between two PSD peaks in FP samples was less significant in FP-HT emulsion-mixtures (Figure 6.5E), which was probably due to the increased solubility and break down of bean particles after HT treatment (Damodaran & Parkin, 2017), which could cause a full starch gelatinization and protein denaturation in beans (Lin & Fernández-Fraguas, under revision). After the oral and gastric phase, less flocculation and entrapment of lipids droplets were observed in HT-FP samples than FP samples (Figure 6.3E2&3) probably, due to the increased hydrolysis of starch (Figure 6.4E1-3) and the loss of viscosity (Figure 6.6C2&C4). CP-emulsion mixtures shifted slightly to smaller particle sizes (Figure 6.5E) after the gastric phase which may be due to an increased digestion of bean starch and protein promoted by HT treatment. After the intestinal stage, almost all the lipid droplets and gelatinized starch disappeared probably because they were fully digested (Figure 6.3-E4 & 6.4-E4). The hydrolysis of starch by pancreatic amylase may release the entrapped lipid droplets and let them exposed to pancreatic lipase. Accordingly, the PSD and d3,2 values of FP-HT samples tend to shift to smaller particle sizes after the intestinal phase (Figure 6.5E & Table D1). Many studies have shown that heating processing could improve the protein and starch digestibility of beans (Edwards et al., 2015; Kaur et al., 2015; Rehman & Shah, 2005). The increased hydrolysis (i.e. digestibility) of starch in HT-beans was
**Figure 6.3** Confocal microscopy images showing the change in microstructure of sunflower oil-in-water emulsions added with structurally different bean flours at three stages of simulated in vitro digestion.

(Lipid droplets (Nile red), lipid droplets depletion flocculation (green arrows), lipid droplets coalescence (blue arrows))
Figure 6.4 Optical microscopy images showing the change in microstructure of sunflower oil-in-water emulsions added with structurally different bean flours at three stages of simulated in vitro digestion.
(entrainment of lipid droplets by fiber or bean particles (green square))
Figure 6.5 Particle size distribution of sunflower oil-in-water emulsions added with microstructurally different bean flours at three stages of simulated *in vitro* digestion.
also shown in one of our previous study which found that HT treatment could significantly decrease RS content in beans (Lin et al., 2020). During HT treatment, starch granules swell with water and the amylose and amylopectin leached out which increased their exposure to hydrolytic enzymes therefore increasing starch digestibility (Pallares et al., 2018). Indigestible DF in HT-FP samples cannot be observed in the optical microscopy after intestinal phase even though they should be the main components remined (since starch and protein were almost hydrolyzed), possibly due to some thermal modification on their solubility.

FP-HHP600/5 samples also showed flocculation and coalescence of lipid droplets initially (Figure 6.3F1). As shown in Figure 6.4F1, despite intact starch granules appear in FP-HHP600/5 samples, their amount is lower compared to FP samples. The polarized structure of starch was also damaged to some extent, indicating some degree of starch gelatinization after HHP600/5 treatment. After the oral phase, coalescence and entrapment of droplets can be seen in the confocal and optical images (Figure 6.3F2 and Figure 6.4F2), and compared to FP-emulsion mixtures, a significant decrease of intact starch granules could be observed (Figure 6.4F2). Also, the structure of starch granules seemed to be ruptured and solubilized to some extent in the digestion fluid. This result was in accordance with our previous study which showed that HHP600/5 could partially gelatinize starch to facilitate starch digestibility and decrease RS content in bean flour (Lin et al., 2020). The solubilization of starch granules resulted in the shifting of PDS to the smaller sizes (~10 µm) after the oral phase (Figure 6.5F) and the decreasing of d3,2 value (Table D1). After the gastric phase, more significant lipid flocculation was shown around those undigested bean components (i.e. bean starch and fiber) (Figure 6.3-F3 and Figure 6.4F3), while the PDS did not show much difference (Figure 6.5F). Possibly, the solubilized starch and partially gelatinized starch by HHP600/5 treatment contribute to the depletion-flocculation of droplets under low pH conditions. After the intestinal phase, some undigested lipids remained (Figure 6.3F4) and were entrapped by undigested bean particles (Figure 6.4F4). This supports the fact that HHP600/5 only partially gelatinize the starch and increase its digestibility. A decrease in the first peak at a smaller size (~10 µm) and an increase in the second peak at a larger size (~100 µm) (Figure 5F) was observed. The decrease of the smaller peak indicates that enzymatic hydrolysis of those solubilized starch is taking place, resulting in a relatively lower peak volume%.

Emulsions added with HHP300/15-treated beans, show a similar microstructure in initial and oral phase with FP samples (Figure 6.3G1 & Figure 6.4G1). Different to FP-HHP600/5-emulsions mixtures, samples treated at HHP300/15 have more intact starch granules, indicating that lower pressurization levels even if applied for more time cause less gelatinization (Lin & Fernández-Fraguas, under revision). After the oral and gastric phase, flocculation was also shown in Figure 6.3G2 and Figure 6.3G3, and more intact starch granules remained (Figure 6.4G2 and 6.4G3) compared to corresponding FP-HHP600/5-emulsions mixtures. After the intestinal phase, only few droplets (Figure 6.3G4) and some undigested starch remained (Figure 6.4G4). The
change of PDS and \(d_{3,2}\) value from initial to intestinal phase were similar to changes in FP and FP-HHP600/15-emulsions mixtures (Figure 6.5G & Table D1).

### 6.3.3. Viscosity of emulsion-fiber/bean flour mixtures during in vitro digestion

The flow curves and the apparent viscosity at 15 s\(^{-1}\) of fiber/bean-added emulsions at each digestion stage are shown in Figure 6.6. Regardless of digestion stage, the viscosity of the control emulsions decreased with the increasing of shear rate from 0.1-5 s\(^{-1}\), presenting a shear-thinning behavior, but then it did not change after 5s\(^{-1}\), showing a Newtonian behavior (Figure 6.6A1). The Newtonian character of oil-in-water emulsions has been reported previously (Karthik et al., 2018). All emulsions added with bean tissue materials (isolated fibers or flours) showed shear-thinning properties (Figure 6.6A2,3&B-C) within the tested shear rate range. Generally, their apparent viscosity progressively decreased as digestion progressed, partially due to the dilution effect of digestive fluids (Figure 6.6A4, B4&C4), which is supported by the decrease of viscosity observed in blank fiber and bean samples during digestion (Figure D3). Other studies have also reported a decrease of viscosity during digestion of emulsions added with pure fibers from diverse sources (Espinal-Ruiz et al., 2014). Comparing emulsions added with different fibers, IDF-emulsion mixtures showed always a higher viscosity than SDF-emulsion mixtures (Figure 6.6A2,3&4), which could be due to the contribution of RS present in IDF fractions (Figure 6.2C1) and the higher amount ratio of IDF than SDF in bean samples.

