STRUCTURAL, RHEOLOGICAL AND BIOLOGICAL FUNCTIONALITY OF FIBER-RICH FRACTIONS FROM BEANS

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

DOCTOR OF PHILOSOPHY

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ABSTRACT

Legumes have been reported as a good source of dietary fiber. This research aimed to understand how soluble dietary fiber (SDF) can be extracted from dry beans to enhance human health. Raw bean samples were ground with a hammer-type mill. Cooked bean samples were boiled to their optimum cooking time, freeze-dried, and ground. Phenolic content was measured by Folin-Coilteau reaction. SDF was extracted by enzymatic digestion (α-amylase, protease, & amyloglucosidase), filtration, SDF flocculation with 95% (v/v) ethanol and freeze-dried. High-Performance Size Exclusion Chromatography (HPSEC) and high-performance anion-exchange chromatography (HPAEC) were used to study SDF composition. Flow behavior was measured at various SDF solution concentrations (2%, 4%, 6% w/v). Immunomodulatory properties were studied *in vitro* by quantifying the levels of selected cytokines released by RAW264.7 cells (macrophages) when treated with SDF. The impact of macrophages conditioned media (MCM) treated with the SDF on 3T3-L1 adipocyte (fat) cells differentiation (adipogenesis) was assessed. The results showed that cooking processing significantly ($p < 0.05$) reduced flour phenolic content and antioxidant capacity. The SDF-rich fractions monosaccharide composition indicated the presence of pectic polysaccharides, hemicelluloses and raffinose family of oligosaccharides (RFOs). The SDF molecular weight distribution showed a significant ($p < 0.05$) reduction of low molecular weight (LMW) fractions after the cooking process. In contrast, the high molecular weight (HMW) fractions were not affected by cooking. Studied SDF solutions were highly viscous, but the viscosity was lost when shear force was applied, thus exhibiting a shear-thinning behavior. For the immunomodulatory properties, the results indicated that the extracted SDF caused a pro-inflammatory response, suggesting its potential use as an immune system stimulator. Regardless of the SDF sample, when TLR-4 signaling inhibitor was added, cytokines

production was significantly ($p < 0.05$) decreased, suggesting the affinity of SDF with the TLR-4 receptor. Finally, a significant ($p < 0.05$) decrease in lipid accumulation and reduced protein expression of adipogenic markers was detected from adipocytes cultured with MCM treated with SDF. The results suggested that the SDF extracted from dry beans has the potential to reduce adipogenesis and could be used as food supplements to reduce or prevent obesity.

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DEDICATION

I would like to dedicate this thesis to my beloved parents,

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who I owe the person I am today.

I love you.

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LIST OF ABBREVIATIONS

FABP4.....Fatty acid-binding protein 4.

IL-1β .....Interleukin 1β.

IL-6 .....Interleukin 6.

MCM.....Macrophage-conditioned media.

ORO .....Oil red O.

PPAR-γ .....Peroxisome proliferator-activated receptor gamma.

RFOs .....Raffinose family of oligosaccharides.

SDF .....Soluble dietary fiber.

TAK-242 .....Resatorvid, TLR-4 signaling inhibitor.

TLR-4.....Toll-like receptor 4.

TNF-α.....Tumor necrosis factor-alpha.

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1. GENERAL INTRODUCTION

The term pulses is defined by the Food and Agriculture Organization (FAO) of the United Nations as annual leguminous crops yielding from one to 12 grains of variable size, shape and color in a pod, which are only harvested as dry grains for food and feed purposes (Calles et al., 2019). Within pulse crops, dry edible beans (*Phaseolus vulgaris* L.) are grown commercially in 18 states, being North Dakota the top producer (Dry Bean Council, 2022). Dry beans are an important source of protein, starch, dietary fiber, minerals and phenolic compounds. Dietary fiber, classified based on its water solubility into insoluble (IDF) and soluble dietary fiber (SDF), is considered a functional ingredient since it promotes various beneficial physiological effects for human health (Bader Ul Ain et al., 2019; Tosh & Yada, 2010). Furthermore, plant-derived polysaccharides, such as bean dietary fiber, have gained growing interest due to their ability to exert effects on immune system functions (Ramberg et al., 2010). Overall, the observed effect of botanical-derived polysaccharides has been the enhancement or activation of macrophage immune responses, which suggests that extracted polysaccharides bind to common surface receptors inducing immunomodulatory responses (Schepetkin & Quinn, 2006). Additionally, supernatants derived from activated macrophages have been studied due to their inhibitory effect on adipose (fat) tissue expansion (Lacasa et al., 2007). It has been hypothesized that the pro-inflammatory molecules secreted by activated macrophages impair lipid accumulation of adipocytes (fat cells) (Ma et al., 2020).

In this sense, the study of dry bean dietary fiber physical properties and composition will be helpful to elucidate its potential as a functional ingredient. Recognizing the nutritional potential of dry bean components' will be helpful for food industries to adopt them as ingredients. Furthermore, the evaluation of its immunomodulatory properties may suggest its use

as a therapeutic agent that mediates pro-inflammatory responses, in addition to exhibiting a potential anti-adipogenic effect. In this way, the enhancement of human health will be promoted while the market value of dry beans is increased.

In this context, the main objectives of this study were as follows,

1. To investigate the impact of the dry bean market class and cooking processing on dry bean flour physicochemical properties
2. To extract and characterize the SDF-rich fraction from dry bean flours.
3. To evaluate the immunomodulatory properties of SDF-rich fraction using cell culture models derived from the immune system cells.
4. To assess the impact on lipid accumulation of products secreted from macrophages stimulated by SDF-rich fractions using fat cell culture models.

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2. LITERATURE REVIEW

Legumes are considered an essential source of nutrients. By being relatively inexpensive and readily available, legumes are part of people's diet worldwide, especially in developing countries and regions where protein-energy malnutrition is prevalent, such as India, Latin America, and Africa (Martin-Cabrejas, 2019; Shimelis & Rakshit, 2005). One of the legume crops consumed as a major source of protein, starch, minerals and dietary fiber is dry beans (*Phaseolus vulgaris* L.) (Xu & Chang, 2008). The *Phaseolus* genus was originated between 4 to 6 million years ago in the Mesoamerican (Mexico-Guatemala) and Andean (Peru-Ecuador) regions, where the domestication of dry beans occurred independently (Delgado-Salinas et al., 2006; Nadeem et al., 2021). The domesticated *Phaseolus* species possess global adaptability, genotypic and phenotypic diversity, and depending on the region and culture, the crop undergoes multiple means of preparation (Siddiq & Uebersax, 2013). However, dry beans are not a staple in the United States. Therefore, opportunities to improve the use of dry beans should encompass the development of value-added products or ingredients that meet modern consumer health-related needs.

2.1. Dry Beans

Dry beans are the world's second most important legume class after soybeans (Xu & Chang, 2008). Legumes are dicotyledonous seeds of flowering plants that belong to the family Fabaceae (formerly known as Leguminosae). The Fabaceae family plant members possess an annual growing cycle. Botanically, these annual plants consist of herbaceous stems, tri-foliate leaves, and flat flowers from which seeds of varying sizes, shapes and colors are produced in a pod (Figure 1) (Nassar et al., 2010; Uebersax & Siddiq, 2013). The seed structure of a dry bean comprises a seed coat, cotyledon, and embryo (Powrie et al., 1960).

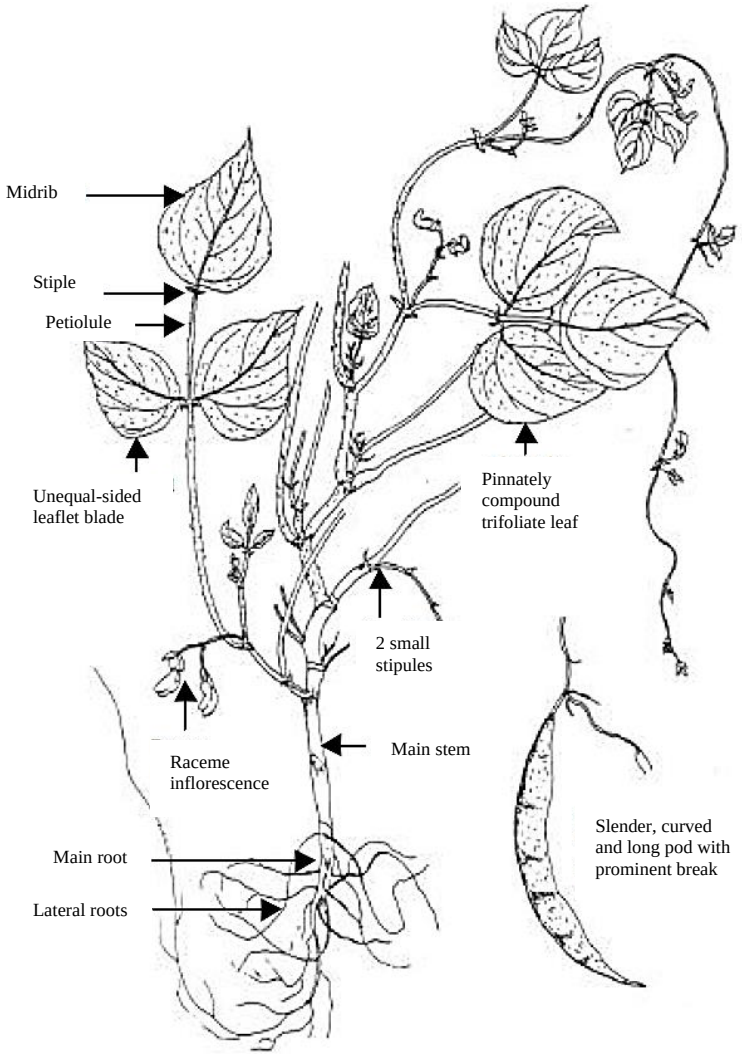

Figure 1. Schematic representation of *Phaseolus vulgaris* L.
(Adapted from Nassar et al., 2010).

2.1.1. Physiology of Dry Bean Seed

The dry bean seed structure is formed by the outer layer seed coat or testa, two cotyledons that serve as the energy storage tissue, and the embryonic cotyledon comprised of epicotyl, hypocotyl, and radicle; in addition to the hilum (stem scar) is the micropyle (minute opening), which allows gas exchange and water absorption (Figure 2) (Uebersax & Siddiq, 2013).

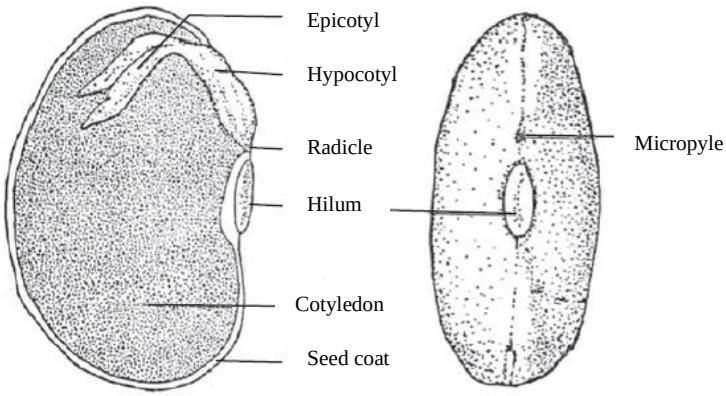

Figure 2. Dry bean seed anatomy.
(Adapted from Uebersax & Siddiq, 2013).

2.1.1.1. Seed coat

The dry bean seed coat accounts for around 8% of the total dry weight of the seed. The seed coat is formed by the outer layer of the waxy cuticle, followed by the palisade cells layer, hourglass cells layer, and the thick cell wall parenchyma (Powrie et al., 1960; Swanson et al., 1985). The seed coat plays a role in cotyledon and embryo protection, as well as in the water permeability of the seed, being the palisade cells layer the one regulating water uptake (Ross et al., 2010; Zeng et al., 2005). The dry bean seed coat is structurally formed by small quantities of lipids, ash, and proteins, contrasting with a major proportion of dietary fiber that ranges from 31 to 50% among different dry bean market classes (Aguilera et al., 1982; Rui & Boye, 2013). In this sense, the seed coat contributes to the majority of the dietary fiber content of dry beans due to its structural polysaccharides composition of cellulose, varying levels of hemicelluloses, and lignin (MacDougall & Selvendran, 2001). Furthermore, the dry bean seed coat color varies depending on the phenolic compounds' composition. The distinctive color of each dry bean market class is imparted by flavonoid pigments (Beninger et al., 1998; Luthria & Pastor-Corrales, 2006), such as anthocyanins, which have been associated with health benefits due to their antioxidant capacity.

2.1.1.2. Cotyledon

The greatest portion of the dry bean seed is the two cotyledons, which comprise around 90% of the total dry weight of the seed. The role of the dry bean cotyledons is to form the embryonic leaf tissue during germination, as they are considered stored energy reserves utilized for the initial seedling growth (Uebersax & Siddiq, 2013). The cotyledons are formed by the outermost epidermal layer, hypodermis, and parenchyma cells bounded by the middle lamella. The parenchyma cells are composed of a matrix of spherical protein bodies in which starch granules of different sizes are embedded (Swanson et al., 1985). The middle lamella is a layer of polysaccharides that comprise the "cotyledon fiber," such as pectin, hemicelluloses, and gums, which give rigidity and strength to the total tissue through the cross-link of divalent cations (Van Buren, 1979).

2.1.1.3. Embryo

The dry bean seed embryo is a living tissue that comprises >2% of the total dry weight of the seed. The embryo is rich in lipids, minerals, and catabolic enzymes, such as proteases, amylases, pectinases, cellulases, and phytases, needed for germination. The enzymatic activity is initiated with water imbibition and mild temperatures (15 to 30°C). Therefore, overnight soaking of dry beans before cooking will reduce the processing time and will yield a tender product due to the breakdown and degradation caused by activated embryonic enzymes (Rebollo-Hernanz et al., 2019).

2.1.2. Composition of Dry Bean Seed

Pulses, such as dry beans, are considered highly nutritious due to their rich protein composition, slow-releasing carbohydrates, dietary fiber, vitamins, minerals, and phytochemicals composition (Tharanathan & Mahadevamma, 2003). This composition has made dry beans to be

considered a source of versatile, functional ingredients with the potential to influence human health (Oomah et al., 2011).

2.1.2.1. Protein

Dry beans contain high amounts of proteins accumulated during seed development in the cotyledon tissue as energy reservoirs. The cotyledonary parenchyma cells contain the protein bodies that survive desiccation during seed maturation, but will be hydrolyzed upon germination to provide ammonia and carbon skeletons to the seedlings (Oomah et al., 2011). The dry bean protein content typically ranges from 20 to 30 % of total dry matter (OECD, 2019). However, dry bean proteins are deficient in some essential amino acids, such as the sulfur-containing methionine and cysteine, and contain anti-nutritional components, such as hydrolase inhibitors and lectins (phytohemagglutinin), that lower protein digestibility (Azarpazhooh & Boye, 2013; Belitz et al., 2004; Oomah et al., 2011).

Albumins (water-soluble), globulins (dilute salt soluble), and glutelins (dilute alkali-soluble) are predominantly present in dry beans (Azarpazhooh & Boye, 2013; Belitz et al., 2004; Osborne, 1924). Globulins are the most abundant storage proteins in dry beans, accounting for 50 to 70 % of the total protein content. Globulins are classified based on their sedimentation coefficients (S) into 7S and 11S (Tang & Sun, 2011). The 7S fraction is a trimeric protein also known as phaseolin, which tends to exhibit a reversible association/dissociation behavior in solutions that depends on the pH and ionic strength. The 7S fraction cannot form disulfide bonds due to the lack of cysteine (Oomah et al., 2011). The 11S fraction is relatively more stable in solutions and typically forms hexamers linked non-covalently (Belitz et al., 2004).