Similar to fiber added emulsions, due to the dilution effect, FP-emulsions mixtures also showed decreased viscosity at each digestive stage (Figure6.6B1). Moreover, the digestion of bean components during the oral, gastric and intestinal phases also could decrease the viscosity (Kim & White, 2012). Other than in-/soluble fibers, bean starch and protein could also contribute to the viscosity of the initial FP samples, thus showing a higher viscosity than IDF and SDF samples (Figure 6.6A4). The limited hydrolysis of starch by \(\alpha\)-amylase in the oral phase and the digestion of protein in the gastric phase slightly decreased the viscosity compared to the initial stage (Figure 6.6B2 & B4). The viscosity of emulsions with bean flours was significantly decreased after the intestinal phase, where much more starch and protein were hydrolyzed by pancreatic enzymes (Figure 6.6-B2 & B4). The slow release of starch and protein components from bean matrix may attenuate the dilution effect on decreasing viscosity. Therefore the decrease of viscosity in bean-emulsions samples was less than fiber-emulsions samples.

Generally, emulsions added with MP and CP flours showed decreased viscosity from initial to intestinal phase (Figure 6.6B2&B3). The viscosity at each stage was higher in the emulsions added with more coarse/larger bean particles (i.e., CP > MP > FP) (Figure 6.6B4). It means larger particles made the samples more viscous, harder to move, and harder to mix with digestion fluids. It has reported that larger particles make up the biggest volume ratio of the fiber or fiber-rich food suspensions and therefore dominate the formation of the network (Tornberg, 2017). Studies have shown that some food suspensions (e.g. apple, tomato, and potato pulp) with larger particles
showed a stronger network and a larger elastic modulu (Bengtsson, 2009). Higher viscosity is usually considered to reduce the diffusion of substance in a fluid, therefore the larger bean particles should reduce the diffusion of nutrients and enzymes during the digestion (Takahashi & Sakata, 2002). After the intestinal stage, the decrease of viscosity in MP and CP-emulsion mixtures was less significant than that observed in FP-emulsion mixtures (Figure 6.6B4), which is likely due to a reduced digestion of bean components (i.e. starch and protein) in coarse particles.

Figure 6.6 Flow curves and viscosity at shear rate of 15 s⁻¹ of emulsions added with bean tissue materials at different stages of simulated in vitro digestion.
FP-HT-emulsion mixtures initially showed a similar viscosity that FP-emulsion mixtures (Figure 6.6C4). However, due to the hydrolysis of starch and protein by α-amylase and gastric pepsin, the viscosity of former samples decreased more significantly in the oral and gastric phases than FP-emulsion samples (Figure 6.6C1&C4). Consequently, after the intestinal phase, the viscosity of FP-HT-emulsion mixtures was significantly lower than FP-emulsion mixtures. The heating process may also affect the starch-fiber networks thus further affect the viscosity during digestion.

FP-HHP600/5-emulsion mixtures also showed a higher viscosity than FP-emulsion mixtures at the initial stage, possibly due to the HHP-induced partial starch gelatinization in beans. The viscosity of FP-HHP600/5-emulsion mixtures was decreased more significantly (Figure 6.5C2 & C4) than FP-emulsion mixtures after oral and gastric digestion because of the increased hydrolysis of starch and protein in the former samples as shown in microscopy (Figure 6.4F2&3). Compared to FP-HT-emulsion mixtures, the starch in FP-HHP600/5 samples was only partially gelatinized and was not completely hydrolyzed after the intestinal stage, as showed in the microstructure (Figure 6.4F4), consequently its viscosity after the intestinal stage was a little bit higher than FP-HT-emulsion mixtures (Figure 6.5C4). As for FP-HHP300/15-emulsion mixtures, the significant decrease of viscosity along with the digestion in HHP600/15 samples was not observed in these samples (Figure 6.4C3). It can be explained by the less loss of starch granules in FP-HHP300/15 samples, as shown in Figure 6.4G1-4. Generally, the viscosity in FP-HHP300/15-emulsion mixtures was only slightly lower than the viscosity of FP-emulsion mixtures (Figure 6.5C4).

6.3.4. Effect of bean tissue materials on the digestion of extrinsic lipids

6.3.4.1 Total free fatty acid release measured by pH-stat method and GC-MS.

The % FFA released during 2 h of intestinal digestion measured by the pH-stat method and by GC-MS is presented in Figure 6.7. The extent and rate of lipolysis was obtained by fitting the data to (Eq.6.2) and is shown in Table 6.1. Generally, the initial release of FFA in all samples was rapid in the first few minutes which was followed by a gradual increase at a longer time until reaching a plateau at 120min.

Different bean tissue materials significantly affected the rate and amount of FFA released from emulsions (Figure 6.7A and Table 6.1). The extent of lipolysis observed in control emulsions, as predicted, was the largest compared to emulsions added with bean fiber or flours. The addition of both, SDF and IDF, to emulsions decreased the % FFA released to a similar extent with IDF showing a non-significant lower release rate (k). Despite IDF-emulsions mixtures showed an increased viscosity as well as a larger d3,2 than SDF mixed emulsions, their effect on lipid digestion were similar. It means viscosity was not the only reason for reducing lipid digestion. A large increase in the macro-viscosity of IDF samples may have little effect on its micro-viscosity since
Figure 6.7. Percentage of free fatty acid released under stimulated in vitro small intestinal digestion for sunflower oil-in-water emulsions added with isolated bean fibers or different bean flours.

small molecules can easily diffuse through the large pores of the polymer network (Espinal-Ruiz et al., 2014). The behavior of lipid droplets within a polymer solution has not shown a linear correlation with the concentration, therefore, lipid digestion do not only rely on the viscosity (Grundy et al., 2017, 2018). These same authors found that a solution of guar gum of low viscosity also caused flocculation or aggregation of lipid droplets and the delay of lipid digestion (Grundy et al., 2017). The reduced lipolysis observed in SDF or IDF-emulsion mixtures can be explained by the entrapment of lipids by the fiber network and by flocculation and coalescence phenomena.
taking place in the presence of DF, as shown in Figure 6.2B&C. Specifically, we propose three possible reasons: First, the entrapment of lipid droplets by DF could decrease the accessibility of bile salts and/or pancreatic lipase to the lipid surface (oil-water interface), where lipid digestion takes place (Espinal-Ruiz et al., 2014). Second, the flocculation and coalescence of droplets would decrease the surface area of lipid available for lipolysis (Grundy et al., 2017). Finally, the increased viscosity generated by DF (Figure 6.6-A4) may slow down the movement of digestive molecules involved in lipid digestion (e.g., bile salts, co-lipase and lipase) towards the droplet surface, consequently, hindering lipolysis (Espinal-Ruiz et al., 2014).

Bean flours (FP) significantly decrease by more than half the amount of FFA released from emulsions (Figure 6.7A and Table 6.1). Since the amounts of FFA released from lipids intrinsically present in bean flour itself (which was small and neglectable) (Figure D4) has been taken into consideration when calculating the % FFA released from the lipolysis of bean-emulsion mixtures, the % FFA reported is exclusively coming from the digestion of the emulsion. The amount of isolated SDF (0.7%) and IDF (2.5%) used in the digestion corresponds to their content in bean flours (10%). FP showed a greater reduction of lipolysis than SDF or IDF, indicating the important role that the bean matrix plays on lipolysis. Similarly, (Grundy et al., 2017) reported that oat flour caused a larger reduction of FFA released from extrinsic lipids than β-glucan, oat main soluble fiber. The possible mechanisms that these authors proposed on oats could be considered in the case of beans. First, the specific interaction of the cell wall with the digestive substrates in the aqueous phase may play a role in reducing the lipolysis rate. For instance, SDF may leach from the bean matrix and surround the bean particles, producing a higher concentration of semi-hydrated polymer, which may interact with digestive enzymes thus inhibiting the lipolysis rate (Grundy et al., 2017). Second, the overall structure of the bean matrix and bean components other than SDF and IDF, present in beans, such as undigested starch may interfere with lipolysis. Figure 6.2D showed that in the FP-emulsion mixture, some undigested lipid droplets were flocculated or surrounded by undigested starch. One of our previous studies found that bean flours showed a higher BS retention than isolated bean fibers, which may further reduce more amount of free BS participating in lipid digestion therefore inducing more reduction on the lipolysis. Finally, the higher viscosity observed in FP-emulsion mixtures (Figure 6.6A4) that contribute by bean matrix may also decrease the lipolysis rate.

MP-emulsion mixtures showed a lower lipolysis rate and extent than FP-emulsion mixtures, while the opposite effect was observed for CP-emulsions (Figure 6.7B and Table 6.1). Despite similar flocculation phenomena and high viscosity were shown in both MP and CP-mixed emulsions, compared to FP (Figure 6.3.C&D & Figure 6.6B4). As deduced from Figure 6.5 D, bean particles from CP flours do not seem to form an homogeneous system when mixed with the emulsion, which may have restricted their ability to interfere with lipolysis. Currently, no studies have reported the effect of different particle sizes of bean flour on the lipolysis rate. Grundy et al.
(2017) reported that the digestion of extrinsic lipids was affected by both, oat flour (small particles) and oat flakes (large particles) but no significant differences were found between the two types of samples. Another study reported that wheat bran with a large particle size (900 μm) showed an increased bile salt (BS)-binding ability than wheat bran with a smaller particle size (200 μm), by increasing water holding capacity, micropores and integrity of the structure. It is possible that in our study, the MP beans with a mean particle size around 900 μm also showed a higher ability to sequester BS and further interfere more significantly with lipolysis, compared to FP beans (200 μm). Further studies testing bean particles of intermediate size (between FP and MP) could help to better explain the impact of bean particle size on lipolysis.

Addition of processed beans to emulsions hindered the digestion of emulsion lipids. (Figure 6.7C) FP-HT beans decreased the lipolysis rate and extent of emulsions; however, the reduction in lipolysis was lower than the caused by FP raw beans in accordance with the microstructural analysis which showed that more lipid droplets remained in the intestinal phase in FP-HT-emulsion mixtures than in the FP-emulsion mixtures (Figure 6.3E4). The lower reduction of lipolysis rate in FP-HT samples may be related to less flocculation and to lower viscosity of these samples (Figure 6.3E and Figure 6.6C1). To be noted, its lipolysis extent was very close to the profile of SDF emulsions (Figure 6.7A), analogous to their similar PDS (Figure 6.2B4 & Figure 6.5E). This suggests that in FP-HT beans, the main component that contributes to the reduction of lipolysis is the SDF fraction, since the bean matrix were damaged due to thermal processing and bean components (e.g. starch and protein) were almost digested. On the other hand, thermal processing may also cause the loss of lipase activity during bean digestion since pancreatic lipase inhibitor are present in raw beans (Ngoh et al., 2017; Padhi & Ramdath, 2017). Cara et al. (1992) studied the effect of various thermal processes (e.g. steaming, autoclaving or popping) of whole-grain or wheat flour on lipase activity, and found that thermal treated flours preserve less than 10% of the inhibitory activity present in the raw flour. Similar to our results, (Abdul-Hamid & Fennema, 1995) have reported a lower reduction of lipolysis in heat-treated wheat bran compared to non-heated, and they pointed that heating may inactivate the lipolysis inhibitor.

Contrary to thermal processing, HHP processing at 600/5min increased the ability of beans to hinder lipid digestion (Figure 6.7C). FP-HHP600/5-emulsion mixtures showed a lower lipolysis rate and extent than FP-emulsions. However, the FP-HHP300/15 samples seemed not to significantly impact the ability of beans to reduce the lipolysis, as compared to FP samples. Despite there is no significant difference in the viscosity between FP and FP-FFP emulsion mixtures (Figure 6.6C4), the FP-HHP600/5-emulsion mixtures tended to show more droplet flocculation (Figure 6.3F) which may contribute to less surface area of lipid droplets available for lipolysis. In our previous studies we found that HHP600/5 treatment induced a more compact starch-protein/fiber complex in bean slurries (Lin et al., 2020). In current study, the same HHP conditions may also manipulate the structures and the starch-protein/fiber complex in whole beans which
probably helped to increase lipid entrapment and promoted more flocculation of lipid droplets (Grundy et al., 2017). Other factors like the ability to sequester more bile salts (Lin et al., 2020) and the increased release of bioactive peptides (i.e., lipase inhibitor) may also contribute to the reduction of lipolysis in FP-HHP600/5-emulsion mixtures (Garcia-Mora et al., 2015; Girgih et al., 2015).

The FFA released during intestinal digestion measured by GC-MS is presented in Figure 6.7. Generally, FFA release followed a similar trend to the profile obtained by the pH-stat method. However, the %FFA released as measured by GC-MS were generally higher than the values obtained with the pH-stat method (Figure 6.7). Consequently, the fitted results on the lipolysis rate and extent were also higher for the GC-MS method (Table 6.1). To be noted, the model fit of GC data provided better regression (R²) values than the pH-stat method (Table 6.1). The lower FFA release values by the pH-stat method may be related to the titration conditions such as pH, pKa of FFA, ionic strength, BS and calcium concentration (Helbig et al., 2012). The pH for the titration must be higher than or equal to the apparent pKa value of FFA, which in turn is highly influenced by the ionic strength of digestion fluids. It was reported that the pKa of oleic acid (the main FFA in the sunflower oil used in current study) is 9 in pure water but shifted to 7.8 in rat pancreatic juice with 1M NaCl (Mattson & Volpenhein, 1966). A further shift to pKa 6.4 was caused when the environment consisted of 0.1M NaCl and 0.5mM Ca²⁺ (Benzonana & Desnuelle, 1968). The titration conditions used in the current study may not fully titrate the released FFA from samples. It is said that pH stat method might underestimate (without back titration, current study) or overestimate (with back titration) the lipid digestion (Heider et al., 2016). The different results obtained between the pH-stat and GC-MS method may also depend on the emulsifier used and the specific methodologies. Helbig et al. (2012) using a similar pH-stat method and FFA extraction method, found that GC-MS analysis gave FFA release values 2-3 times higher than the values obtained by the pH-stat-method in whey protein-stabilized emulsions, whereas these values were 10% higher in guar gum stabilized emulsions. Another study from Grundy et al. (2015) using solid-phase extraction to extract FFA samples showed lower values with the GC method than with the pH-stat method. The commonly used pH-stat method although provides a fast means to obtain a general result on lipid digestion, its results are strongly influenced by the local environment, the pKa of fatty acids and the emulsifier type used at the oil-water interface (Heider et al., 2016; Helbig et al., 2012). Therefore, a combination of this method with GC-MS is encouraged for more accurate FFA determination.
Table 6.1 Parameters describing the lipolysis rate and extent of digestion of sunflower oil-in-water emulsions added with isolated bean fiber and different bean flours measured by pH-stat and GC-MS method