2.1.2.2. Lipids

The lipid content in dry beans ranges from 1 to 3 %, and includes triglycerides, and minor amounts of free fatty acids, sterol esters, phospholipids, and glycolipids (Azarpazhooh & Boye, 2013; Hayat et al., 2014). The main polyunsaturated fatty acids found in dry beans are linolenic ($C_{18:3}$), palmitic ($C_{16:0}$), linoleic ($C_{18:2}$), and oleic ($C_{18:1}$) acid (Campos-Vega et al., 2010; Grela & Günter, 1995). Therefore, dry beans are regarded as a good source of free unsaturated fatty acids (Campos-Vega et al., 2010).

2.1.2.3. Carbohydrates

Dry beans' principal constituent is carbohydrates, with contents range from 50 to 60 % of dry matter (Oomah et al., 2011; Ovando-Martínez et al., 2011). Starch and non-starch polysaccharides comprise the dry beans' carbohydrate fraction (Bravo et al., 1998).

Starch is the dry beans' main storage carbohydrate (Oomah et al., 2011). Starch is composed of amylose and amylopectin. Amylose, which constitutes 15 to 30 % of the total starch, is a linear macromolecule of α-D-glucopyranose residues linked by α-(1→4) bonds, and its molecular weight ranges from 100 to 1000 kDa. In comparison, amylopectin is highly branched with α-D-glucopyranose residues linked by both α-(1→4) and α-(1→6) linkages with a molecular weight greater than 10000 kDa (Cui, 2005; Hoover et al., 2010). Regarding digestibility, based on the rate of glucose release and absorption by the gastrointestinal tract, starch has been classified into rapidly digestible (RDS), slowly digestible (SDS), and resistant starch (RS) (Englyst et al., 1992). After ingestion, RDS causes a sudden rise in blood glucose; SDS is completely absorbed in the small intestine but at a lower rate than RDS, while RS is fermented in the large intestine. In this sense, SDS and RS are potentially beneficial to human health as their consumption results in a low glycemic index. It has been recognized that native

pulse starches are more readily digestible than potato or high amylose maize starches but less rapidly digestible than cereal starches. (Hayat et al., 2014; Q. Liu et al., 2006). The reduced digestibility of pulse starches is attributed to the absence of pores on the granule surface, a high amylose content, and strong interactions between amylose chains (Hoover et al., 2010).

Dry beans are rich in non-starch polysaccharides, which constitute dietary fiber, a complex mixture of polysaccharides that resist enzymatic digestion and absorption in the small intestine, passing down to the large intestine where bacteria convert them into short-chain fatty acids (Bader Ul Ain et al., 2019). Dietary fiber was defined by the Cereals and Grains Association (formerly known as AACC International) as "the remnants of the edible part of plants and analogous carbohydrates that are resistant to digestion and absorption in the human small intestine with complete or partial fermentation in the human large intestine. It includes polysaccharides, oligosaccharides, lignin, and associated plant substances. Dietary fiber exhibits one or more of the following functions: laxation (fecal bulking and softening; increased frequency; and regularity), blood cholesterol attenuation, and blood glucose attenuation" (DeVries et al., 2001). Dietary fiber can be classified based on its water solubility into insoluble (IDF) and soluble dietary fiber (SDF) (Bader Ul Ain et al., 2019; Tosh & Yada, 2010). IDF consists of lignin, cellulose, and hemicellulose, while SDF includes pectin, gums, mucilages, and inulin-type fructans (Dhingra et al., 2012). Typically the ratio of SDF and IDF in foods is 1:3, respectively (Cummings, 1981). Regarding dry beans, their total dietary fiber content reported in the literature ranges from 23 to 32 %, from which 20 to 28 % is IDF and 3 to 6 % is accounted as SDF (Tosh & Yada, 2010). Dry beans' IDF and SDF contents reported in the literature vary depending on the employed analytical method and the applied treatment, SDF content being more sensitive to processing conditions (Kutoš et al., 2003). For instance, Shiga & Lajolo (2006)

reported that cooking beans until cotyledon's softness led to increased free fragments of arabinans and the degradation of pectic material. During cooking, the structure of the polysaccharides is depolymerized due to the high temperature (Mattson, 1946).

2.1.2.4. Minerals

Dry beans have the highest mineral content compared to other pulses (Hayat et al., 2014). Among pulses, dry beans have the highest content of iron and zinc, found in the range of 62 to 150 µg/g and 10 to 109 µg/g, respectively (Vadivel & Janardhanan, 2000). Other minerals such as calcium, copper, magnesium, potassium, and phosphorus are found in dry beans. Phosphorus in dry beans is present as phytic acid (Azarpazhooh & Boye, 2013). Phytic acid (myo-inositol hexakisphosphate or IP6) is the plant's main phosphorus storage form, located in the endosperm protein bodies as salts (phytates). Humans cannot absorb phytate due to its insolubility at the intestine's pH; therefore, it is not considered a source of either inositol or phosphate. Furthermore, phytate is considered an anti-nutrient because it binds to minerals due to its chelating property, thus inhibiting minerals (iron, zinc, and calcium) bioavailability (Sandberg, 2002a). So to improve mineral absorption, phytate degradation should be achieved with processing methods that enhance the phytase enzyme synthesis and activity, such as soaking, germination, hydrothermal processing, and fermentation (Sandberg, 2002b).

2.1.2.5. Phenolic compounds

The major phenolic compounds in dry beans consist of flavonoids, flavonols, tannins, proanthocyanidins, anthocyanins, glycosides, and phenolic acids (Campos-Vega et al., 2010). Most phenolic compounds are found in the seed coat, while lesser amounts are accounted for in the dry bean cotyledons (De Mejía et al., 1999). The level of phenolic compounds in dry beans is determined by environmental and genetic factors, such as growing location and cultivar,

respectively (Rocha-Guzmán et al., 2007). In addition, previous studies suggest that dry beans' seed coats with darker colors contain higher amounts of phenolics than seed coats with light colors (Barampama & Simard, 1993). Feenstra (1960) was one of the first researchers to extract and separate phenolic compounds in dry beans' seed coats. He concluded that dry beans' seed coat color was determined by the differences in the composition of procyanidins, flavonol glycosides, and anthocyanidins. Differences in phenolic compound composition have been seen between two U.S. market classes of beans: pinto and black beans, differentiated in their seed coat color, thus nutraceutical potential. Pinto beans mainly contain 3-O-glucosides of the flavonol kaempferol. On the other hand, black beans contain 3-O-glucosides of the anthocyanidins malvidin, petunidin, and delphinidin (Lin et al., 2008). However, it is important to note that dry beans are cooked before consumption so that the thermal treatment will modify the phenolic composition. For instance, López et al. (2013) reported a reduction of 68% of anthocyanins content in black beans after boiling them for 60 minutes.

2.2. Inflammation

Inflammation is a response of the immune system characterized by coordinated interactions between cells and soluble factors, and triggered by trauma, microbial infection, or a toxic or autoimmune injury (Nathan, 2002). A controlled inflammatory process is beneficial as it aims to resolve infection or injury and restore the homeostatic state (Barton, 2008; Medzhitov, 2008). Furthermore, inflammation can be considered life-preserving as people, with genetic deficiencies in the principal components of the inflammatory process, have an increased risk of having severe infections (Nathan, 2002). However, inflammation can become detrimental if dysregulated, causing damage to the host tissues (Medzhitov, 2008).

2.2.1. Inflammatory Response Through Pattern Recognition

The innate immune system initiates an inflammatory response after detecting highly conserved microbial molecular structures, known as pathogen-associated molecular patterns (PAMPs) (Kumar et al., 2009; Medzhitov, 2001). Innate immune cells express pattern-recognition receptors (PRRs) that target the PAMPs. However, the PRRs have broad specificity; thus, they can bind various molecules with common structural motifs or patterns (Medzhitov, 2007). Examples of PAMPs include the bacterial cell wall components lipopolysaccharides (LPS), peptidoglycans, lipoteichoic acids, and lipoproteins, as well as fungal cell wall β-glucans (Abbas et al., 2019; Medzhitov, 2007). Once the PRRs detect the PAMPs, a series of inflammatory signaling molecules are released to initiate the host defense.

Several classes of PRRs have been identified, including Toll-like receptors (TLRs), Retinoic acid-inducible gene-I-like receptors (RLRs), and Nucleotide-binding oligomerization domain-like receptors (NLRs) (Abbas et al., 2019; Kumar et al., 2009). One of the best characterized PRRs families is the TLRs one. The TLRs family is formed by type I transmembrane glycoprotein receptors, characterized by two domains: 1) extracellular leucine-rich repeats (LRRs) N-terminal domain; and 2) the intracellular Toll/IL-1 receptor (TIR) C-terminal domain (Akira et al., 2006). The members of the TLR family are expressed in different cell compartments, with TLR-1, TLR-2, TLR-4, TLR-5, TLR-6, and TLR-11 being expressed on the cell surface, while TLR-3, TLR-7, TLR-8, and TLR-9 are expressed in intracellular vesicles (Akira et al., 2006; Kumar et al., 2009). The surface-expressed TLRs recognize specific microbial proteins, lipids, and polysaccharides, whereas intracellular TLRs recognize microbial nucleic acids (Abbas et al., 2019). For instance, TLR-2 recognizes bacterial peptidoglycan and lipopeptides, and TLR-5 recognizes bacterial flagellar proteins. However, some TLRs, such as

TLR-4, can recognize structurally unrelated ligands, such as bacterial lipopolysaccharide (LPS), fungal mannan and glucuronoxylomannan, and parasitic glycoinositolphospholipids (Akira et al., 2006). In general, the TLRs play a vital role in establishing inflammatory responses.

The typical stages of an inflammatory response are recognition of infection, followed by recruitment of cells to the sites of infection, then elimination of the microbe, and final resolution of inflammation and return to homeostasis (Barton, 2008). In this sense, tissue-resident macrophages and mast cells recognize the infection through their PRRs, such as TLRs, expressed on their cell surface. The pathogen recognition and consequent activation of tissue-resident cells produce and release pro-inflammatory cytokines and chemokines (Medzhitov, 2008). For instance, tissue-resident macrophages activated through TLRs produce cytokines such as tumor necrosis factor α (TNF- α), interleukin-1β (IL-1β), and IL-6 that coordinate systemic inflammatory responses. TNF- α and IL-1β induce vasodilation while increasing blood vessel permeability, allowing plasma, neutrophils and monocytes to migrate to the infected or wounded site (Barton, 2008; Medzhitov, 2007). Neutrophils are activated at the site of infection or injury through direct pathogen or DAMP encounter (Medzhitov, 2008). Together, the release of IL-1β and IL-6 leads to the expression of collectins and pentraxins, which activate the complement system by opsonizing pathogens and enhancing macrophages and neutrophils phagocytosis (Medzhitov, 2007). Neutrophils produce reactive oxygen species (ROS) and reactive nitrogen species (RNS) while also possessing granules with proteases that degrade any phagocyted material (Barton, 2008; Medzhitov, 2008). In addition, neutrophils become activated in the presence of TNF- α, leading to the release of their granules into the extracellular spaces to produce a toxic environment that causes collateral damage to the host (Barton, 2008). Finally, if the inflammatory response is able to eliminate the microbial infection, the resolution of

inflammation starts (Medzhitov, 2008). Neutrophils die by apoptosis (programmed cell death), and macrophages clear apoptotic neutrophils by phagocytosis. Overall, the cells start producing anti-inflammatory signals, like lipoxins which stop the influx of neutrophils while promoting monocytes recruitment to aid in dead cell clearance (Barton, 2008). The termination of the inflammatory response will lead to homeostasis reestablishment.

2.2.2. Signaling Molecules in Inflammation

As previously stated, inflammation triggers the immune system cells to produce pro-inflammatory cytokines as immune response regulators to protect the host against infection (Luo & Zheng, 2016). It is generally accepted that TNF- α, IL-1β and IL-6 are pro-inflammatory cytokines.

2.2.2.1. Tumor-necrosis factor α (TNF- α)

Different cells can synthesize TNF- α, with macrophages being the major producer of this cytokine (Parameswaran & Patial, 2010). TNF- α has been reported to be one of the most abundant early mediators during inflammation as it is rapidly released after trauma, infection, or LPS bacterial exposure (Feldmann et al., 1994). For instance, Toll-like receptors cause macrophages to produce TNF- α, among other pro-inflammatory agents, to activate other macrophages (Parameswaran & Patial, 2010). TNF- α activates cells through two transmembrane cell surface receptors, TNF receptor 1 (TNFR1) and TNF receptor 2 (TNFR2). The binding of TNF- α to TNFR1 is considered irreversible (Tartaglia et al., 1993). This activation has been stated as responsible for inflammatory responses like cell growth, cytotoxicity, and upregulation of adhesion and cytokine genes (Gehr et al., 1992). Furthermore, macrophages activation through TNFR2 has been reported to be important for the proliferation of lymphoid cells and cytotoxicity (Holler et al., 1992). Activated macrophages migrate to inflammation sites to lyse pathogens by

producing nitric oxide (NO), through the expression and activity of inducible nitric oxide synthase (iNOS) (Magez et al., 2007). TNF- α is considered to be a master regulator since it plays an important role during inflammatory cell activation and recruitment, and regulates the pro-inflammatory cytokine cascade production (Clark, 2007; Parameswaran & Patial, 2010).

2.2.2.2. Interleukin-1β (IL-1β)

Various cell types, including monocytes and macrophages, can synthesize and secrete IL-1β (Lopez-Castejon & Brough, 2011). IL-1β is a pro-inflammatory cytokine that mediates the host's response to injury and infection (Dinarello, 1996). IL-1β is produced in response to microbial products detected by PRRs on macrophages (Takeuchi & Akira, 2010). The expression of pro- IL-1β (IL-1β precursor) is induced, then pro- IL-1β is processed (cleaved and activated) by caspase-1. The intracellular multiprotein complex, inflammasome, activates the caspase-1; thus, the inflammasome is considered an important regulator of inflammation (Kaneko et al., 2019; Lopez-Castejon & Brough, 2011; Ren & Torres, 2009). Activated IL-1β starts its biological functions following the interaction with cell surface receptors. IL-1β can bind and signal through the cell membrane IL-1 type 1 receptor (IL-1RI) (Dinarello, 2018; Ren & Torres, 2009). IL-1β is a master regulator of inflammation, through the expression of genes such as cyclooxygenase type 2 (COX-2) and iNOS, the resulting production of nitric oxide (NO) and the increase expression of adhesion molecules to promote infiltration of cells from the circulation to the inflamed zone (Dinarello, 2009; Kaneko et al., 2019).

2.2.2.3. Interleukin-6 (IL-6)

IL-6 is a cytokine that affects inflammation and hematopoiesis (formation of blood cellular components). When immune cells, such as monocytes and macrophages, detect an exogenous pathogen through their PRRs, IL-6 is synthesized and released through the

bloodstream to trigger the host's defense mechanisms (Tanaka et al., 2014). IL-6 can bind and signal through the immune cell membrane receptor IL-6 receptor (IL-6R), which can also be cleaved from the cell membrane, thus be known as soluble IL-6R (sIL-6R), without losing its binding affinity for IL-6 (Luo & Zheng, 2016). At the site of inflammation, IL-6 is the chief stimulator of acute-phase proteins, including C-reactive protein (CRP), fibrinogen and haptoglobin, among others (Gabay, 2006). The binding of IL-6 to sIL-6R activates monocyte chemoattractant protein (MCP)-1, induces the expression of adhesion molecule, and enhances leukocyte infiltration, thus increasing vascular permeability (Romano et al., 1997). Furthermore, platelets are released when activated IL-6 reaches the bone marrow, and platelet count is used to evaluate severity of inflammation in clinical settings (Ishibashi et al., 1989; Tanaka et al., 2014).

2.2.2.4. Nitric oxide (NO)

NO is a signaling molecule member of reactive oxygen species (ROS) containing one nitrogen atom covalently bonded to an oxygen atom with an unpaired electron (Sharma et al., 2007). NO generation is catalyzed by nitric oxide synthase (NOS) by converting L-arginine and molecular oxygen into NO and L-citrulline, utilizing electrons donated by NADPH (Tripathi et al., 2007). There are three types of NOS, two constitutively expressed in cells, known as neuronal NOS (nNOS) and endothelial NOS (eNOS), and an isoform only expressed in activated immune cells, known as inducible NOS (iNOS) (Förstermann & Kleinert, 1995). The activation of the iNOS gene promoter is regulated by cytokines; thus, cytokines determine the *de novo* synthesis and stability of iNOS mRNA. The expression of iNOS following inflammation is considered to be a vital component of the host's defense against noxious stimuli (Tripathi et al., 2007). For instance, NO can induce vasodilation and modulate neutrophil adhesion, thus

contributing to inflammation; NO also regulates leukocyte cytokine synthesis and stimulates macrophage motility (Bogdan et al., 2000; Madan & Rao, 1996).