<table>
<thead>
<tr>
<th>Samples</th>
<th>pH-stat method</th>
<th>GC-MS method</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$Y_m$ (%)</td>
<td>$k$ (× 10^{-3} s^{-1})</td>
</tr>
<tr>
<td>Control Emulsion</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fiber fractions</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SDF</td>
<td>33.30±0.09$^{bc}$</td>
<td>3.58±1.15$^{ab}$</td>
</tr>
<tr>
<td>IDF</td>
<td>34.82±0.50$^{bc}$</td>
<td>2.15±0.11$^{ab}$</td>
</tr>
<tr>
<td>Raw bean flours</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FP</td>
<td>20.66±2.22$^{d}$</td>
<td>2.58±1.03$^{ab}$</td>
</tr>
<tr>
<td>MP</td>
<td>17.47±1.96$^{d}$</td>
<td>5.09±0.03$^{ab}$</td>
</tr>
<tr>
<td>CP</td>
<td>39.16±1.07$^{bc}$</td>
<td>4.63±0.57$^{ab}$</td>
</tr>
<tr>
<td>Processed bean flours</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FP-HT</td>
<td>32.74±4.32$^{bc}$</td>
<td>5.96±2.41$^{a}$</td>
</tr>
<tr>
<td>FP-HHP600/5</td>
<td>18.77±2.90$^{d}$</td>
<td>1.84±0.59$^{b}$</td>
</tr>
<tr>
<td>FP-HHP300/15</td>
<td>30.73±0.45$^{c}$</td>
<td>1.27±0.07$^{a}$</td>
</tr>
</tbody>
</table>

$^a$: Parameters fitted to Eq.6.2: $Y = Y_m \times (1 - e^{-kt})$, where $Y_m$ is the maximum percentage of the total FFA present that is released at the end of the reaction (extent of lipolysis), $k$ is the FFA release rate constant. $t$ is the lipid digestion time (s).

Data are expressed as the mean values (n=3) ± standard error (SE). Comparisons for all pairs were made using Tukey-Kramer HSD; Along the column, mean values with different letters are significantly different (P < 0.05).
6.3.4.2 Profile of individual FFA released measured by GC-MS

The % of major individual FFA in total FFA released from emulsions or fiber/bean mixed emulsions under simulated in vitro intestinal digestion obtained by GC-MS are shown in Table 6. The major FA present in sunflower oil is also listed for comparison. The FA profile of sunflower oil is also shown in Figure 5. In sunflower oil, the main FFA are palmitic acid (C16:0), octadecanoic acid (C18:0), oleic acid (C18:1 n-9) and linoleic acid (C18:2 n-6). The sunflower oil used in our study is a medium oleic acid oil, or called as NuSun oil. Their percentage in an increasing order is C18:1 n-9 (~60%) > C18:2 n-6 (~30%) > C16:0 (5.3%) > C18:0 (4.7%). This is a new type of sunflower oil in the market, which is developed by standard hybrid procedures to have an oleic acid ratio at 55-75%. This type of oil is shown to works well in commercial frying applications and had fewer oxidation issues, compared to traditional linoleic sunflower oil (~69% of linoleic acid/polyunsaturated fatty acids) (National Sunflower Association, n.d.).

Table 6.2 Individual free fatty acids release percentage of total free fatty acids released (GC-MS method) under stimulated in vitro small intestinal digestion for sunflower oil-in-water emulsions added with isolated bean fibers or different bean flours, compared to the fatty acids percentage in original sunflower oil.

<table>
<thead>
<tr>
<th>Oil and Samples</th>
<th>C16:0</th>
<th>C18:0</th>
<th>C18:1 n-9</th>
<th>C18:2 n-6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sunflower oil</td>
<td>5.27±0.27&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.74±0.03&lt;sup&gt;a&lt;/sup&gt;</td>
<td>59.01±0.41&lt;sup&gt;c&lt;/sup&gt;</td>
<td>30.99±0.17&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Control Emulsion</td>
<td>6.81±0.03&lt;sup&gt;a&lt;/sup&gt;b</td>
<td>2.80±0.63&lt;sup&gt;a&lt;/sup&gt;</td>
<td>67.53±0.23&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>22.87±0.42&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Fiber fractions</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SDF</td>
<td>7.99±0.94&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.39±0.32&lt;sup&gt;a&lt;/sup&gt;</td>
<td>69.77±1.92&lt;sup&gt;a&lt;/sup&gt;</td>
<td>18.86±3.18&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>IDF</td>
<td>7.75±0.99&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>2.83±0.76&lt;sup&gt;a&lt;/sup&gt;</td>
<td>69.78±1.07&lt;sup&gt;a&lt;/sup&gt;</td>
<td>19.65±0.67&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Raw bean flours</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FP</td>
<td>7.63±0.39&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>2.47±0.32&lt;sup&gt;a&lt;/sup&gt;</td>
<td>69.45±0.92&lt;sup&gt;a&lt;/sup&gt;</td>
<td>20.46±0.85&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>MP</td>
<td>7.73±0.35&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>3.15±0.62&lt;sup&gt;a&lt;/sup&gt;</td>
<td>68.51±1.79&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>20.62±2.74&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>CP</td>
<td>7.69±0.36&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>3.95±0.13&lt;sup&gt;a&lt;/sup&gt;</td>
<td>63.85±0.43&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>24.53±0.19&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Processed bean flours</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FP-HT</td>
<td>7.45±0.95&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>2.96±1.92&lt;sup&gt;a&lt;/sup&gt;</td>
<td>66.94±2.58&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>22.66±0.29&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>FP-HHP600/5</td>
<td>6.94±0.82&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>3.88±0.71&lt;sup&gt;a&lt;/sup&gt;</td>
<td>66.46±1.64&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>22.72±0.10&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>FP-HHP300/15</td>
<td>7.05±0.74&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>3.41±0.04&lt;sup&gt;a&lt;/sup&gt;</td>
<td>67.78±0.87&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>21.77±1.57&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

C16:0, Palmitic acid; C18:0, Octadecanoic acid; C18:1 n-9, oleic acid; C18:2 n-6, Linoleic acid. Data are expressed as the mean values (n=3) ± standard error (SE). Comparisons for all pairs were made using Tukey-Kramer HSD; along the column, mean values with different letters are significantly different (P < 0.05).

The ratio of FFA released from sunflower oil-in-water emulsions (control) was changed to some extent as compared to the FFA profile of original sunflower oil (Table 6.2). Specifically, C16:0 and C18:1 were released to a higher extent than C18:0 and C18:2, resulting in an increased percentage of C16:0 and C18:1 to 6.8% and 67%, and a decreased percentage of C18:0 and C18:2 to 2.8% and 22.8% in the final digested samples. The higher release of C16:0 and C18:1 could be related to the hydrolysis preference or specificity of pancreatic lipase to these two FA. First, the pancreatic lipase activity may depend on the number and positions of the FA unsaturation (Mukherjee et al., 1993). For instance, it is reported that pancreatic lipase is less active on long-chain polyunsaturated FA, possible due to the short distance between the first double bond and the ester linkage (Giang et al., 2016; Yang et al., 1990). Therefore, FA with shorter chains, C16:0, is
easier to be hydrolyzed than C18:0, while polyunsaturated FA, C18:2, is more resistant than C18:1 to be hydrolyzed by the enzyme. Second, pancreatic lipase preferentially hydrolyzes FAs in the sn-1 and sn-3 positions resulting in free FA and sn-2 monoacylglycerols (MAG). The stereospecific position of fatty acid in TAG from the NuSun sunflower oil has not been found, however some studies have analyzed the FA position from regular sunflower oils rich in linoleic acid. Their study showed that C16:0 is exclusively in sn-1,3 position and most of C18:1 is in sn-1,3 position (Yoshida et al., 2001). The hydride procedure in NuSun oil may further transform some C18:2 FA at sn-1,3 positions into C18:1 FA. All these factors above would make C16:0 and 18:1 easier to be hydrolyzed, resulting in higher release percentage. Regarding the FFA release profile of fiber/bean-emulsions, it seems that fiber and beans did not change the profile significantly compared to control emulsions (Table 6.2), even though they could change the FFA release rate and extent. This could be due to the fact that the FFA hydrolysis preference is mainly related to the specificity of lipase and the type of oil used in the study.

6.4 Conclusions
This study has evaluated the effect of isolated bean fibers and bean tissue materials with different structural integrity on the digestion of extrinsic lipids. Both, isolated bean fibers and bean flours, modulated lipolysis to a different extent, but had little effect on the profile of FFA released from the digestion of sunflower oil emulsions. Bean cell wall fiber reduced the lipolysis rate and extent by increasing the viscosity of digesta, entrapping lipid droplets in their polymer network, and causing depletion-flocculation and/or coalescence of lipid droplets. The higher reduction that bean flours had on lipolysis compared to isolated fiber fractions indicates that not only dietary fiber, but other components in beans and/or the complex networks formed between fiber polymers and other components within bean matrix, also could interfere with the lipolysis process. Moreover, maintaining the integrity of bean structures by means of less severe mechanical treatment or non-thermal processing, HHP, is important to reduce the lipolysis rate and extent. On the contrary, HT treatment, which dramatically damage the integrity of bean matrix, had a negative effect on beans to reduce lipolysis rate. Overall, these findings could provide more fundamental knowledges for the food industry to produce dry beans products or ingredients while preserving their lipid-lowering effect, to fulfill the U.S. dietary guidelines “My Plate” and improve the overall health.

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Conflicts of interest
All the authors declare no conflict of interest.
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Chapter 7: Conclusions and Future Work

The overarching goal of this work was to understand how hydrostatic pressure (HHP) (as compared with hydrothermal (HT)) processing, beans components, and bean matrix influence the abilities of dry beans to retain bile salts (BS) and modulate lipid digestion in vitro, which are closely related to the cholesterol and lipid-lowering effects of dry beans. It is the first work that investigated the effect of non-thermal processing (i.e., HHP) technology on the ability of dry beans to retain BS and modulate lipid digestion. Also, non-works have been done before to have a comprehensive study on the BS-binding ability of bean fractions, including soluble and insoluble dietary fiber, starch, and protein. Our study working on the effect of the integrity of bean structures (i.e. different bean particle size) on reducing lipid digestion is also novel. To achieve the overall goal of this work, a variety of techniques (e.g., HPLC and GC-MS) and characterization methods (e.g., microscopy, rheology, DSC, and particle size analyzer) were applied with the standardized in vitro experiment.

Previous studies have shown that HHP processing could modify the composition, microstructure and physicochemical properties of plant foods such as fruits and vegetables which further impact the nutritional quality and biological functionality of foods. Therefore, our first study was designed to identify the effect of HHP on the thermo-rheological and functional properties of dry bean flours, compared with HT treatment. We mechanically processed whole beans into flours and then treated them by HT or HHP treatment at various conditions. We systematically characterized the rheological, thermal, pasting and resulting functional properties of these differently processed bean flours. The results from Chapter 3 showed that severe HT treatment caused complete starch gelatinization and protein denaturation of beans which could markedly decrease the protein solubility and emulsifying activity of bean flours while increasing the water absorption capacity and cold-paste viscosity. HHP induced partial or no gelatinization of starch in beans. The HHP treatment contributed to the increase of viscoelastic properties of bean flours at a different extent. In addition, less protein denaturation was shown in HHP-treated bean flours, resulting in superior protein solubility and emulsifying activity/stability. This study provides good proof that HHP processing is a promising non-thermal technology to produce minimally processed bean flours with improved functionality, which could be applied in a range of food formulations for the food industry.

In order to investigate how physico-chemical changes occurred on beans during processing affect the physiological properties of dry beans, in the second study (Chapter 4 and 5), we investigated the role of bean components and the effect of HHP on the in vitro BS-binding ability of dry beans. Specifically, by examining the content of soluble and insoluble DF, the microstructure, and the digest viscosity of dry beans treated by HT and HHP treatment, we evaluated the impact of these modifications on the capacity of beans to retain BS in vitro. HT treatment was shown to disrupt the bean cell wall integrity, protein matrices, and starch granules more severely than HHP-treated bean flours, resulting in superior protein solubility and emulsifying activity/stability. This study provides good proof that HHP processing is a promising non-thermal technology to produce minimally processed bean flours with improved functionality, which could be applied in a range of food formulations for the food industry.

We further investigated the retention ability of beans to all the four primary BS in humans with different conjugation group and hydroxylation degree by using dialysis model and HPLC analysis. Dry beans that treated with an initial mechanical treatment before being subjected to HT or HHP
(Chapter 4) and that treated by preceding HT or HHP processing followed by a mechanical treatment (Chapter 5) showed different impacts on the digest viscosity and BS retention ability of dry beans, indicating the important role of processing sequences on affecting the bean matrix. To evaluate the contribution of bean fractions to the retention of primary BS, we isolated different bean fractions (SDF, IDF, starch, and protein). It shows that in addition to SDF, the contribution of other bean components (e.g., protein) to retain BS was also significant and cannot be ignored.

The structure of the primary BS could also affect the BS binding ability of beans, which showed higher retention of hydrophobic di-hydroxy-BS than tri-hydroxy-BS. It also revealed that SDF could retain BS by both viscous entrapments while protein and starch may retain BS by other molecular interactions. Future efforts could be directed towards exploring the structural modification of cell wall polysaccharides during bean processing to provide deeper insight into the mechanisms of BS-binding of beans. Also, it would be worth investigating how processing sequences impact the integrity of bean structures and the release of bean components during in vitro digestion which further interact with BS.

The main findings from Chapter 4 and Chapter 5 proved that HHP processing and the change of bean matrix could modify the BS-binding ability of beans. Since BS are key components in lipid digestion, the final study (Chapter 6) was aimed at exploring whether and how bean fiber, bean matrix and HHP processing influence the ability of dry beans to modulate the lipid digestion in vitro. It was observed that both SDF and IDF were shown to reduce lipolysis by increasing viscosity, entrapping lipid droplets and causing flocculation of lipid droplets. Compared to isolated bean fibers, bean flour matrix showed a higher reduction on the lipolysis. The integrity of bean structures through less severe mechanical treatment and HHP treatment appeared to be important for dry beans to maintain or even improve their ability to reduce lipid digestion. HT treatment, which decreased the integrity of bean structure and induced starch gelatinization and protein denaturation most largely, decreased the ability of dry beans in reducing lipid digestion. To gain a deeper understanding on the effect of integrity of bean structure on reducing lipolysis, future work should be performed to determine how different bean particle size could affect the viscosity and components (starch, protein, and lipid) digestibility of bean-emulsions mixtures.