2.3. Adipose Tissue

Obesity is defined by the World Health Organization (WHO) as abnormal or excessive fat accumulation that brings risks to health (World Health Organization, 2021). Obesity is a risk factor for chronic diseases, such as cardiovascular diseases, type 2 diabetes, respiratory disorders, and hormone-dependent cancers. Multiple factors result in obesity, including impaired energy expenditure versus energy consumed. Therefore, the excess energy is stored as lipids, in the form of triacylglycerol, in adipose tissues (Finer, 2015; Otto & Lane, 2005). Apart from the role of energy source, the adipose tissue serves as an endocrine organ and physiological cushion (Sarantopoulos et al., 2018).

The adipose tissue, commonly called “fat,” is a loose connective tissue located beneath the skin or surrounding organs, comprised of collagen fibers, blood vessels, immune cells, and lipid-filled cells (adipocytes) (Kershaw & Flier, 2004). Thus, adipose tissue exhibits cellular heterogeneity. Regarding adipocytes, both pre-adipocytes (stem cells committed to adipocyte lineage) and mature functioning adipocytes are found in the adipose tissue (Ambele et al., 2020). The adipocyte number and volume govern the changes in the adipose tissue mass. The three events that impact the adipocyte number are pre-adipocyte maturation by adipogenesis, pre-adipocyte replication, and cell apoptosis (Warne, 2003). At the same time, adipocyte volume is impacted by the enlargement of existing adipocytes, known as hypertrophy (Ambele et al., 2020). These processes are controlled by various factors, including prostaglandins, glucocorticoids, glycoproteins, and cytokines (MacDougald & Mandrup, 2002).

2.3.1. Adipogenesis

Once a multipotent stem cell is restricted to the adipogenic lineage, it develops into a pre-adipocyte that undergoes adipogenesis. Adipogenesis is the process by which pre-adipocytes mature and fully develop into functioning adipocytes (Warne, 2003). Adipogenesis comprises growth arrest, followed by one or two rounds of mitotic clonal expansion, and then transcriptional activation of adipocyte genes that lead to the adipocyte phenotype differentiation (MacDougald & Mandrup, 2002; Otto & Lane, 2005). The key transcriptional regulators of adipose tissue are the CCAAT/enhancer-binding protein-α (C/EBP-α) and peroxisome proliferator-activated receptors-γ (PPAR-γ) (Sarantopoulos et al., 2018). C/EBP-α is a transcriptional activator of adipocyte-specific genes, which has an anti-mitotic activity, so its expression is delayed until the pre-adipocytes undergo the required mitotic clonal expansion for subsequent differentiation (Otto & Lane, 2005). Even though C/EBP-α is required for differentiation, evidence suggests that PPAR-γ is adipogenesis's central regulator. PPAR-γ is responsible for activating genes involved in the binding, uptake and storage of fatty acids, such as lipoprotein lipase, acyl-coenzyme A synthase and phosphoenolpyruvate carboxykinase (Schoonjans et al., 1996; Tontonoz et al., 1994). Once expressed, C/EBP-α and PPAR-γ regulate each other's expression (Otto & Lane, 2005).

2.3.1.1. Adipogenesis inhibition by cytokines

Several agents have been shown to repress adipogenesis, including inflammatory cytokines. Exposure of pre-adipocytes to the inflammatory cytokines TNF-α, IL-1, IL-6, and IL-11, has demonstrated inhibition of pre-adipocyte differentiation *in vitro* (Grégoire et al., 1992; MacDougald & Mandrup, 2002; Ohsumi et al., 1994). For instance, TNF-α has been shown to downregulate C/EBP-α and PPAR-γ encoding genes (B. Zhang et al., 1996). This disruption can

be explained by TNF-α blocking the mitotic clonal expansion required for adipogenesis (Lyle et al., 1998). In addition, other studies have demonstrated that TNF-α might be involved in the activation of the classic mitogen-activated protein (MAP) kinase, extracellular regulated kinase (ERK) 1 and 2, a pathway that causes phosphorylation of PPAR-γ, consequently inhibition of adipogenesis (Font de Mora et al., 1997; Rosen & Spiegelman, 2000; Warne, 2003). Therefore, TNF-α may promote weight loss and reduce the triglyceride pool by decreasing the adipocyte number through adipogenesis inhibition (Warne, 2003).

2.4. Immunomodulatory Dietary Polysaccharides

Polysaccharides are an abundant group of biopolymers implicated in biological processes, such as cell to cell communication and cellular immunity (Liu et al., 2015). Furthermore, these interactions have been associated with potential stimulatory effects on the immune system, strengthening innate and adaptive immune responses (Ferreira et al., 2015). Therefore, it is thought that the mechanisms involved in the dietary polysaccharides' immunomodulatory effects rely on enhancing or activating macrophage immune responses through their surface receptors, such as toll-like receptor 4 (TLR-4) (Schepetkin & Quinn, 2006; Zhang et al., 2016). In this sense, polysaccharides can work as biological response modifiers, regulating activities such as inflammation, thus pathogen infection response (Zhang et al., 2016).

Different extracted plant polysaccharides have been reported to mediate the activation of macrophages through TLR-4. These extracted polysaccharide fractions include botanical sources like elderberries (*Sambucus nigra* L.) (Ho et al., 2015), safflower (*Carthamus tinctorius* L.) (Ando et al., 2002), *Dendrobium huoshanense* (Xie et al., 2016), *Acanthopanax senticosus* (Han et al., 2003), *Solanum nigrum* (Razali et al., 2014), and *Tinospora cordifolia* (Gupta et al., 2016). In particular, these compounds have been shown to increase macrophage cytotoxic activity by

the increased production of reactive oxygen species, like NO, as well as the enhanced secretion of pro-inflammatory cytokines (TNF-α, IL-1β, IL-6, IL-8 and IL-12) (Schepetkin & Quinn, 2006). It has been hypothesized that various factors may be implicated in the polysaccharide's immunomodulatory activity, such as the monosaccharide composition, glucosidal bonds, molecular size, branching degree and the overall molecular conformation (Zhang et al., 2016). Due to the potential of dietary polysaccharides as ingredients with preventive or therapeutical purposes, future research should encompass the extraction of polysaccharides, such as fibers, from crops that are not stapled in America but may increase their value if they are fractionated, for instance, dry beans.

2.5. References

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3. PHYSICOCHEMICAL PROPERTIES OF DRY BEAN FLOURS AND THEIR SOLUBLE DIETARY FIBER EXTRACTION AND CHARACTERIZATION

3.1. Abstract

Dry beans are rich in proteins, starch, dietary fiber, and phenolic compounds. Dry bean fractions, like soluble dietary fiber (SDF), can be considered functional food ingredients due to their potential positive diet-related health effects. This study aimed first to investigate the impact of the dry bean market class and cooking processing on dry bean flour physicochemical properties; and second to extract and characterize the SDF-rich fraction from the dry bean flours. The cooking processing significantly ($p < 0.05$) impacted macronutrient composition, enhancing dietary fiber content in dry bean flours. Extractable phenols and antioxidant activity were significantly ($p < 0.05$) higher in raw dry bean flours, whereas hydrolyzable phenols and their antioxidant activity were significantly ($p < 0.05$) higher in the cooked counterparts. Regarding market class, pinto beans had significantly ($p < 0.05$) higher slowly digestible starch (23.76 %) and resistant starch contents (5.24 %) than black beans (20.63 % and 3.22 %, respectively). Then, the SDF-rich fraction extracted from cooked dry bean flours had significantly ($p < 0.05$) lower residual protein content. The extracted SDF-rich fractions sugar composition indicated the presence of pectic polysaccharides, hemicelluloses and RFOs. SDF-rich fractions molecular weight distribution was significantly ($p < 0.05$) affected by cooking processing. Overall, SDF-rich fraction solutions exhibited a shear-thinning behavior. The obtained results contribute to the knowledge of dry beans' potential health benefits and how they are affected by the traditional cooking process and market classes.

3.2. Introduction

Legumes have been recognized as important sources of protein, minerals, starch and non-starch carbohydrates (dietary fiber), and phytochemicals for human nutrition (Aguilera et al., 2009; Dalgetty & Baik, 2003; Siddiq & Uebersax, 2013; Tharanathan & Mahadevamma, 2003). Dry beans (*Phaseolus vulgaris* L.) are the most extensively produced crops within legumes (Celmeli et al., 2018). According to the United States Department of Agriculture (USDA), dry bean production has decreased within the last five years, going from 36 million hundredweight (cwt) in 2017 to 22.7 million cwt in 2021, having a record-high production in 2018 of 37.5 million cwt in contrast with the record-low production in 2019 of 20.8 million cwt (NASS & USDA, 2022a). Of the eighteen states that produce dry beans on a commercial scale, North Dakota, Michigan, Nebraska, Idaho, and Minnesota stand out as the top producers (Ag Marketing Resource Center, 2022). Regarding market classes, pinto and navy beans have historically been the leading produced classes in the United States; however, black beans have dramatically increased their production (Siddiq & Uebersax, 2013). For instance, pinto beans represented 32.73 % and black beans 20.44 % of the total American dry bean production in 2021, followed by 15.59 % from navy beans (NASS & USDA, 2022a). From the leading dry bean market classes, North Dakota has been the number one producer of pinto beans from 2017 to 2021 and the second major black bean producer within the same period with slightly lower production than Michigan (NASS & USDA, 2022b).

As dry beans are typically consumed as whole seeds, they are subjected to a constant but low market value. In this sense, Dalgetty & Baik (2003) proposed that by fractioning legumes into their major components, like starch, protein and dietary fiber, their utilization and market value will be increased. Furthermore, increasing attention to legumes' nutritional value has been

gained due to the consumers' trend to look for healthy foods (Aguilera et al., 2009). Therefore, the supplementation of food products with dry bean components is expected to improve the nutritional value of the final products (Rui & Boye, 2013). Additionally, dry beans are unique among protein-rich foods due to their low-fat content and rich micronutrient, phenolic, dietary fiber, and resistant starch contents (Messina, 2014).

Some of the potential health benefits attributed to dry beans derived from their phytochemical content since the inverse association between the risk of chronic human diseases and the consumption of a diet rich in phenolic compounds has been observed in previous studies (García-Lafuente et al., 2014; López et al., 2013; Quirós-Sauceda et al., 2014). During oxidative stress, the formation of free radicals known as Reactive Oxygen Species (ROS) induces damage such as lipid peroxidation, protein disruption, and DNA crosslinking (Pietta, 2000). The reduction of chronic disease is due to increased plasma antioxidant capacity, which confers cells protection against oxidative damage (López et al., 2013). Previous research has shown that phenolic compounds in dry beans restrain the development of initiating free radical species through metal ions (e.g., iron) chelation or enzyme inhibition (Rocha-Guzmán et al., 2007). For instance, Oomah et al. (2010) reported that phenolic compounds extracted from four different dry bean market classes, including pinto and black beans, inhibited the formation of key enzymes needed for ROS production, such as xanthine oxidase, lipoxygenase, cyclooxygenase, and NADH oxidase. In this sense, it is assumed that dietary phenols, like flavonoids, display their first antioxidant defense by limiting ROS formation, followed by scavenging them (Pietta, 2000). Therefore, the consumption of dietary antioxidants is relevant to attenuating the harmful effects of oxidative processes in living organisms (Nordberg & Arnér, 2001).

Additionally, dietary fiber is a functional ingredient of great interest because it has been associated with the risk reduction of some chronic diseases, thus conferring health benefits. Dietary fiber can be classified by its water solubility into insoluble dietary fiber (IDF) and soluble dietary fiber (SDF) (Chawla & Patil, 2010). The IDF mainly consists of cell wall components, like cellulose and lignin (Dreher, 2001). The porous IDF is considered metabolically inert and resistant to colonic fermentation (Bader Ul Ain et al., 2019). Therefore, several benefits have been positively correlated with the consumption of IDF, such as the inhibition of pancreatic lipase activity and the stimulation of bowel evacuation, which leads to the detoxification of the digestive tract (He et al., 2015). On the other hand, SDF consists of non-cellulosic polysaccharides, such as pectins, gums and mucilages (Dreher, 2001). Therefore, it has been reported to have functional properties as an emulsifier, a gelling agent, and even an antioxidant. These functional properties confer beneficial effects like reducing glycemic response, microflora build-up leading to a greater gut fermentability, preventing gastrointestinal problems by reducing constipation, and anti-inflammatory activity on the digestive tract (Bader Ul Ain et al., 2019; Chawla & Patil, 2010). However, even when dry beans have the highest dietary fiber content when compared to chickpeas, peas and lentils (Kutoš et al., 2003), few studies (Hooper et al., 2017; Kutoš et al., 2003; Tosh & Yada, 2010) have been performed to isolate dietary fiber from dry beans and characterize its composition and physicochemical properties.

Due to the potential positive diet-related health effects, dry bean fractions can be considered functional food ingredients. Therefore, assessing the physicochemical properties of dry bean fractions is needed to fully understand them as ingredients and their impact on physical and functional properties in product formulations. In this regard, the aim of this study was first to

investigate the impact of the dry bean market class and cooking processing on dry bean flours physicochemical and antioxidant properties; and second to extract and characterize the SDF-rich fraction from the analyzed dry bean flours.

3.3. Materials and Methods

3.3.1. Materials

The two dry bean varieties, Monterrey (Pinto bean) and Eclipse (Black bean) were used to test, two samples from each variety. Both varieties were grown in 2018 at three different North Dakota Agricultural Experiment Stations located at Forest River, Hatton, and Prosper, ND. The North Dakota State University (NDSU) Dry Bean Breeding Program kindly provided all samples.

3.3.2. Preparation of Raw and Cooked Bean Samples

Raw and cooked bean samples were milled into flour. First, samples were cleaned by removing foreign material and damaged seeds. Then, raw dry bean flour of each variety was prepared as described by Ovando-Martínez et al. (2011). Briefly, 25 g of raw dry bean seeds were milled with a hammer-type cyclone mill (Laboratory Mill 3100, Perten Instruments, Springfield IL) with a standard sieve of 0.8 mm.

For the cooked samples, cooking time was determined as described by Hooper et al. (2017). Briefly, a Mattson pin drop cooker, which consists of 25 stainless steel 70 g piercing tip rods, was placed in contact with the middle surface of 25 dry beans. The Mattson cooker was placed on a hot plate into a 6 L beaker containing 2 L of boiling distilled water. Cooking time was recorded as the time required for 90% of the 2 mm diameter piercing tip rods to pass through each bean under a low steady boiling. Then, 100 g of dry bean samples were cooked according to their optimum cooking time. Cooked bean samples were freeze-dried, then grounded with a

commercial grain grinder (CGOLDENWALL, US) at 28000 rpm passing through a standard sieve of 0.8 mm. All flour samples were kept in airtight plastic bags in refrigeration until their analysis.

3.3.3. Proximate Analysis of Raw and Cooked Beans

Moisture and ash were determined gravimetrically with the AACC-approved methods 44-15.02 and 08-01.01, respectively (AACC, 2010). Nitrogen (N) was determined using a Leco (LECO Corp. St. Joseph, MI, USA) combustion nitrogen analyzer (AACC approved method 46-30.01, AACC, 2010). Crude protein content was calculated as N×6.25. Lipid content was determined by extracting the ground sample with hexane for three hours using a Soxhlet apparatus according to the AOCS (1998) official method Ba 3-38. A total starch assay kit (Megazyme International, Co. Wicklow, Ireland) was used to analyze total starch content on a dry weight basis for each sample following the approved method 76-13.01 (AACC, 2010).