Overall, this study has increased our understanding of how bean components (i.e., fiber, protein, and starch), bean matrices, and bean processing (i.e., HHP) influence dry beans to retain bile salts and modulating lipid digestion in vitro. Our work demonstrated that dry beans, with high content of dietary fiber and resistant starch, have significant health benefits related to lowering cholesterol and lipid levels. Increasing the consumption of dry beans would definitely help to improve the overall health. HHP, as an non-thermal processing technology, showed the potential to produce minimally processed bean products with enhanced health benefits and diverse application properties. Our findings shed the light on the significance of food structure (not only nutrient content) on impacting health benefits of foods, especially the foods like dry beans having complex matrix. Our studies also revealed that not only SDF but also IDF pay a role in retaining BS as well as the significant contribution of bean protein in retaining BS possibly through molecular interactions rather than viscosity. As a result, we were able to better understand the mechanisms for the cholesterol and lipid-lowering effect of dry beans and provide further knowledge of rational design of food matrix structures to maximize the health efficacy of dry beans. These findings could be extended through continuing research into the influence of beans on the gut from different aspects, such as the secondary BS profile, the short-chain fatty acid profile, and the gut microbiota, which are also closely related to the cholesterol and lipid metabolism regulation. In vivo study could also be performed to testify the effect of different bean matrix on BS excretion.
and blood lipid levels. Even though many questions remain regarding the cholesterol and lipid-lowering effects of beans, these studies highlight the significance of advanced understanding of HHP processing and food structures on modifying the digestibility and functionality of fiber-rich foods or complex food matrix during the gastrointestinal tract.
Appendix A: Supplementary Information for Chapter 3

Table A1
Linear regression ($R^2$) of rheological models corresponding to untreated, hydrothermal (HT) and high hydrostatic pressure (HHP)-treated bean flour dispersions.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Herchel-Bulkley$^a$</th>
<th>Newtonian$^b$</th>
<th>Bingham$^c$</th>
<th>Power law$^d$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raw</td>
<td>0.999</td>
<td>0.784</td>
<td>0.838</td>
<td>0.994</td>
</tr>
<tr>
<td>HT15</td>
<td>0.990</td>
<td>0.726</td>
<td>0.826</td>
<td>0.926</td>
</tr>
<tr>
<td>HT120</td>
<td>0.999</td>
<td>0.686</td>
<td>0.717</td>
<td>0.899</td>
</tr>
<tr>
<td>HHP150/5</td>
<td>0.998</td>
<td>0.797</td>
<td>0.922</td>
<td>0.967</td>
</tr>
<tr>
<td>HHP300/5</td>
<td>0.998</td>
<td>0.802</td>
<td>0.912</td>
<td>0.981</td>
</tr>
<tr>
<td>HHP450/5</td>
<td>0.996</td>
<td>0.705</td>
<td>0.884</td>
<td>0.987</td>
</tr>
<tr>
<td>HHP600/5</td>
<td>0.998</td>
<td>0.584</td>
<td>0.854</td>
<td>0.991</td>
</tr>
<tr>
<td>HHP150/10</td>
<td>0.998</td>
<td>0.509</td>
<td>0.877</td>
<td>0.982</td>
</tr>
<tr>
<td>HHP300/10</td>
<td>0.986</td>
<td>0.729</td>
<td>0.897</td>
<td>0.973</td>
</tr>
<tr>
<td>HHP450/10</td>
<td>0.994</td>
<td>0.754</td>
<td>0.896</td>
<td>0.974</td>
</tr>
<tr>
<td>HHP150/15</td>
<td>0.999</td>
<td>0.454</td>
<td>0.871</td>
<td>0.981</td>
</tr>
<tr>
<td>HHP300/15</td>
<td>0.997</td>
<td>0.685</td>
<td>0.872</td>
<td>0.981</td>
</tr>
<tr>
<td>HHP450/15</td>
<td>0.997</td>
<td>0.752</td>
<td>0.897</td>
<td>0.982</td>
</tr>
</tbody>
</table>

$^a$Herchel-Bulkley: $\sigma = \sigma_0 + K\dot{\gamma}^n$ (Eq. 3.3)  
$^b$Newtonian: $\sigma = K\dot{\gamma}$ (Eq. 3.4)  
$^c$Bingham: $\sigma = \sigma_0 + K\dot{\gamma}$ (Eq. 3.5)  
$^d$Power law: $\sigma = K\dot{\gamma}^n$ (Eq. 3.6)  
$\sigma$: the shear stress (Pa); $\sigma_0$: Yield stress (Pa); $K$: Consistency coefficient(Pa s$^n$); $n$: Flow behavior index; $\dot{\gamma}$: shear rate (s$^{-1}$).
Table A2
Effect of hydrothermal and hydrostatic pressure treatments on the pasting parameters of bean flours

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Initial viscosity (Pa)</th>
<th>Peak viscosity (Pa)</th>
<th>Hot paste viscosity (Pa)</th>
<th>Cold paste viscosity (Pa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raw</td>
<td>530±15d</td>
<td>9970±299c</td>
<td>1869±56a</td>
<td>9736±292d</td>
</tr>
<tr>
<td>HT15</td>
<td>33325±999b</td>
<td>-</td>
<td>15243±457a</td>
<td>59122±1172a</td>
</tr>
<tr>
<td>HT120</td>
<td>39192±1175a</td>
<td>-</td>
<td>7954±238c</td>
<td>22375±627b</td>
</tr>
<tr>
<td>HHP150/5</td>
<td>63±2d</td>
<td>7599±228ef</td>
<td>4803±144e</td>
<td>2301±62f</td>
</tr>
<tr>
<td>HHP300/5</td>
<td>21±0.6d</td>
<td>7629±220ef</td>
<td>3374±101g</td>
<td>156±5g</td>
</tr>
<tr>
<td>HHP450/5</td>
<td>23±0.7d</td>
<td>8576±215d</td>
<td>6196±185d</td>
<td>32±1g</td>
</tr>
<tr>
<td>HHP600/5</td>
<td>1844±55c</td>
<td>19222±527a</td>
<td>11962±358b</td>
<td>15101±343c</td>
</tr>
<tr>
<td>HHP150/10</td>
<td>208±6d</td>
<td>11476±344b</td>
<td>4649±139c</td>
<td>5591±126c</td>
</tr>
<tr>
<td>HHP300/10</td>
<td>63±2d</td>
<td>6993±209f</td>
<td>4507±135e</td>
<td>30±1g</td>
</tr>
<tr>
<td>HHP450/10</td>
<td>22±0.7d</td>
<td>7971±213de</td>
<td>6282±127d</td>
<td>122±4g</td>
</tr>
<tr>
<td>HHP150/15</td>
<td>45±1d</td>
<td>8052±241de</td>
<td>3829±188g</td>
<td>7053±121c</td>
</tr>
<tr>
<td>HHP300/15</td>
<td>36±2d</td>
<td>7909±237de</td>
<td>4190±114ef</td>
<td>60±2g</td>
</tr>
<tr>
<td>HHP450/15</td>
<td>18±0.5d</td>
<td>7370±221ef</td>
<td>5917±125d</td>
<td>37±1g</td>
</tr>
</tbody>
</table>
Appendix B: Supplementary Information for Chapter 4

Table B1
Composition (g/100g DM)\(^a\) of untreated, hydrothermal (HT) treated and high hydrostatic pressure (HHP) treated dry beans.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Moisture</th>
<th>Ash</th>
<th>Fat</th>
<th>Protein</th>
<th>Klason lignin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>--</td>
<td>4.27±0.27(^{cd})</td>
<td>2.61±0.75(^a)</td>
<td>1.20±0.00(^{ab})</td>
<td>25.35±1.11(^a)</td>
</tr>
<tr>
<td>HT 90°C/2h</td>
<td>5.28±1.03(^{bcd})</td>
<td>4.21±1.45(^a)</td>
<td>1.38±0.07(^a)</td>
<td>26.88±0.22(^a)</td>
<td>11.53±3.26(^a)</td>
</tr>
<tr>
<td>150MPa/5min</td>
<td>4.82±0.37(^{bcd})</td>
<td>2.63±0.75(^a)</td>
<td>1.33±0.06(^{ab})</td>
<td>26.51±0.14(^a)</td>
<td>10.20±0.55(^a)</td>
</tr>
<tr>
<td>300MPa/5min</td>
<td>5.63±0.02(^{bcd})</td>
<td>4.24±0.00(^a)</td>
<td>1.19±0.13(^{ab})</td>
<td>25.48±0.9(^a)</td>
<td>11.07±3.72(^a)</td>
</tr>
<tr>
<td>450MPa/5min</td>
<td>6.43±0.44(^{ab})</td>
<td>3.74±0.77(^a)</td>
<td>1.23±0.06(^{ab})</td>
<td>25.17±0.28(^a)</td>
<td>12.68±1.91(^a)</td>
</tr>
<tr>
<td>600MPa/5min</td>
<td>5.64±0.00(^{bcd})</td>
<td>3.18±1.50(^a)</td>
<td>1.16±0.01(^{ab})</td>
<td>25.19±0.32(^a)</td>
<td>12.08±1.08(^a)</td>
</tr>
<tr>
<td>HHP 150MPa/10min</td>
<td>3.96±0.15(^{d})</td>
<td>4.17±1.48(^a)</td>
<td>0.82±0.01(^{de})</td>
<td>25.56±0.29(^a)</td>
<td>10.41±1.13(^a)</td>
</tr>
<tr>
<td>300MPa/10min</td>
<td>5.59±0.71(^{bcd})</td>
<td>3.71±0.78(^a)</td>
<td>0.77±0.02(^e)</td>
<td>25.82±0.25(^a)</td>
<td>9.89±0.16(^a)</td>
</tr>
<tr>
<td>450MPa/10min</td>
<td>5.11±0.29(^{bcd})</td>
<td>3.16±0.01(^a)</td>
<td>0.95±0.11(^{cd})</td>
<td>25.47±0.21(^a)</td>
<td>11.05±0.72(^a)</td>
</tr>
<tr>
<td>150MPa/15min</td>
<td>5.99±0.15(^{bc})</td>
<td>3.19±1.51(^a)</td>
<td>1.09±0.13(^{bc})</td>
<td>26.24±0.08(^a)</td>
<td>10.37±0.32(^a)</td>
</tr>
<tr>
<td>300MPa/15min</td>
<td>6.66±0.04(^{bcd})</td>
<td>2.68±0.76(^a)</td>
<td>0.72±0.02(^e)</td>
<td>26.06±0.30(^a)</td>
<td>14.06±1.68(^a)</td>
</tr>
<tr>
<td>450MPa/15min</td>
<td>4.22±0.22(^{cd})</td>
<td>3.65±0.75(^a)</td>
<td>1.10±0.00(^{bc})</td>
<td>25.93±0.00(^a)</td>
<td>13.13±2.53(^a)</td>
</tr>
</tbody>
</table>

\(^a\)DM: dry matter.
Data are expressed as the mean values (n=3) ± standard deviation (SD). Comparisons for all pairs were made using Tukey-Kramer HSD; Along the column, mean values with different letters are significantly different (P < 0.05).
Figure B1 Scanning Electron Microscopy (SEM) of (A-C) beans samples treated at 300MPa for 10min at magnification of 300x, 1Kx, and 3Kx, signal: lnLens; (D&E) untreated beans and beans samples treated at 600MPa/5min (damaged starch granules) at magnification of 5Kx, signal: lnLens; (a-d) beans samples treated at 150MPa, 300MPa, 450MPa, 600MPa for 5min, at magnification of 10Kx, signal: SE. (P: protein or protein complex; S: starch; C: cell wall or cell wall fiber; Po: pores)
Appendix C: Supplementary Information for Chapter 5

A. NaGC

B. NaTC

C. NaGCDC

D. NaTCDC

Figure C1 HPLC chromatograms of individual primary bile salts (20mM) using a water/acetonitrile/0.5M tetrabutylammonium phosphate (45:50:1, v/v/v) mobile phase.
Figure C2 Ability of bean flour and isolated bean fractions to retain a BS mixture (A) and individual primary bile salts (B) in vitro. The % BS bound/retained has been expressed on an equal dry weight basis. Data are expressed as the mean values (n=3) ± standard deviation (SD) bar. Comparisons for all pairs were made using Tukey-Kramer HSD. Mean values with different letters are significantly different (p < 0.05).
**Figure C3** Correlation between viscosity at shear rate 15 s\(^{-1}\) of digested samples and % BS bound by beans treated by hydrothermal (HT) and high hydrostatic pressure (HHP) processing.

\[
Y = 102.2X + 27.58 \\
R^2 = 0.47 \\
p = 0.01
\]

**Figure C4** The correlation between viscosity at shear rate 15 s\(^{-1}\) of digested processed bean samples and (A) \(k\), release rate constant and (B) \(C_f\), concentration of BS released after reaching equilibrium, when considering the bile salts (BS) mixture.

\[
Y = -2.352X + 0.7874 \\
R^2 = 0.35 \\
p = 0.04
\]

\[
Y = -28.12X + 91.97 \\
R^2 = 0.23 \\
p = 0.09
\]
Table C1 HPLC Standard curve used for quantification of unbound Bile Salts

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Linear Regression</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaTC</td>
<td>$y = 1017.4x - 0.3618$</td>
<td>1</td>
</tr>
<tr>
<td>NaGC</td>
<td>$y = 891.1x - 0.4986$</td>
<td>0.99</td>
</tr>
<tr>
<td>NaTCDC</td>
<td>$y = 944.78x - 0.9641$</td>
<td>0.99</td>
</tr>
<tr>
<td>NaGCDC</td>
<td>$y = 947.92x + 0.3971$</td>
<td>0.99</td>
</tr>
<tr>
<td>Total BS</td>
<td>$y = 951.91x - 1.4274$</td>
<td>1</td>
</tr>
</tbody>
</table>

Table C2 Enthalpy change values ($\Delta H$, J/kg) obtained from the thermograms of 1% (w/v) bean flour and its isolated starch and protein fractions in the absence or presence of 5 or 10mM BS (NaTC or NaTCDC).