3.3.4. Phytic Acid Content

The phytic acid content of raw and cooked dry bean flour samples was determined according to Haug & Lantzsch (1983) with modifications by Guttieri et al. (2006). Phytic acid was extracted with 0.2 M hydrochloric acid overnight. The sample extracts and standard solutions (ranging from 1.5 to 25 µg/mL) were boiled before adding 1 mL 415 µM ferric ammonium chloride solution. After cooling, the samples were added to microplates along with 150 µL of 2,2-bipyridine-thioglycolic acid, and the absorbance was read at 530 nm (Guttieri et al., 2006). The phytic acid content was determined by plotting the absorbance of the standard curve against concentration.

3.3.5. In Vitro Starch Digestibility

In vitro starch digestibility of raw and cooked beans was analyzed using the method described by Englyst et al. (1992). The samples were weighed (0.3 g) into 50 mL plastic centrifuge tubes. The samples were incubated at 37°C with an enzyme mix (amyloglucosidase, invertase, and pancreatin) for 180 min. Aliquots of the digest were taken every 20 min to determine the amount of glucose released by reaction with glucose oxidase/peroxidase (GOPOD). A glucose standard curve was used to calculate the amount of glucose released. Then the rapidly digestible starch (RDS), slowly digestible starch (SDS), and resistant starch (RS) were determined from the amounts of glucose at 20 and 120 min.

Hydrolysis index (HI) was obtained by dividing the area under the hydrolysis curve of the sample by the area obtained for white bread. Then, the expected glycemic index (eGI) was calculated using the equation described by Ovando-Martínez et al. (2011): $eGI = 8.198 + 0.862HI$.

3.3.6. Determination of Phenols

Raw and cooked dry bean flour samples were subjected to constant shaking at room temperature with methanol/water acidified with HCl (50:50 v/v, pH 2, 50 mL/g of sample, 60 min, room temperature, constant shaking) and acetone/water (70:30 v/v, 50 mL/g of sample, 60 min, room temperature). After centrifugation (15 min, 25°C, 3,000 × g), supernatants were combined and used to determine extractable phenols. Gallic acid was used to prepare a standard curve (ranging from 25 to 250 ppm). Extractable phenols were determined by the Folin–Ciocalteu procedure (Singleton et al., 1999). The results were expressed as gallic acid equivalents.

Hydrolyzable phenols were extracted by a methanol/18.4 M H_2SO_4 90:10 (v/v) hydrolysis at 85°C for 20 h from the residues of methanol/acetone/water extraction that were left from the determination of soluble phenols (Hartzfeld et al., 2002); after two centrifugation steps (15 min, 25°C, 3,000 × g), supernatants were combined and used to determine the hydrolyzable phenols by the Folin–Ciocalteu method with a gallic acid standard curve (ranging from 25 to 250 ppm) (Singleton et al., 1999). The results were expressed as gallic acid equivalents.

3.3.6.1. Metal ions chelating activity

Metal ions chelating activity was measured according to the method described by (Xie et al., 2008). Briefly, 1 mL of sample solution was pre-mixed with 0.05 mL of iron dichloride solution (2 mmol/L) and 1.1 mL of distilled water. Then, 0.4 mL of ferrozine solution (5 mmol/L) was added and mixed vigorously. The mixture was incubated at room temperature for 10 min. Absorbance was measured at 562 nm. EDTA was used to prepare a standard curve (ranging from 0 to 250 ppm). The results were expressed as mg of EDTA per g of dry matter.

3.3.6.2. Free radical scavenging assay (ABTS)

The antioxidant capacity was estimated in terms of radical scavenging activity as described by (Ovando-Martínez et al. (2014). Briefly, ABTS radical cation (ABTS+•) was produced by reacting 7 mmol/L ABTS stock solution with 2.45 mmol/L potassium persulphate in the dark at room temperature for 12 - 16 h before use. First, the ABTS+• solution was diluted with methanol to an absorbance of 0.70 ± 0.02 at 750 nm. Then, after adding 100 µL of sample to 390 µL of diluted ABTS+• solution, absorbance was read at 750 nm. Finally, the results were expressed as mg of Trolox equivalents per g of dry matter.

3.3.6.3. Ferric-reducing antioxidant power (FRAP)

The antioxidant capacity was also estimated in terms of the reduction of a ferric-tripyridyl triazine complex to its ferrous-blue-colored form due to the presence of antioxidants, as described by Benzie & Strain (1996). Briefly, the FRAP reagent consisted of TPTZ (2,4,6-tripyridyl-S-triazine) solution (10 mmol L−1) in HCl (40 mmol L−1) plus $FeCl_3$ (20 mmol L−1). In a microplate, 20 µL of the sample were mixed with 280 µL of FRAP reagent. The mixture was incubated in the dark at room temperature for 10 min. Absorbance was measured at 595 nm. The results were expressed as mg of Trolox equivalents per g of dry matter.

3.3.7. Total, Soluble, and Insoluble Dietary Fiber

Dietary fiber was measured according to the AACC official method 32-07.01. The MES (2-(N-morpholino) ethane sulfonic acid)-TRIS (tris (hydroxymethyl) aminomethane) buffer was used. Duplicate 1 g of raw and cooked dry bean flour samples were subjected to sequential enzymatic digestion by heat-stable alpha-amylase, protease, and amyloglucosidase. Insoluble dietary fiber (IDF) was filtered, and the residue was washed with warm distilled water. A solution combining filtrate and water washings was precipitated with four volumes of 95% ethanol for soluble dietary fiber (SDF) determination. The precipitate was then filtered and dried. Both SDF and IDF residues were corrected for protein, ash, and blank for the final SDF and IDF values. TDF value was the sum of SDF and IDF values.

3.3.8. Soluble Dietary Fiber (SDF) Rich Fractions Extraction

The SDF-rich fraction was extracted by the enzymatic-gravimetric procedure according to Feng et al. (2017) with slight modifications. Briefly, six grams of raw and cooked dry bean flour samples were dispersed in 240 mL MES-TRIS buffer (pH 8.2) mixed with magnetic stirring for one hour. Then 300 µL of a-amylase solution were added at 95°C - 100°C under

constant shaking for 35 min. After the temperature of the hydrolysate decreased to 60°C, 600 μL of protease solution was added for further hydrolysis under constant shaking for 30 min. Next, the pH of the mixture was adjusted to 4.5 by acetic acid (3 M). Next, amyloglucosidase (600 μL) was added for final hydrolysis at 60°C for 30 min under constant shaking. The hydrolysate was filtrated, and the sediment was washed twice with distilled water. The filtrate was collected and condensed to about one-tenth in a vacuum rotary evaporation system. Then, the concentrated filtrate was mixed with 95% (v/v) ethanol at 60°C for one hour, followed by centrifugation at 4500 rpm for 10 min. Finally, the precipitated flocculate was freeze-dried and saved as the SDF-rich fraction.

3.3.9. Modified Lowry Protein Method Determination

The determination of protein in the extracted SDF-rich fraction was done according to the Thermo Scientific modified Lowry protein assay kit (23240), which combines a stabilized formulation of the original Lowry reagent and the Folin-Ciocalteu Reagent. Bovine Serum Albumin (BSA) (1−1500 mg/L) from Thermo Scientific was used as the standard for calibration curve preparation. First, SDF-rich fraction samples were diluted to 0.75 mg/mL with distilled water. Then, 200 μL of standard or sample were added to 1.0 mL of the Modified Lowry reagent at 15 seconds intervals. After 10 min of incubation at room temperature, 0.1 mL of Folin-Ciocalteu Phenol reagent was added at 15 seconds intervals. Finally, the samples were incubated at room temperature for 30 min, and the absorbance of all the samples was measured at 750 nm.

3.3.10. Carbohydrate Analysis by HPAEC-PAD

The sugar composition was determined by high-performance anion-exchange chromatography (HPAEC) with pulsed-amperometric detection (PAD). The samples were hydrolyzed according to Nishitsuji et al. (2020) method, with some modifications. The sample

(2-3 mg) was weighed into a glass screw cap test tube. A 0.5 mL aliquot of 1 M HCl was added, and the sample was heated at 100°C for 1 hour. Next, the sample was cooled to room temperature before adding 0.5 mL of 1 M NaOH to neutralize the sample. The analysis was conducted according to Nagel et al. (2014) method. The sample was diluted with 1 mL of ASTM Type II deionized water before being filtered through a 0.45 µm nylon syringe filter. The samples were analyzed with a Thermo Fisher DIONEX ICS5000+ using a CarboPac PA-20 analytical column and a CarboPac PA-20 guard column. The standard curve was prepared using arabinose, galactose, glucose, xylose, fructose, and galacturonic acid.

3.3.11. Molecular Weight Determination

The samples were prepared according to Simsek et al. (2015) to determine SDF-rich fraction molecular mass. The SDF was dissolved in a 1:10 (v/v) solution of 6 M urea and 1 M KOH and heated for 90 min at 100°C. The samples were then neutralized using 1 M HCl and filtered through 0.45 µm nylon syringe filters before analysis by high-performance size exclusion chromatography (HPSEC, Agilent Technologies, USA) with multi-angle light scattering (MALS, Wyatt Technology, USA). The results were fitted to a first-order polynomial model. For further details about the method refer to Appendix A.

3.3.12. Rheological Measurements

The rheological measurements were carried out using a Discover HR2 rheometer (TA Instruments, USA) equipped with a water circulator as the temperature controller. The rheological studies were performed using a parallel plate (40 mm diameter with a gap of 500 mm).

The flow behavior was studied as described by Feng et al. (2017). Briefly, SDF-rich fraction aqueous solutions were prepared as follows: 2%, 4%, 6% (w/v) of samples were

prepared with distilled water at 55°C under continuous magnetic stirring for one hour. After totally dissolved, the solutions were equilibrated at 4°C overnight to allow the elimination of entrapped air. The apparent viscosity was recorded by a function of shear rate ranging from 0.1 to 1000 s^{-1} from the logarithmic ramp at 25°C.

3.3.13. Statistical Analysis

The statistical analyses were conducted with SAS software (version 9.4, SAS Institute, Cary, NC, USA). The experimental design was a split-plot design, where the main plot is the growing location, and the sub-plot is a combination of dry bean variety market class and the processing treatment. The analysis of variance (ANOVA) for the main effects and interactions was determined by the 'MIXED' procedure with the 'CONTRAST' option in SAS. The least significant difference (LSD) value was used to separate means, and the significance was accepted at $p<0.05$. The different parameters were correlated by Pearson's two-tailed significant correlation using the 'CORR' procedure.

3.4. Results and Discussion

3.4.1. Chemical Composition of Raw and Cooked Bean Flours

In order to improve digestibility or even improve the suitability for particular product applications, such as specific ingredients extraction, dry beans are treated with thermal processes. The thermal processes use controlled temperature to modify raw whole seeds, flours, concentrates or isolates (Rebollo-Hernanz et al., 2019). One of the most common dry bean thermal processes is cooking in boiling water at atmospheric pressure (AP) without pre-soaking (Ovando-Martínez, Osorio-Díaz, et al., 2011; Rebollo-Hernanz et al., 2019). The chemical composition of raw and cooked (with boiling water at AP without pre-soaking) dry bean flour samples is presented in Table 1.

Significant ($p < 0.05$) differences in the beans composition were found between dry bean market classes and processing conditions (Table 1). The moisture content was significantly ($p < 0.05$) higher in raw samples (8.03 – 8.65 %) compared to the cooked samples (2.62 – 3.10 %). The loss of moisture observed in the cooked samples might be caused by the freeze-drying process the cooked samples underwent before being milled, whereas the dry bean flour from raw samples reflected the moisture content of the whole seeds. Furthermore, the pinto dry bean samples had the highest moisture contents within both processing conditions. This trend was observed in the results reported by Choe et al. (2022), where both raw and boiled pinto dry bean derived flours had higher moisture contents when compared with their black bean counterparts. The observed differences could be correlated to the dry bean market class seed size, as pinto dry bean seeds per pound ranged from 1200-1600 compared to the range of 2100-2500 black dry bean seeds per pound (Kandel & Endres, 2019); thus the larger the seed size, the more moisture it will accumulate.

The ash content decreased significantly ($p < 0.05$) in flours produced from cooked samples compared to flours from raw dry beans. A decrease in ash content after dry beans underwent a cooking process was also reported by Choe et al. (2022), Piecyk et al. (2012), and Wang et al. (2010). The ash content decreases due to the loss of water-soluble minerals in the cooking water. Within treatments, the ash contents did not significantly ($p < 0.05$) differ among the cooked samples, whereas the raw pinto dry beans had a significantly ($p < 0.05$) higher ash content (3.83 %) compared to the raw black dry bean samples (3.59 %). These results agree with Wang et al. (2010) study, which described a higher mineral content in pinto beans, where calcium, iron, potassium, magnesium, and zinc were the minerals with the highest concentration in raw samples.

The protein content values for raw and cooked pinto and black dry bean flour samples agreed with protein contents reported by Choe et al. (2022) and Ovando-Martínez, Bello-Pérez et al. (2011) of more than 20 % for the same studied dry bean market classes. However, the obtained results contrast with those reported by Ovando-Martínez, Bello-Pérez, et al. (2011), where the protein content of beans decreased after thermal processing. In contrast, our results showed that the cooking process resulted in a significant ($p < 0.05$) increase in protein content, a trend also reported by Choe et al. (2022) and Wang et al. (2010). The observed increase has been attributed to the loss of soluble solids during cooking. Thermal processing has been suggested to increase the insoluble protein fractions due to increased crosslinking between phytic acid, condensed tannins and proteins, along with the formation of Maillard products (Azarpazhooh & Boye, 2013; Rebollo-Hernanz et al., 2019). Therefore, the formation of the complexes and the reduction of soluble solids due to cooking may explain the observed protein content increase.

Lipid content in dry beans is low, typically ranging from 2 to 5 % (Azarpazhooh & Boye, 2013). In this sense, the obtained lipid contents fall within the expected range for dry beans. No significant ($p < 0.05$) differences were observed among dry bean market class or processing.

Dry beans have a starch content of around 40 % on a dry-weight basis (Rui & Boye, 2013). The obtained values (37.47 – 40.24 %) agree with the expected starch content. The pinto dry bean samples had the significantly ($p < 0.05$) highest starch content regardless of the processing. Regarding processing, the starch content values increased after the samples were cooked. The obtained results are in line with Wang et al. (2010) study, which described an increase in starch content after different dry bean market classes were cooked, reporting that the pinto bean starch contents were higher than those from the black bean samples.

Phytic acid (myo-inositol hexaphosphate or $InsP_6$) is plants' primary phosphorus storage form. It is highly abundant in dry beans, located in the protein bodies of the endosperm (Campos-Vega et al., 2013). The assessment of $InsP_6$ levels is relevant since it is regarded as an antinutrient due to its mineral, protein and even starch binding capacity, thus impairing nutrients bioavailability (Grases et al., 2001). From the analyzed samples, it was observed that within raw samples, the pinto beans (9.15 mg/g) had a significantly ($p < 0.05$) higher $InsP_6$ content when compared to black bean samples (4.38 mg/g). The observed results agree with Oomah et al. (2008), which reported greater $InsP_6$ levels in pinto than in black dry beans. Furthermore, a significant ($p < 0.05$) increase in the $InsP_6$ content was seen after dry bean samples underwent cooking, however, no significant ($p < 0.05$) differences were found between cooked dry bean classes. The documented $InsP_6$ increase contrasts with Campos-Vega et al. (2010) observations that show $InsP_6$ concentrations tend to be higher in raw dry beans. Despite this, the observed $InsP_6$ values from the cooked samples were comparable to previously reported results (Wang et al., 2010; Wang et al., 2017).

Table 1. Dry bean flour proximate analysis and phytic acid content.

Sample	Moisture (%)	Ash (% db[a])	Protein (% db)	Lipid (% db)	Starch (% db)	Phytic acid (mg/g)
Raw						
Pinto	8.65 a	3.83 a	22.58 c	4.15 a	39.84 a	9.15 b
Black	8.03 b	3.59 b	20.65 d	4.03 a	37.47 c	4.38 c
Cooked						
Pinto	3.10 c	2.63 c	24.60 a	4.35 a	40.24 a	12.15 a
Black	2.62 d	2.61 c	22.75 b	4.43 a	38.47 b	12.15 a

Means with the same letter within columns are not significantly different ($p < 0.05$). The data are the means of two independent replicate experiments ($n = 2$).
[a] % db: Results expressed on a dry weight basis.

Dietary fiber content is a relevant parameter for determining dry beans' processing quality and physiological value (De Almeida Costa et al., 2006). Total dietary fiber (TDF) consists of soluble (SDF) and insoluble dietary fiber (IDF), which are likely to change during processing (Azarpazhooh & Boye, 2013; Brummer et al., 2015). The analyzed raw and cooked dry bean flours IDF, SDF, and TDF composition are shown in Table 2. Significant ($p < 0.05$) increases in IDF, SDF, and TDF contents after the samples underwent the cooking process, regardless of the dry bean market class, were found. The observed increments in fiber contents of cooked beans were reported by Wang et al. (2010) in seven different market classes, including pinto and black beans. Interestingly, the TDF contents within the processing treatments were higher in black dry beans than in pinto dry beans. Moghaddam et al. (2018) reported higher dietary fiber contents from black beans when compared to pinto, pink and white dry beans. Furthermore, the higher TDF content might be attributed to the seed size. As smaller seeds, like black bean ones, have a bigger surface to volume ratio, thus a higher seed coat to cotyledon proportion (Wang et al., 2003), then smaller seeds will exhibit higher fiber content concentration associated with the seed coat than larger size seeds will do (Wang et al., 2010). Regarding the SDF content, the observed increase after the cooking process will be due to the depolymerization and solubilization of hemicelluloses and insoluble pectic substances from the middle lamella of the cotyledon cell walls (De Almeida Costa et al., 2006; Kutoš et al., 2003; Tosh & Yada, 2010) and increased content of resistant starch induced by the thermal processing, causing amylose retrogradation (Tharanathan & Mahadevamma, 2003). The observed increase in IDF may be attributed to the formation of protein-fiber complexes during the thermal processing (Wang et al., 2010), known to impair protein solubility. Despite the observed IDF increase in the cooked dry bean flours, the IDF to SDF ratio decreased in the cooked samples. In other words, around 80 % of the TDF

corresponded to the IDF fraction in the raw dry bean samples, whereas in the cooked samples, the IDF fraction represented 75 % of the TDF. The 3:1 IDF to SDF ratio is typical of cooked dry beans without pre-soaking. Due to extensive material solubilization, soaking dry beans before cooking has been observed to decrease the SDF content (Kutoš et al., 2003).

Table 2. Dry bean flour content of insoluble, soluble and total dietary fiber, and its distribution.

Sample	IDF [a] (%)	SDF [b] (%)	TDF [c] (%)	IDF:SDF
Raw				
Pinto	21.83 c	5.10 c	26.93 d	4:1
Black	24.23 ab	4.63 c	28.82 c	5:1
Cooked				
Pinto	23.57 b	7.62 b	31.20 b	3:1
Black	25.25 a	8.53 a	33.78 a	3:1

Means with the same letter within columns are not significantly different ($p < 0.05$). The data are the means of two independent replicate experiments (n = 2).
[a] IDF: Insoluble dietary fiber.
[b] SDF: Soluble dietary fiber.
[c] TDF: Total dietary fiber.

3.4.2. *In Vitro* Starch Digestibility

Legumes, such as dry beans, are considered starchy foods with a low glycemic index compared to cereal starches (Rebollo-Hernanz et al., 2019; Tharanathan & Mahadevamma, 2003). Since starch is mainly packed between the cotyledon protein bodies, Choe et al. (2022) suggested that starch digestibility is determined by the protein structure attached to the intracellular starch granules, thus working as a barrier against starch digestion. Furthermore, uncooked starches are less susceptible to enzymatic hydrolysis due to their highly ordered crystalline structure (Oomah et al., 2011). Since dry beans are generally cooked before being consumed (Hooper et al., 2017), the starch fractions, hydrolysis index (HI) and estimated glycemic index (eGI) of the analyzed cooked dry bean market classes obtained by *in vitro* starch digestion are shown in Table 3.

Significant ($p < 0.05$) differences between dry bean market classes were found for all starch fractions. Pinto bean samples had a significantly ($p < 0.05$) lower rapidly digestible starch (RDS) fraction (12.53 %) compared to black bean samples (15.66 %). In contrast, both slowly digestible starch (SDS) and resistant starch (RS) fractions were significantly ($p < 0.05$) higher in pinto bean samples (23.76 % and 5.24 %, respectively) than in black bean samples (20.63 % and 3.22 %, respectively). The obtained RDS contents agree with Güzel & Sayar (2012). They reported RDS contents of 12 to 16 % for different pulses, including dry beans. The RDS contents were lower than those reported for other starchy foods, including corn, tapioca, rice, potato, and wheat, ranging from 60 to 80 % (Simsek et al., 2015). In contrast with RDS, SDS is slowly digested in the small intestine, resulting in a slow release of glucose into the bloodstream, thus a low glycemic response (Miao et al., 2015). The SDS contents were slightly higher than those reported by Choe et al. (2022) for cooked pinto and black beans (16.68 and 16.65 %, respectively). The RS is not digested in the small intestine but fermented in the colon as dietary fiber, resulting in the production of short-chain fatty acids (Güzel & Sayar, 2012; Miao et al., 2015). The RS contents were lower than the cooked dry bean ones reported by Choe et al. (2022), Güzel & Sayar (2012) and Ovando-Martínez, Bello-Pérez, et al. (2011), ranging from 10 to 28 %. However, the SDS and RS obtained contents are considerably higher than those reported for starchy foods (Simsek et al., 2015), thus considered nutritionally beneficial for their potential consumers.

Furthermore, the HI and eGI were significantly ($p < 0.05$) higher for pinto beans (64.65 % and 69.93 %, respectively) than for black beans (60.81 % and 60.62 %, respectively). Differences in starch digestibility have been attributed to granule size, amylose/amylopectin ratio, and amylopectin chain length distribution (Ovando-Martínez, Bello-Pérez, et al., 2011); so,

the variability of these features will result in different glycemic responses. In this sense, Ovando-Martínez, Bello-Pérez, et al. (2011) reported that a higher HI from cooked pinto bean samples was due to a higher proportion of B2 and B3 amylopectin long chains when compared to black bean samples. Despite the significant ($p < 0.05$) differences for eGI observed between dry bean market classes, both values corresponded to a medium GI category, where values range from 56 to 59 (Pruthi, 2020). Therefore, no dramatic differences in the rise of blood glucose would be observed after the consumption of the analyzed samples.

Table 3. Cooked dry bean flour starch fractions determined by *in vitro* starch digestion.

Market class	RDS [a] (%)	SDS [b] (%)	RS [c] (%)	HI [d]	eGI [e]
Pinto	12.53 b	23.76 a	5.24 a	64.65 a	63.93 a
Black	15.66 a	20.63 b	3.22 b	60.81 b	60.62 b

Means with the same letter within columns are not significantly different ($p < 0.05$). The data are the means of two independent replicate experiments ($n = 2$).
[a] RDS: Rapidly digestible starch.
[b] SDS: Slowly digestible starch.
[c] RS: Resistant starch.
[d] HI: Hydrolysis index.
[e] eGI: Estimated glycemic index.

3.4.3. Dry Bean Flour Phenolic Content and Antioxidant Capacity

Some of the potential health benefits attributed to dry beans are associated with secondary metabolites such as phenolic compounds (Oomah et al., 2010). Phenolic compounds can scavenge free radicals or chelate metal ions, thus playing an important role in antioxidant activity (Hayat et al., 2014). In this sense, Table 4 presents the extractable phenols (EP) and hydrolyzable phenol (HP) content in raw and cooked dry bean flours, as well as their metal-chelating activity and antioxidant capacity measured by ABTS and FRAP.

Regarding the content of EP and HP, significant ($p < 0.05$) differences were detected between the raw and cooked samples. The raw pinto and black dry bean flours exhibited

significantly ($p < 0.05$) higher EP values than their cooked counterparts. This dry bean EP content reduction after cooking with boiling water has been reported by Ovando-Martínez et al. (2014), Saini et al. (2016), and Xu & Chang (2008). Some explanations for this content decrease have been proposed, including the degradation of phenolic compounds into monomers and their later diffusion into the cooking water (Ovando-Martínez et al., 2014; Rocha-Guzmán et al., 2007), or their chemical transformation into other forms that reduce their ability to be measured, such as phenolic-protein complexes (Mastura et al., 2017; Xu & Chang, 2008). Unfortunately, the cooking water of the present research was discarded; however, the significantly ($p < 0.05$) higher HP contents obtained from the cooked samples suggest that cooking processing allowed some phenolic complexes formation that were released and detected after acid hydrolysis. Furthermore, it was seen that raw beans also have naturally occurring phenolic complexes as reflected in their HP content, which could correspond to phenolic-protein or phenolic-IDF complexes mainly found in dry bean hulls (Oomah et al., 2010). Even when the identification of phenolic extracts was out of the scope of the present study, extensive research has been conducted regarding pinto and black dry bean phenolic components (Câmara et al., 2013; Campos-Vega et al., 2010; Dueñas et al., 2009; L.-Z. Lin et al., 2008). Therefore, in EP fractions, flavonoids, such as Kaempferol 3-*O*-glucoside, were found in both pinto and black dry beans, as well as hydroxycinnamic acid derivatives, such as ferulic acid, galactaric acid, glucaric acid and altaric acid (Câmara et al., 2013; L.-Z. Lin et al., 2008; Pitura & Arntfield, 2019). The HP fractions of both pinto and black dry beans were mainly composed of hydrocinnamic acids, such as sinapic acid, *p*-coumaric acid and isoferulic acid (L.-Z. Lin et al., 2008). Only the black dry bean EP extracts contained anthocyanins (3-*O*-glucosides of delphinidin, petunidin and malvidin), whereas black dry bean HP extracts had anthocyanins aglycone forms (Campos-Vega

et al., 2010; L.-Z. Lin et al., 2008). Unfortunately, the cited studies lack the discussion of the impact of bean processing on the detected phenolic components.

The metal chelating activity was significantly ($p < 0.05$) higher in both EP and HP cooked dry beans fractions than in the raw counterparts. However, a significant ($p < 0.05$) difference was observed in the EP fraction between raw dry bean market classes, being the black bean sample the one with the lowest chelating capacity. Interestingly, both dry bean samples' EP and HP fractions exhibited good chelating capacity after the cooking process, even when raw beans' EP total content was higher. Therefore, the metal chelating activity was negatively and significantly ($p < 0.01$) correlated with EP content, while being positively and highly significantly ($p < 0.001$) correlated with the HP content (Table 5). The improvement of the chelating activity in beans after cooking processes was described by Saini et al. (2016), suggesting that the formation of products from the Maillard reaction may be involved in the observed improvement. The described metal chelating activity in the studied samples is relevant since chelating agents stabilize transition metals, thus inhibiting free radicals' presence and their further damage (Siddhuraju, 2006).

Antioxidant compounds react with radicals through different mechanisms (Mastura et al., 2017), so assessing antioxidant capacities with more than one test will be better for understanding the dry bean antioxidant potential. In this sense, ABTS radical cation scavenging and ferric reducing antioxidant power (FRAP) activities for raw and cooked dry bean samples EP and HP fractions are presented in Table 4. The antioxidant capacity of EP from raw dry beans was significantly ($p < 0.05$) higher than that from cooked dry beans. A decrease in antioxidant capacity from phenolic extractable fractions after cooking dry beans was reported by Mastura et al. (2017), Ovando-Martínez et al. (2014), and Siddhuraju (2006). Furthermore, Hagerman et al.

(1998) described that phenolic compounds' number of aromatic rings, number of substituting hydroxyl groups, as well as molecular weight determined their ability to scavenge free radicals. Therefore, the obtained results implied a change in the native form of the phenolic compounds after the cooking process, thus impairing the efficiency of their antioxidant capacity. In contrast, the antioxidant activity for HP fractions was significantly ($p < 0.05$) higher in cooked dry bean samples than in the raw counterparts. These findings agree with Ovando-Martínez et al. (2014) study, where the antioxidant activity of HP was higher in cooked pinto and black beans than in HP fraction from raw samples. Interestingly, the studied antioxidant activities by ABTS and FRAP were highly significantly ($p < 0.001$) and positively correlated with the type of phenolic extract content (Table 5). In this sense, the total EP and HP content of bean flour can serve as a predictor of antioxidant capacity.

Table 4. Dry bean flour phenolic content and antioxidant properties.

Sample	Extractable phenols				Hydrolyzable phenols			
	Total (mg GAE [d] / g)	MCA [a] (mg EDTAE [e]/ g)	ABTS [b] (mg TroloxE [f]/ g)	FRAP [c] (mg TroloxE / g)	Total (mg GAE / g)	MCA (mg EDTAE / g)	ABTS (mg TroloxE / g)	FRAP (mg Trolox E / g)
Raw								
Pinto	7.00 a	10.37 a	11.44 b	7.62 b	5.41 b	91.62 b	12.02 c	2.47 d
Black	6.59 a	7.17 b	13.95 a	9.79 a	5.16 b	82.10 b	11.29 c	3.70 c
Cooked								
Pinto	1.37 b	11.36 a	2.55 c	1.45 d	9.20 a	320.97 a	13.11 b	9.94 b
Black	1.54 b	11.46 a	3.38 c	1.96 c	9.44 a	340.83 a	16.36 a	12.32 a

Means with the same letter within columns are not significantly different ($p < 0.05$). The data are the means of two independent replicate experiments ($n = 2$).

[a] MCA: Metal chelating activity.

[b] ABTS: Free radical scavenging assay.

[c] FRAP: Ferric reducing antioxidant power.

[d] GAE: Gallic acid equivalents.

[e] EDTAE: Ethylenediaminetetraacetic acid.

[f] TroloxE: Trolox equivalents.

Table 5. Simple linear correlation coefficients between dry bean flour phenolic content and antioxidant properties.

	Metal chelating activity (mg EDTAE [c]/ g)	ABTS [a] (mg TroloxE [d]/ g)	FRAP [b] (mg TroloxE / g)
Extractable phenols (mg GAE [e]/ g)	-0.575 **	0.966 ***	0.955 ***
Hydrolyzable phenols (mg GAE / g)	0.947 ***	0.768 ***	0.883 ***

NS: nonsignificant; *: $p > 0.05$; **: $p \leq 0.01$; and ***: $p \leq 0.001$; n= 24.
[a] ABTS: Free radical scavenging assay.
[b] FRAP: Ferric reducing antioxidant power
[c] EDTAE: Ethylenediaminetetraacetic acid.
[d] TroloxE: Trolox equivalents.
[e] GAE: Gallic acid equivalents.

3.4.4. Sugar Composition of Soluble Dietary Fiber-Rich Fraction

The extraction of the soluble dietary fiber (SDF)-rich fraction from raw and cooked dry bean flours was done as Feng et al. (2017) described. The total neutral sugars and protein contents of the obtained extracts are presented in Table 6. Significant ($p < 0.05$) differences were seen between dry bean market classes and processing conditions. The SDF-rich fraction sugars concentration was significantly ($p < 0.05$) higher in samples extracted from cooked dry beans than from the raw counterparts, which is reflected in the protein content, being cooked dry bean extracted samples the ones with the significantly ($p < 0.05$) lowest protein content. The lower protein content in samples extracted from cooked beans might be due to the formation of less stable structures upon the thermal treatment, which increases digestibility by proteolysis, as reported by Carbonaro et al. (2012), who studied the dry beans' protein secondary structures and their changes after cooking. Previous studies (Brummer et al., 2015; Feng et al., 2017; Ruiz-Ruiz et al., 2012; Shiga & Lajolo, 2006) have extracted and characterized the SDF-rich fraction from dry beans, but the protein content of the extracted fraction has not been reported. However, Dalgetty & Baik (2003) reported soluble fiber concentrates with purities ranging from 64.5 to

70.6 % extracted from peas, lentils and chickpeas. In addition, Njoroge et al. (2014) extracted the insoluble fiber-rich fraction from pinto dry beans reporting residual protein contents from 24.95 to 34.77 %. Overall, the present study's residual protein contents of the extracted SDF-rich fractions ranged from 13.8 to 21.3 %. The relatively high contents of residual protein might be due to the inability of the protease enzyme to break down all the proteins, in addition to the existing crosslinking between proteins and fiber components (Njoroge et al., 2014).