<table>
<thead>
<tr>
<th>Bean materials</th>
<th>BS concentration</th>
<th>Peak1</th>
<th>Peak2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bean flour</td>
<td>0</td>
<td>0.02±0.0005$^a$</td>
<td>0.012±0.001$^a$</td>
</tr>
<tr>
<td></td>
<td>5mM NaTC</td>
<td>0.019±0.001$^a$</td>
<td>0.008±0.001$^b$</td>
</tr>
<tr>
<td></td>
<td>10mM NaT</td>
<td>0.016±0.001$^b$</td>
<td>0.006±0.0006$^c$</td>
</tr>
<tr>
<td></td>
<td>5mM NaTCDC</td>
<td>0.018±0.001$^{ab}$</td>
<td>0.01±0.0005$^b$</td>
</tr>
<tr>
<td></td>
<td>10mM NaTCDC</td>
<td>0.013±0.001$^c$</td>
<td>0.005±0.001$^c$</td>
</tr>
<tr>
<td>Isolated Starch fraction</td>
<td>0</td>
<td>0.127±0.002$^a$</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>5mM NaTC</td>
<td>0.075±0.002$^b$</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>10mM NaT</td>
<td>0.05±0.005$^c$</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>5mM NaTCDC</td>
<td>0.071±0.002$^b$</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>10mM NaTCDC</td>
<td>0.04±0.01$^c$</td>
<td>-</td>
</tr>
<tr>
<td>Isolated Protein fraction</td>
<td>0</td>
<td>-</td>
<td>0.088±0.002$^a$</td>
</tr>
<tr>
<td></td>
<td>5mM NaTC</td>
<td>-</td>
<td>0.079±0.003$^a$</td>
</tr>
<tr>
<td></td>
<td>10mM NaTC</td>
<td>-</td>
<td>0.046±0.01$^b$</td>
</tr>
<tr>
<td></td>
<td>5mM NaTCDC</td>
<td>-</td>
<td>0.024±0.003$^c$</td>
</tr>
<tr>
<td></td>
<td>10mM NaTCDC</td>
<td>-</td>
<td>0.023±0.002$^c$</td>
</tr>
</tbody>
</table>

Peak1: starch gelatinization
Peak2: protein denaturation
Figure D1 Particle size distribution of original samples of isolated bean fiber and different bean flours.
Figure D2 Particle size distribution of samples blanks of isolated bean fiber and bean flours as they passed through the stimulated *in vitro* gastrointestinal tract.
Figure D3 Flow curves of samples blanks of isolated bean fiber and bean flours as they passed through the stimulated in vitro gastrointestinal tract.
**Figure D4** Percentage of free fatty acid released under stimulated *in vitro* small intestinal digestion for sample blanks of isolated bean fiber and bean flours.

**Figure D5** Fatty acids profile graph analyzed by GC-MS in original sunflower oil.
Table D1 The surface mean particle diameter \( d_{3,2} \) on of sunflower oil-in-water emulsions added with bean fiber fractions and bean flours as they passed through the stimulated \textit{in vitro} gastrointestinal tract, as compared to original emulsion/fiber/bean samples.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Original sample</th>
<th>Emulsion or fiber/bean added-emulsion, ( d_{3,2} (\mu m) )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( d_{50} (\mu m) )</td>
<td>( d_{3,2} (\mu m) )</td>
</tr>
<tr>
<td>Control Emulsion</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fiber SDF</td>
<td>189.57±28.52\textsuperscript{c}</td>
<td>65.48±4.35\textsuperscript{c}</td>
</tr>
<tr>
<td>Fiber IDF</td>
<td>889.80±13.71\textsuperscript{b}</td>
<td>304.90±14.77\textsuperscript{a}</td>
</tr>
<tr>
<td>Raw FP</td>
<td>172.80±18.83\textsuperscript{cd}</td>
<td>27.05±0.11\textsuperscript{d}</td>
</tr>
<tr>
<td>Raw MP</td>
<td>938.83±45.85\textsuperscript{b}</td>
<td>165.00±11.61\textsuperscript{b}</td>
</tr>
<tr>
<td>Raw CP</td>
<td>1452.00±183.51\textsuperscript{a}</td>
<td>62.69±1.34\textsuperscript{c}</td>
</tr>
<tr>
<td>Processed FP-HHT</td>
<td>199.30±3.29\textsuperscript{c}</td>
<td>21.70±0.21\textsuperscript{d}</td>
</tr>
<tr>
<td>Processed FP-HHP600/5</td>
<td>168.87±3.70\textsuperscript{cd}</td>
<td>24.38±1.17\textsuperscript{d}</td>
</tr>
<tr>
<td>Processed FP-HHP300/15</td>
<td>228.97±3.58\textsuperscript{e}</td>
<td>30.10±0.48\textsuperscript{d}</td>
</tr>
</tbody>
</table>

\( d_{50} \): median particle size (\( \mu m \))

Sample denotation, applied here and elsewhere:

- SDF, soluble dietary fiber fraction of raw beans
- IDF, insoluble dietary fiber fraction of raw beans
- FP, fine particles of raw bean flour
- MP, medium particles of raw bean flour
- CP, coarse particles of raw bean flour
- FP-HT, fine particles of hydrothermal treated bean flour
- FP-HHP600/5, fine particles of High hydrostatic pressure (600MPa/5min) treated bean flour
- FP-HHP300/15, fine particles of High hydrostatic pressure (300MPa/15min) treated bean flour

Data are expressed as the mean values (\( n=3 \)) ± standard error (SE). Comparisons for all pairs were made using Tukey-Kramer HSD; Along the column, mean values with different letters are significantly different (\( p < 0.05 \)).
**Table D2** The surface mean particle diameter ($d_{3,2}$) on of samples blanks of isolated bean fiber and bean flours as they passed through the stimulated *in vitro* gastrointestinal tract.

<table>
<thead>
<tr>
<th>Blank samples</th>
<th>Initial (μm)</th>
<th>Oral (μm)</th>
<th>Gastric (μm)</th>
<th>Intestinal (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SDF</td>
<td>65.48±4.35&lt;sup&gt;b&lt;/sup&gt;</td>
<td>75.13±8.09&lt;sup&gt;b&lt;/sup&gt;</td>
<td>56.41±1.48&lt;sup&gt;b&lt;/sup&gt;</td>
<td>14.94±0.27&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>IDF</td>
<td>304.9±14.77&lt;sup&gt;a&lt;/sup&gt;</td>
<td>292.57±7.76&lt;sup&gt;a&lt;/sup&gt;</td>
<td>229.70±25.95&lt;sup&gt;a&lt;/sup&gt;</td>
<td>61.67±1.77&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>FP</td>
<td>20.38±5.69&lt;sup&gt;c&lt;/sup&gt;</td>
<td>33.98±0.97&lt;sup&gt;c&lt;/sup&gt;</td>
<td>31.77±0.43&lt;sup&gt;b&lt;/sup&gt;</td>
<td>60.60±2.33&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Data are expressed as the mean values (n=3) ± standard error (SE). Comparisons for all pairs were made using Tukey-Kramer HSD; Along the column, mean values with different letters are significantly different ($p < 0.05$).