Legume SDF is comprised of arabinose and galacturonic acid-rich pectins (xylogalacturonans, rhamnogalacturonans, and homogalacturonans), hemicelluloses (xyloglucans and xylans), and the raffinose family of oligosaccharides (RFOs) (stachyose, raffinose and verbascose) (Brummer et al., 2015; Capuano, 2017). In contrast with cereal grains, arabinoxylans are not present in dry beans in appreciable amounts (Dodevska et al., 2013; Naivikul & D'Appolonia, 1979). The sugar composition of the SDF-rich fractions was similar to that obtained by Brummer et al. (2015), Njoroge et al. (2014), and Shiga et al. (2003) from dry bean cell walls and cotyledons, agreeing on the predominance of arabinose in its composition. Significant ($p < 0.05$) differences in sugar composition were detected between dry bean market classes extracted fractions, as well as processing conditions. Significantly ($p < 0.05$) lower sugar contents were found in the SDF-rich fraction from black beans within each processing condition. The galacturonic acid constitutes the backbone of pectic polysaccharides (Chawla & Patil, 2010), therefore, the results suggest the SDF-rich fraction from pinto dry beans, especially after the cooking process, displays longer pectic structures. Interestingly, higher contents of arabinose in fractions extracted from pinto dry beans when compared to other dry bean market classes were reported by Njoroge et al. (2014), suggesting more branched rhamnogalacturonan pectins in the form of arabinan in pinto dry beans. Additionally, the detection of galactose, glucose and

fructose significantly ($p < 0.05$) higher contents in SDF-rich fraction from pinto dry beans suggests more stachyose and raffinose, which are the major RFOs oligosaccharides in dry beans (Aguilera et al., 2009). The levels of glucose and xylose indicate the presence of xyloglucans and xylans (Njoroge et al., 2014). Therefore, a significant ($p < 0.05$) xylose content decrease in the cooked black dry bean SDF-rich sample suggests some hemicellulose degradation. The substitution of the pectin backbone with mannose side chains may occur (Chawla & Patil, 2010). In this sense, mannose was detected in all SDF-rich fractions but in lesser amounts than arabinose and xylose, agreeing with Martín-Cabrejas et al. (2004) and Njoroge et al. (2014) findings.

Table 6. Dry bean soluble fiber-rich fraction composition.

Sample	Carbohydrates							Total sugars (%)	Protein (%)
	Arabinose (%)	Galactose (%)	Glucose (%)	Xylose (%)	Mannose (%)	Fructose (%)	Galacturonic acid (%)		
Raw									
Pinto	25.5 bc	9.4 b	5.4 c	9.3 b	7.2 a	12.6 c	5.3 b	74.8 c	19.1 ab
Black	24.6 c	8.7 c	5.2 d	9.4 b	5.9 b	17.3 b	4.9 bc	76.0 bc	21.3 a
Cooked									
Pinto	27.9 a	10.2 a	6.7 a	10.0 a	7.2 a	12.9 c	7.9 a	82.8 a	13.8 c
Black	26.8 ab	7.5 d	6.3 b	7.8 c	3.8 c	22.6 a	4.4 c	79.3 ab	17.2 bc

Means with the same letter within columns are not significantly different ($P < 0.05$). The data are the means of two independent replicate experiments ($n = 2$).

3.4.5. Soluble Dietary Fiber-Rich Fraction Molecular Weight Distribution

The SDF molecular weight plays a major role in its functional and nutritional properties (Aguilera et al., 2009). Therefore, the distribution of the weight-averaged molecular weight and the HPSEC chromatograms of the extracted SDF-rich fractions from cooked and dry beans are shown in Table 7 and Figure 3. Two peaks representing high and low molecular (HMW and LMW, respectively) fractions were detected from each analyzed SDF-rich fraction. The HMW fraction was significantly ($p < 0.05$) impacted by the dry bean market class, as no significant ($p < 0.05$) differences were seen due to the cooking processing. In this sense, the SDF-rich fractions extracted from black beans had a significantly ($p < 0.05$) higher HMW than the pinto dry bean counterparts. On the other hand, the LMW fraction was significantly ($p < 0.05$) impacted by the cooking processing rather than the dry bean market class. Thus the LMW of SDF-rich fractions extracted from raw dry beans was significantly ($p < 0.05$) higher than the fractions from cooked dry beans. The described differences explain the HMW and LMW percental changes (Table 7). For the molecular weight distribution of SDF-rich fractions extracted from raw samples, around 60 % corresponds to LMW polymers, but the HMW and LMW percental proportion changes when samples were extracted from cooked samples as significantly ($p < 0.05$) lower LMW polymers were present. Presumably, the HMW fraction encompasses pectin polymers, as the obtained weights (4840.5 – 5061.08 kDa) are in accordance with the HMW reported by Brummer et al. (2015) for pinto and navy beans (2146 – 2600 kDa) and the HMW reported by Bibbins-Martínez et al. (2011) for SDF extracted from apples (a pectin-rich extract ranging from 1000 – 5000 kDa). In addition, Shiga & Lajolo (2006) suggested that HMW (>3000 kDa) dry bean polymers are pectic polysaccharides with strong interactions that provide stability versus harsh conditions; this explains why the cooking process did not cause significant ($p < 0.05$)

changes in HMW fractions. Furthermore, the LMW fraction comprises shorter pectic polymers and RFOs (Njoroge et al., 2014; Ovando-Martínez et al., 2011). In this sense, the decrease in LMW observed in the SDF-rich fractions extracted from cooked dry beans was due to the cooking process causing the depolymerization and consequent reduction of RFOs (Aguilera et al., 2009).

Table 7. Dry bean soluble fiber-rich fraction molecular weight distribution.

Sample	HMW [a] (kDa)	LMW [b] (kDa)	HMW (%)	LMW (%)
Raw				
Pinto	4840.50 b	424.35 a	37.33 b	62.67 a
Black	5002.58 a	440.56 a	41.93 b	58.07 b
Cooked				
Pinto	4845.50 b	290.57 b	51.99 a	48.01 c
Black	5061.08 a	337.75 b	50.71 a	49.29 c

Means with the same letter within columns are not significantly different ($p < 0.05$). The data are the means of two independent replicate experiments ($n = 2$).
[a] HMW: High molecular weight.
[b] LMW: Low molecular weight.

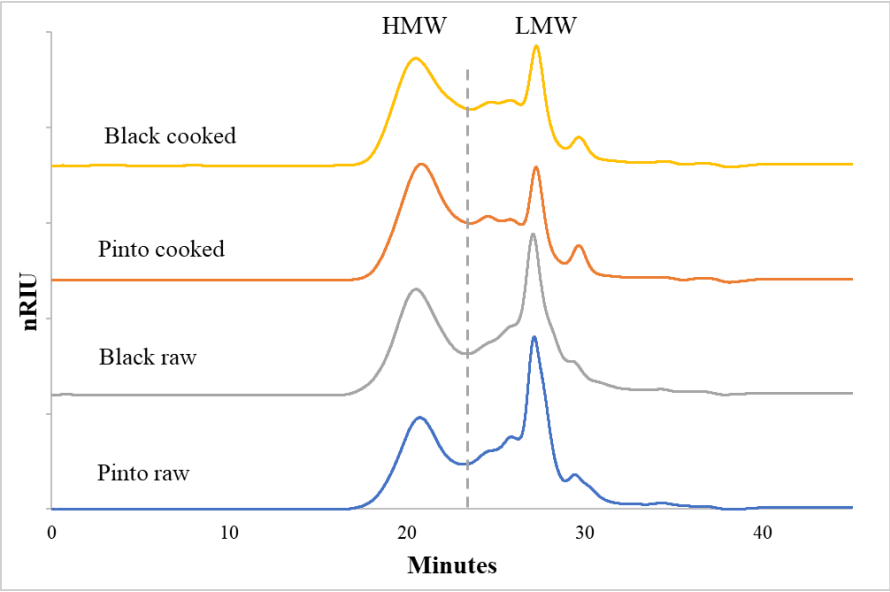

Figure 3. HPSEC chromatograms of dry bean soluble fiber-rich fraction.
HMW: High molecular weight; LMW: Low molecular weight; nRIU: nano refractive index units.

3.4.6. Rheological Properties

The flow properties of the solutions with varying concentrations (2 %, 4 %, and 6 % w/v) of the extracted SDF-rich fraction from raw and cooked beans were analyzed by using the power-law model $\tau = K\gamma^n$, where τ is the shear stress (N/m^2 or Pa), γ is the steady shear rate (s^{-1}), K is the consistency index ($Pa \cdot s^n$), and n is the flow behavior index (dimensionless) (Mezger, 2014). In this sense, the power-law parameters, K and n, were calculated by linear regression from a plot of log τ and log γ. All the correlation coefficients (r^2) were >0.9. The obtained K and n values are presented in Table 8. The n value equal to 1 corresponds to a Newtonian fluid, whereas lower n values correspond to a shear-thinning behavior (Song et al., 2006). Hence, all the samples exhibited a shear-thinning behavior (n<0.8); thus, the viscosity of the solutions decreased with increasing shear rate (Sousa et al., 2017). This pseudoplastic behavior was expected as it has been previously reported from solutions rich in hemicelluloses and pectic material (Brummer et al., 2015; Feng et al., 2017; Niu et al., 2018; Pongsawatmanit et al., 2006). However, K and n values significant ($P < 0.05$) differences were detected between the SDF-rich solutions from different dry bean market classes and processing conditions. Overall, significantly ($p < 0.05$) lower K values and significantly ($p < 0.05$) higher n values corresponded to the solutions prepared with SDF-rich fractions extracted from cooked samples, being the 2 % solution of SDF-rich fraction from cooked pinto dry bean the one with the lowest K value and highest n value, which suggests this solution resembled a Newtonian fluid. On the other hand, the highest K and lowest n values corresponded to the 6 % solution of SDF-rich fraction from raw black bean, which indicates a large level of polysaccharide entanglement lost under increasing shear rate (Simsek et al., 2009). Even when the analyzed SDF-rich samples demonstrated their ability to make viscous solutions at low concentrations (2 %), especially from

samples extracted from raw beans, it is clear that *n* values increase with concentration. This may be a consequence of the development of a stronger network (chain-chain entanglement) due to a higher concentration of closer molecules with restricted movement (increased viscosity) (Y. Lin et al., 2021).

Significant ($p < 0.05$) correlations were detected between some of the SDF-rich fraction composition traits and the power-law parameters (Table 9). The obtained results suggest that the lower degradation of LMW fractions, as seen in the SDF-rich fractions extracted from raw dry beans (Table 7), had an impact on increasing viscosity due to the highly significant ($p < 0.001$) and negative correlations found between LMW (%) and *n* values. This result contrasts with Brummer et al. (2015) findings that larger molecular weight fibers cause higher viscosities. A possible explanation is that the HMW fraction from SDF-rich extracts was not degraded by the cooking process as was the LMW fraction, meaning that a higher concentration of both fractions resulted in enhanced interaction between the polysaccharides, thus greater entanglement, causing more viscous solutions. This agrees with Wang et al. (2016) report that low molecular weight pectic material (around 400 kDa) and higher neutral sugars concentration increase viscosity. Furthermore, the highly significant ($p < 0.001$) and positive correlations found between arabinose (%), glucose (%), total sugars (%) and *n* values suggest that the monosaccharide composition, mainly from arabinose-rich pectins and hemicelluloses, are closely related to the rheological properties.

Table 8. Dry bean soluble fiber-rich fraction solutions consistency index (*K*) and flow behavior index (*n*).

	2 % solution		4 % solution		6 % solution	
Sample	*K* (Pa·s^n)	*n*	*K* (Pa·s^n)	*n*	*K* (Pa·s^n)	*n*
Raw						
Pinto	0.34 b	0.62 c	5.82 a	0.36 c	8.99 b	0.35 c
Black	0.51 a	0.60 d	4.08 b	0.36 c	18.91 a	0.28 d
Cooked						
Pinto	0.07 d	0.86 a	0.64 d	0.69 a	3.08 d	0.57 a
Black	0.12 c	0.82 b	1.27 c	0.64 b	6.17 c	0.51 b

Means with the same letter within columns are not significantly different (P < 0.05). The data are the means of two independent replicate experiments (n = 2).

Table 9. Simple linear correlation coefficients between dry bean soluble fiber-rich fraction flow properties and composition.

	2 % solution		4 % solution		6 % solution	
Sample	*K* [a] (Pa·s^n)	*n* [b]	*K* (Pa·s^n)	*n*	*K* (Pa·s^n)	*n*
Arabinose (%)	-0.554**	0.563**	-0.680***	0.591**	-0.628**	0.638***
Galactose (%)	NS	NS	NS	NS	NS	NS
Glucose (%)	-0.794***	0.831***	-0.804***	0.821***	-0.757***	0.826***
Xylose (%)	NS	NS	NS	NS	NS	NS
Mannose (%)	NS	NS	NS	NS	NS	NS
Fructose (%)	NS	NS	NS	NS	NS	NS
Galacturonic acid (%)	NS	NS	NS	NS	-0.438*	0.437*
Total sugars (%)	-0.513*	0.635***	-0.602**	0.643***	-0.485*	0.638***
HMW [c] (%)	-0.679***	0.835***	-0.728***	0.851***	-0.533**	0.792***
LMW [d] (%)	0.679***	-0.835***	0.728***	-0.851***	0.533**	-0.792***

NS: nonsignificant; *: p > 0.05; **: p ≤ 0.01; and ***: p ≤ 0.001; n= 24.
[a] *K*: consistency index.
[b] *n*: flow behavior index.
[c] HMW: High molecular weight.
[d] LMW: Low molecular weight.

3.5. Conclusions

The present study demonstrated that the chemical composition of macronutrients and antioxidant properties of dry beans can be affected by market class and cooking processing. Overall, the cooking process of boiling at atmospheric pressure without pre-soaking dry beans decreased ash content, but increased protein, starch, phytic acid, and dietary fiber contents. Pinto dry beans had the highest protein and starch contents, but black beans had higher dietary fiber content. The raw dry beans had a higher extractable phenol content with significantly higher antioxidant activity. Dry bean market class significantly affected the starch digestibility, being pinto dry beans the ones with the highest SDS and RS content. However, both dry bean classes had higher RS when compared to cereals, highlighting their potential nutritional benefits. Furthermore, SDF-rich fractions were successfully extracted from the analyzed dry bean flours. SDF-rich fraction extracted from cooked dry bean flours had less residual protein content. The monosaccharide composition of the extracted SDF-rich fractions suggested the presence of pectic polysaccharides, hemicelluloses and RFOs. The molecular weight distribution suggested the degradation of LMW fractions by the cooking process. The degradation of these fractions resulted in solutions of SDF extracted from cooked beans with lower viscosity than those from their raw counterparts. Overall, the SDF-rich fraction solutions had a shear-thinning behavior. The obtained results highlight the nutritional potential of dry bean components, which is valuable information for food industries to adopt them as ingredients.

3.6. References

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4. DRY BEAN SOLUBLE DIETARY FIBER AS INFLAMMATORY IMMUNOMODULATOR

4.1. Abstract

Immunomodulatory polysaccharides are compounds able to modulate the immune system by activating specific mechanisms of the host response. In macrophages, which are cells of the innate immune system, the toll-like receptors (TLR) are involved in the detection of pathogens and subsequent production of inflammatory mediators. TLR-4 has been identified as the receptor with affinity for polysaccharides extracted from various sources. Therefore, the present study investigated the immunomodulating properties of soluble dietary fiber (SDF) rich fractions extracted from dry beans on the RAW 264.7 murine macrophage cell line. When the macrophages were cultured with SDF-rich fractions, the secretion of pro-inflammatory molecules, such as NO, TNF-α, IL-6, and IL-1β, was detected. In addition, enhanced transcript levels of TNF-α, IL-6, and IL-1β were observed. Furthermore, by using the TLR-4 antagonist TAK-242, TLR-4 was identified as one receptor-activated in macrophages upon SDF-rich fraction modulation. The SDF-rich fractions' monosaccharide composition revealed that glucose, arabinose, xylose, mannose and galacturonic acid were significantly ($p < 0.05$) correlated to the immune response. The results suggest the potential of SDF-rich fractions as immunomodulators.

4.2. Introduction

Dry beans (*Phaseolus vulgaris* L.), apart from being valuable sources of proteins, starch, minerals, and phytochemicals, are rich in soluble (SDF) and insoluble dietary fibers (IDF). Therefore, dietary fiber content is one of the most important parameters to evaluate dry beans' technological quality and physiological value (Vasic et al., 2009). However, even when dietary fiber is being considered as a valuable non-food and food ingredient that aids in the texture

modification, freeze/thaw stability, and nutritional value enhancement of food products (Dzudie et al., 2002; Talukder, 2015), its isolation from dry bean whole seeds has been limitedly explored (Rui & Boye, 2013). In general, legume extraction and fractionation technologies mainly focus on protein isolation (Tassoni et al., 2020). Therefore, to enhance the utilization and market value of the dry bean whole seeds, the identification of fiber fraction utilization is essential. Furthermore, as dietary fibers consist of heterogeneous polysaccharides, including non-structural hemicelluloses, gums, and pectins (Rui & Boye, 2013), the study of dry bean polysaccharides' immunomodulatory activity would reinforce the purpose of use of the fractionation process.

Polysaccharides extracted from botanical sources have gained attention due to their potential immunostimulatory properties and low toxicity (Razali et al., 2014; Schepetkin & Quinn, 2006). Glucans, mannans, pectic polysaccharides, and arabinoxylans are examples of polysaccharides with reported immunostimulatory activity (Ferreira et al., 2015). Immunostimulation is part of the body's defense strategy, through which the immune system is activated, triggering several cellular events (Yu et al., 2018). Neutrophils, monocytes, and macrophages are key cell participants of the innate and adaptive immune response; they represent the first line of defense of the host once the epithelial barrier has been breached (Schepetkin & Quinn, 2006). The polysaccharides' immunostimulatory effect is thought to be mediated through macrophage stimulation and activation. Macrophages possess surface pattern recognition receptors (PRRs) that detect pathogen-associated molecular structures to activate macrophages, thus triggering the innate immune response (Ferreira et al., 2015). There are several functionally different PRRs, but one reported to mediate the activation of macrophages by polysaccharides is the toll-like receptor 4 (TLR-4) (Zhang et al., 2016). The activation of macrophages by polysaccharides through TLR-4 triggers signaling pathways that lead to the

activation of pro-inflammatory cytokines genes, such as TNF-α, interleukin-1β (IL-1β), IL-6, as well as the activation of transcription factors and enzymes that induce the expression of inflammatory mediators, such as inducible nitric oxide synthase (iNOS) (Gupta et al., 2016; Ho et al., 2015; Xie et al., 2016). Therefore, since plant polysaccharides potentially enhance macrophages' function, they have been considered as prospective therapeutic candidates to strengthen immune responses or as health-improving ingredients in functional foods (Ferreira et al., 2015; Gupta et al., 2017).

The studies that reported the activation of macrophages through TLR-4 by extracted plant polysaccharides include plant sources such as safflower (*Carthamus tinctorius* L.) (Ando et al., 2002), *Acanthopanax senticosus* (Han et al., 2003), *Solanum nigrum* (Razali et al., 2014), elderberries (*Sambucus nigra* L.) (Ho et al., 2015), *Dendrobium huoshanense* (Xie et al., 2016), and *Tinospora cordifolia* (Gupta et al., 2016). These plant polysaccharides mediate immunostimulation by the production of different pro-inflammatory cytokines and nitric oxide (NO). However, the polysaccharide-rich fraction, or dietary fiber, has not been considered when dry beans' immunomodulatory properties have been studied. Dry bean studies have focused on extracted fractions from either the whole seeds or the dry bean hulls, which included phenolic rich fractions (Boudjou et al., 2013; García-Lafuente et al., 2014; Oomah et al., 2010), and protein hydrolysates (López-Barrios et al., 2016; Oseguera-Toledo et al., 2011). The aforementioned dry bean fractions have been demonstrated to exhibit an *in vitro* anti-inflammatory activity, reflected in the reduced expression of NO and pro-inflammatory cytokines via the inactivation of the NF-κB pathway (García-Lafuente et al., 2014; Oseguera-Toledo et al., 2011) or cyclooxygenases (COX-1 and COX-2) inhibition (Boudjou et al., 2013; Oomah et al., 2010; Oseguera-Toledo et al., 2011).

Therefore, exploring the immunostimulation properties of dietary fiber fractions extracted from dry beans will add value to dry beans as a source of health promotion ingredients and will enhance their utilization. Thus, the aim of the present study was to investigate the immunomodulating properties of SDF-rich fractions, extracted from dry beans, and possessing different monosaccharide compositions, as affected by dry bean type and cooking, using the RAW 264.7 mouse macrophage cell line *in vitro* assays.

4.3. Materials and Methods

4.3.1. Materials

The RAW 264.7 murine macrophages were purchased from the American Type Culture Collection (ATCC) (Manassas, VA). Dulbecco's Modified Eagle's Medium (DMEM) (ATCC®. 30-2002) and Fetal Bovine Serum (FBS) (ATCC®. 30-2020) were purchased from ATCC (Manassas, VA). Trypsin (0.25%) EDTA (1X) (VWR, 02-0154-0100) was purchased from VWR (Solon, OH). Lipopolysaccharides (LPS) from *E. coli*, Serotype O111:B4 (1 mg/mL, ALX-581-012-L002) was obtained from Enzo Life Sciences (Farmingdale, NY). TAK-242 (TLR 4 signaling inhibitor, A3850) was purchased from APExBIO (Houston, TX). Genscript ToxinSensor™ Chromogenic LAL Endotoxin Assay Kit (L00350) was purchased from Genscript (Piscataway, NJ). The mouse TNF-α Quantikine ELISA kit (MTA00B) (detection limit of 10.9-700 pg/mL), mouse IL-6 Quantikine ELISA kit (M6000B) (detection limit of 7.8-250 pg/mL), and mouse IL-1β Quantikine ELISA kit (MLB00C) (detection limit of 12.5-400 pg/mL) were purchased from R&D Systems, Inc. (Minneapolis, MN). The Griess Reagent System (G2930) was obtained from Promega Co. (Madison, WI). The PureLink RNA Mini Kit (12183018A) was obtained from Invitrogen (Waltham, MA). The First Strand cDNA Synthesis (Quick Protocol) (#M0253) components were purchased from New England BioLabs Inc.

(Ipswich, MA). The 5 x HOT FIREPol EvaGreen qPCR Mix Plus (ROX) was obtained from Solis BioDyne (Tartu, Estonia).

4.3.2. Production and Characterization of SDF-Rich Fractions

The SDF-rich fraction was extracted from dry beans by the enzymatic-gravimetric procedure according to Feng et al. (2017) with slight modifications as described in Chapter 2. Briefly, the raw and cooked bean (pinto and black) flour samples underwent stepwise enzymatic hydrolysis of α-amylase, protease, and amyloglucosidase. Then, the hydrolysate was filtrated. Next, the filtrate was collected and condensed to about one-tenth in a vacuum rotary evaporation system. Then, the concentrated filtrate was mixed with 95% (v/v) ethanol at 60°C for one hour, followed by centrifugation at 4500 rpm for 10 min. Finally, the precipitated flocculate was freeze-dried and saved as the SDF-rich fraction.

The determination of carbohydrate molecular weight and composition was carried out according to the methods described in Chapter 2 using HPSEC-MALS and HPAEC-PAD techniques, respectively.

4.3.3. Determination of Endotoxin Contamination in SDF-Rich Fractions

The amount of endotoxin present in SDF-rich fraction sample solutions (500 µg/mL) was determined using the ToxinSensor™ Chromogenic LAL Endotoxin Assay Kit (GenScript, Piscataway, NJ, USA; L00350). The sample solutions were assayed for endotoxin levels using the kit according to the manufacturer's instructions. The absorbance was measured and compared to the standard curve (0.05 – 1 EU/mL) at 545 nm.

4.3.4. Determination of Immunomodulatory Properties of SDF-Rich Fractions

The SDF-rich fraction samples were evaluated using RAW 264.7 mouse macrophages for their immunomodulatory properties. RAW 264.7 cells were grown in DMEM supplemented with

10% FBS, penicillin (100 U/mL) and streptomycin (100 µg/mL) at 37°C under a humidified atmosphere of 95% air and 5% CO_2. The cells were cultured in the media described above until they reached 90% confluency. The number of viable cells was determined by counting manually on a hemocytometer after staining with 0.4% (w/v) trypan blue (Mediatech, Inc., Manassas, VA).

RAW 264.7 cells were plated at 3×10^5 cells per well in a 24 well-plate and incubated overnight. Then, 2000 µg/mL SDF-rich fraction solutions were prepared with serum-free and antibiotic-free DMEM, and 200 µL of the solutions were added to each well. The media control (RAW 264.7 macrophages cultured only with DMEM) and LPS control (LPS stimulated RAW 264.7 macrophages) wells received 200 µL of serum-free and antibiotic-free DMEM. After a two-hour incubation, the LPS control wells received 10 µL of LPS (80 µg/mL) dissolved in serum-free and antibiotic-free DMEM. The rest of the wells received 10 µL of serum-free and antibiotic-free DMEM. Therefore, the final concentration in the wells was 500 µg/mL for SDF-rich fraction samples and 1 µg/mL of LPS. The cells were incubated for 24 hours. The same procedure was carried out when the TLR-4 signaling inhibitor TAK-242 was used, with the following modifications. After the RAW 264.7 cells were incubated overnight in the 24 well-plates, 20 µL of TAK-242 (400 µM) dissolved in serum-free and antibiotic-free DMEM was added to the wells that received after two hours 200 µL of the SDF-rich fraction solutions and 10 µL of dissolved LPS, as described above. The final concentration of TAK-242 in the wells was 10 µM.

4.3.5. Determination of TNF-α, IL-6 and IL-1β by ELISA

The media of the cells treated as described above was collected and centrifuged at 13000 rpm for two minutes to pellet any cell debris. Then, the supernatants were aliquoted and

stored at -20°C until their analysis. The presence of each cytokine in the collected media was determined following the manufacturer's instructions for each ELISA kit.

4.3.6. Nitric Oxide Determination

The RAW 264.7 cells were treated as described above but were seeded in 96 well-plates (1×10^5cells per well) instead of 24 well-plates. The final working concentration of each compound was: SDF-rich fractions (500 µg/mL), LPS (1 µg/mL), and TAK-242 (10 µM). From each well, 50 µL of culture media was collected and transferred to a new 96 well-plate to carry out the Griess reaction. Then, sulfanilamide solution was added and cells were incubated for ten minutes in the dark. Next, N-1-naphthyl ethylenediamine dihydrochloride (NED) was added and cells were incubated again for ten minutes in the dark until the color developed. Finally, absorbance was read at 535 nm. The amount of nitric oxide (NO) produced was determined based on the amount of nitrite measured during the assay using a standard curve.

4.3.7. Quantitative Reverse Transcriptase Real-Time Polymerase Chain Reaction (qRT-PCR)

The treated cells were detached using a cell scraper and washed with cold PBS. Then, the PureLink® RNA Mini Kit (Invitrogen) for total RNA isolation was used to purify cells' RNAs, according to the manufacturer's instructions. The RNA samples were stored at −80°C until use. The reverse transcription system (M0277) (New England BioLabs Inc.) was used for the production of cDNAs. The cDNA samples were stored at −20°C until use. The qRT-PCR mixture system was set up as follows according to the manufacturer's instructions with some modifications: 10 µL of 5× HOT FIREPol EvaGreen qPCR Mix Plus (ROX) (Solis BioDyne), 2 µL of the primer mix (2.5 µM of forward primer and 2.5 µM of reverse primer), 1 µL of cDNA

(5 ng/μL) and 7 μL of nuclease-free PCR grade water. The final volume of reaction was 20 μl. The primers were as follows:

- **Actin β** (forward) CATTGCTGACAGGATGCAGAAGG,
  (reverse) TGCTGGAAGGTGGACAGTGAGG.
- **TNF-α** (forward) CAGACCCTCACACTCAGATCA,
  (reverse) TTGTCTTTGAGATCCATGCC.
- **IL-6** (forward) CCAAGAGGTGAGTGCTTCCC,
  (reverse) CTGTTGTTCAGACTCTCTCCCT.
- **IL-1β** (forward) GAAATGCCACCTTTTGACAGTG
  (reverse) TGGATGCTCTCATCAGGACAG.
- **iNOS** (forward) GTTCTCAGCCCAACAATACAAGA,
  (reverse) GTGGACGGGTCGATGTCAC.
- **TLR-4** (forward) ACACCAGGAAGCTTGAATCC,
  (reverse) GAGGTGGTGTAAGCCATGC.

The amplification protocol was as follows: 95 °C for 10 min; 40 cycles consisting of 95°C for 30 s, 60°C for 1 min, and 72°C for 1 min; a melting step consisting of 95°C for 1 min; 55 °C for 30 s; 95°C for 30 s was added at the end of the cycles. Ct values were determined by the software of the real-time PCR instrument (Stratagene Mx3000p). ΔCt values were calculated using actin β as the housekeeping gene. The fold of change was calculated as $2^{\Delta(\Delta Ct)}$.

4.3.8. Statistical Analysis

All the cell culture experiments were performed in duplicate. The analysis of variance (ANOVA) was conducted using the SAS software (version 9.4, SAS Institute, Cary, NC, USA). The least significant difference (LSD) value was used to separate means, and the significance

was accepted at $p<0.05$. In addition, the immunological outcomes and SDF-rich fraction composition details were analyzed to evaluate relationships by Pearson's two-tailed significant correlation using the 'CORR' procedure.

4.4. Results and Discussion

4.4.1. Endotoxin Contents in SDF-Rich Fractions Solutions

Endotoxin is frequently a contaminant in biological preparations and possesses macrophage-stimulating properties (Schepetkin & Quinn, 2006). Therefore, the ToxinSensor™ Chromogenic LAL Endotoxin Assay Kit was used to assess the amount of endotoxin in SDF-rich fraction sample solutions (500 µg/mL). The kit can detect as little as 0.05 endotoxin units per mL (EU/mL), an EU/mL being a measure of endotoxin activity equivalent to 0.1 ng endotoxin per mL of solution (GenScript, 2022). In this sense, the endotoxin levels in the prepared solutions ranged from 0.08 to 0.12 ng/mL. Previous research has shown that at least 0.5 ng/mL endotoxin concentration is needed to induce *in vitro* cytokines production in macrophages (Corning, 2022) and dendritic cells (Tynan et al., 2012). In addition, none of the obtained results were significantly ($p <0.05$) correlated with the endotoxin concentration, which suggests that the detected immunomodulatory activity can be attributed to the studied SDF-rich fraction samples.

4.4.2. Immunological Activity of SDF-Rich Fractions

Macrophages are one of the innate immune system sentinel cells. When they are activated, they release reactive oxygen species (ROS) and inflammatory cytokines for defense against microbial invasion (Gupta et al., 2017). For this reason, macrophages (RAW 264.7) have been utilized to investigate the immunomodulatory effects of diverse plant-derived extracts (García-Lafuente et al., 2014; Mendis et al., 2016; Oseguera-Toledo et al., 2011; Tang et al., 2018). Furthermore, polysaccharides extracted from botanical sources have been seen to

stimulate the immune system by activating macrophages, increasing NO and cytokines production (Gupta et al., 2017; Razali et al., 2014; Schepetkin & Quinn, 2006). For these reasons, the cell model of macrophages cultured with the SDF-rich fractions, as described in the methods of the present chapter, was implemented. In addition, since it has been described that plant polysaccharides activate macrophages through the pattern recognition receptor toll-like receptor 4 (TLR-4), a TLR-4 antagonist was used to elucidate if the analyzed dry bean extracts act through TLR-4. The selected TLR-4 antagonist was TAK-242 (resatorvid), a small molecule known to selectively bind to TLR-4, causing the inhibition of TLR-4 mediated signaling, thus suppressing pathogen-induced inflammatory cytokines (Samarpita et al., 2020).

4.4.2.1. Effect of SDF-rich fractions on NO production

Nitric oxide (NO) is a member of the reactive oxygen species (ROS) radical entities, and it contains a nitrogen atom covalently bonded to an oxygen atom with one unpaired electron (Sharma et al., 2007). NO is a short-lived free radical able to induce vasodilatation, consequently playing a role in the inflammatory process (Tripathi et al., 2007). Additionally, NO is recognized for its ability to kill phagocytosed microbes in macrophages, thus being a killing mechanism of activated macrophages (Razali et al., 2014). For this reason, nitrite concentrations in the supernatant of SDF-rich fraction stimulated macrophages with and without TAK-242 were measured to reflect NO production (Figure 4). We observed significantly ($p < 0.05$) higher levels of nitrite from macrophages treated with the dry bean extracts than from control macrophages (treated with media only). In addition, higher nitrite levels were produced when macrophages were treated with SDF black raw bean fraction than with SDF black cooked bean fraction. Furthermore, we observed that treatment of macrophages with SDF bean extracts (black or pinto, raw or cooked) in the presence of TAK-242 resulted in significantly ($p < 0.05$) lower nitrite

concentrations than treatment with the SDF extracts alone. Macrophages treated with LPS were used as control conditions, and showed higher levels of nitrites than in non-treated macrophages. The LPS-mediated increase in nitrites could be partially reversed by the addition of the TLR-4 inhibitor. Overall, the results suggest that the SDF-rich fractions interact with TLR-4 to stimulate the macrophages, since the NO levels from macrophages stimulated with SDF-rich fractions were significantly ($p < 0.05$) reduced with the TLR-4 inhibitor.

The enhanced NO production from RAW 264.7 macrophages cultured with extracted polysaccharides has also been reported by Martins et al. (2017) and Ren et al. (2017). Martins et al. (2017) studied extracts from a Fabaceae family plant, *Pterospartum tridentatum*, which contained pectic polysaccharides, arabinogalactans, galactomannans, and xyloglucans, which presents similarities to the composition we proposed for the SDF-rich extracts in Chapter 2. In addition, Ren et al. (2017) studied a polysaccharide extract from *Hericium erinaceus* containing glucose, galactose, mannose and arabinose monosaccharides, resembling the composition of the SDF-rich fractions from dry beans. Previous studies assessing polysaccharides' immunomodulatory activity did not utilize a TLR-4 antagonist. However, Kawamoto et al. (2008) demonstrated the inhibition of NO production from RAW 264.7 cells stimulated with LPS, reporting significantly ($p < 0.05$) lower nitrite levels from LPS stimulated macrophages with TAK-242 than without TAK-242, agreeing with the obtained results.

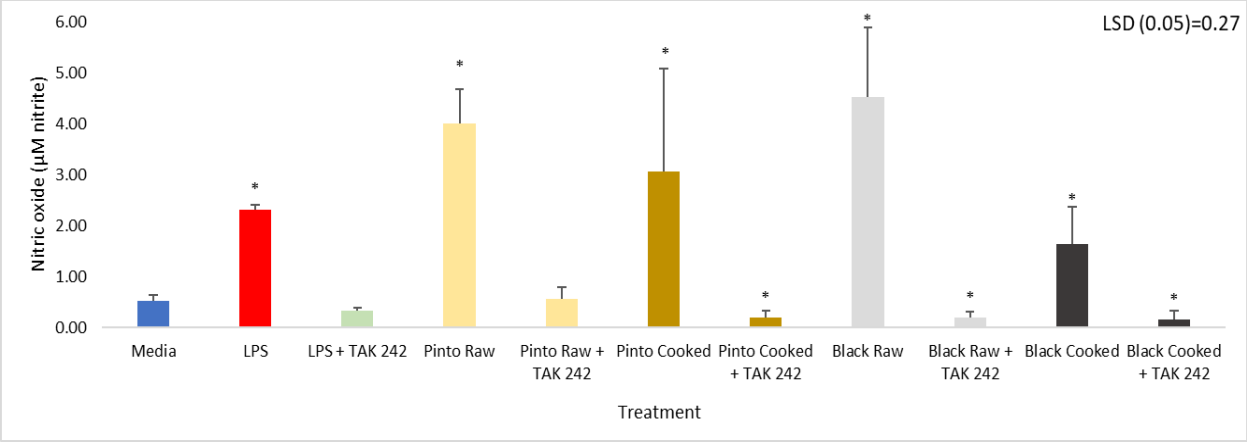

Figure 4. Immunomodulatory effect of SDF-rich fractions on nitric oxide production in RAW 264.7 macrophages.
Media: cells incubated with media only; LPS: cells incubated with LPS; LPS+TAK 242: cells with LPS and the TLR-4 antagonist TAK 242; other treatment conditions include the bean derived SDF (black or pinto) cooked or raw, with or without treatment with the TLR-4 inhibitor. Data are presented as means ± SD. LSD: least significant difference ($p < 0.05$). Differences from cells treated with media only are marked with an asterisk (*). Values differing more than LSD are significantly different from each other.

NO is synthesized from L-arginine and molecular oxygen by an enzymatic process catalyzed by the inducible nitric oxide synthase (iNOS), utilizing electrons donated by NADPH, converting L-arginine into L-citrulline and NO (Sharma et al., 2007; Tripathi et al., 2007). Therefore, to support the NO data, changes in iNOS gene expression were assessed by detecting changes in iNOS mRNA levels in macrophages (Figure 5). The results showed that macrophages cultured with SDF-rich extracts from raw beans significantly ($p <0.05$) promoted the iNOS mRNA expression compared to non-stimulated cells (media control). In addition, the expression of iNOS mRNA was significantly ($p <0.05$) decreased in each analyzed SDF-rich fiber fraction treatment when TAK-242 was utilized. The LPS control also resulted in increased expression of iNOS. This increase was partially reverted in the presence of the TLR-4 inhibitor. Our results thus suggest that TLR-4 stimulation by LPS leads to a signaling cascade resulting in increased expression of iNOS. A similar mechanism was previously suggested by Razali et al. (2014).

Overall, the iNOS mRNA expression results were consistent with the accounted NO levels produced. These results suggest that SDF-rich fractions, especially from raw dry beans, were able to stimulate NO production, as well as iNOS expression through TLR-4 activation.

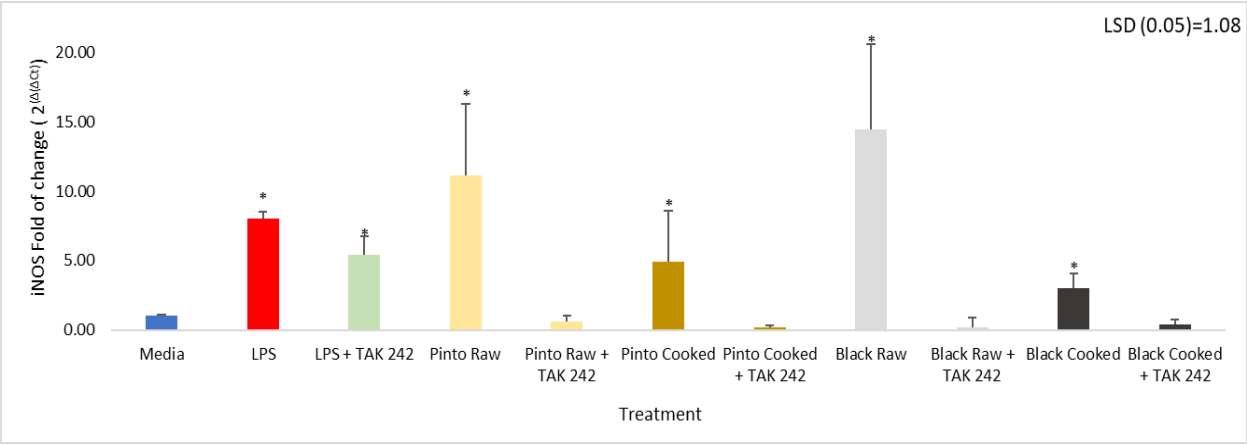

Figure 5. Immunomodulatory effect of SDF-rich fractions on inducible nitric oxide synthase (iNOS) mRNA production in RAW 264.7 macrophages.
Media: cells incubated with media only; LPS: cells incubated with LPS; LPS+TAK 242: cells with LPS and the TLR-4 antagonist TAK 242; other treatment conditions include the bean derived SDF (black or pinto) cooked or raw, with or without treatment with the TLR-4 inhibitor. Data are presented as means ± SD. LSD: least significant difference ($p < 0.05$). Differences from cells treated with media only are marked with an asterisk (*). Values differing more than LSD are significantly different from each other.

4.4.2.2. Effect of SDF-rich fractions on TNF-α production

Cytokines are small proteins secreted by activated macrophages and other immune cells, and mediate and regulate immune responses (Ren et al., 2017). Therefore, cytokine production is oftentimes used to measure macrophage activation (Razali et al., 2014). Tumor necrosis factor α (TNF-α) is a pro-inflammatory cytokine released after trauma, infection or LPS recognition by macrophages (Feldmann et al., 1994). TNF-α is regarded as a master regulator since it can activate other macrophages to enhance functional responses like the production of pro-inflammatory cytokines and prostaglandins, as well as to promote cell recruitment to the inflammation site (He et al., 2012; Parameswaran & Patial, 2010). In the present study, the SDF-rich fractions were able to activate macrophages as reflected in the significantly ($p < 0.05$) higher

levels of TNF-α produced compared to the unstimulated macrophages (Figure 6). Interestingly, the SDF extracted from raw dry beans stimulated significantly ($p < 0.05$) higher levels of TNF-α than the SDF extracted from cooked beans. Similar findings were reported by Yao et al. (2015) in their study of water-extractable polysaccharide fractions from raw adzuki beans. The studied adzuki bean extracts were composed of rhamnose, arabinose, mannose, galactose and glucose, which is a very similar monosaccharide composition to our SDF-rich fractions, except for the presence of rhamnose. When the macrophages were treated with the SDF-rich fractions and the TLR-4 inhibitor, a significant ($p < 0.05$) decrease in the production of TNF-α was observed compared to macrophages treated with the SDF-rich fraction alone. Likewise, Kawamoto et al. (2008) reported the successful suppression of TNF-α production in LPS-stimulated RAW 264.7 macrophages due to TAK-242 selective TLR-4 inhibitor activity. The macrophages' dramatic decrease in TNF-α secretion indicates that SDF-rich fractions interact with TLR-4, thus mediating TLR-4 signaling pathways. TNF-α has been pointed out as the main product secreted in response to LPS-mediated TLR-4 stimulation (Kawai & Akira, 2006; Rahimifard et al., 2017). Interestingly, the level of TNF-α produced is significantly ($p < 0.05$) higher than the levels obtained with LPS-stimulation.

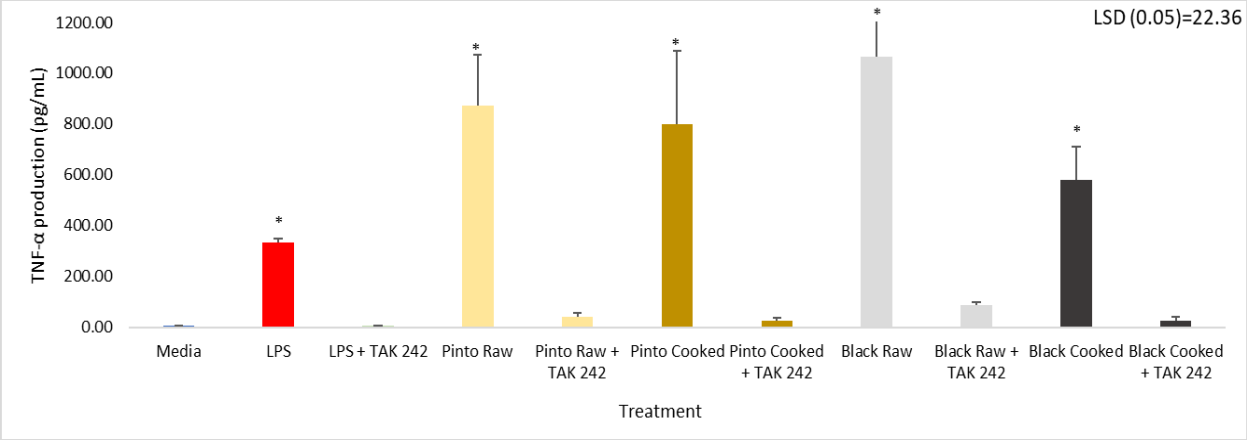

Figure 6. Immunomodulatory effect of SDF-rich fractions on TNF-α production in RAW 264.7 macrophages.
Media: cells incubated with media only; LPS: cells incubated with LPS; LPS+TAK 242: cells with LPS and the TLR-4 antagonist TAK 242; other treatment conditions include the bean derived SDF (black or pinto) cooked or raw, with or without treatment with the TLR-4 inhibitor. Data are presented as means ± SD. LSD: least significant difference ($p < 0.05$). Differences from cells treated with media only are marked with an asterisk (*). Values differing more than LSD are significantly different from each other.

After observing an increase in cytokine levels in the SDF stimulated macrophages, we determined if the mRNA levels of this cytokine were also increased. The TNF-α mRNA levels were significantly ($p < 0.05$) higher in the cells cultured with SDF-rich extracts compared to the unstimulated macrophages (Figure 7). Furthermore, the enhanced TNF-α mRNA levels observed in the macrophages treated with the SDF-rich extracts are in agreement with the enhanced TNF-α levels measured by ELISA. On the other hand, macrophages cultured with TAK-242 showed similar TNF-α mRNA levels to the control macrophages. These results indicate that the macrophages are activated upon TLR-4 receptor recognition by the bean extracted SDF. It is worth noting that the TNF-α mRNA levels were significantly ($p < 0.05$) higher in macrophages treated with raw bean SDF-rich extracts than with LPS.

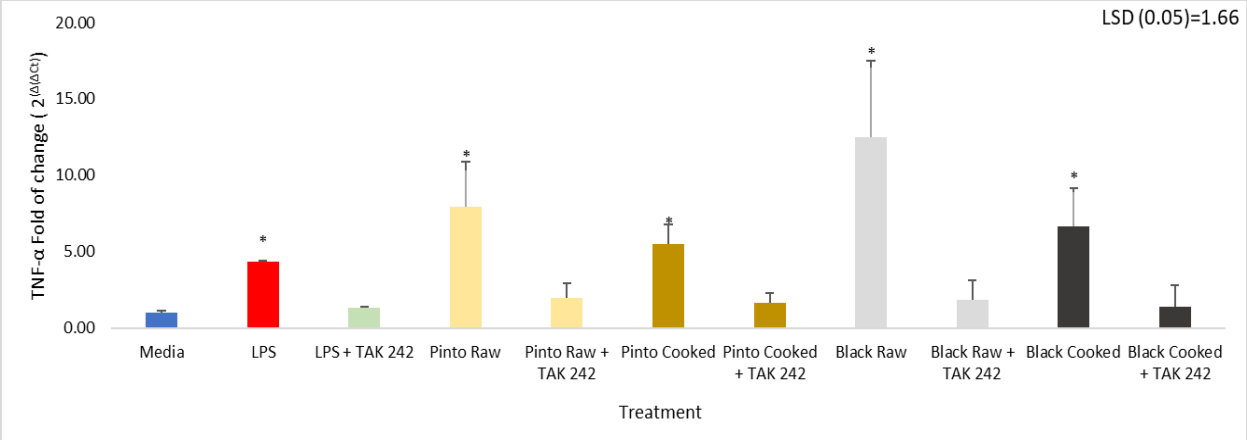

Figure 7. Immunomodulatory effect of SDF-rich fractions on TNF-α mRNA production in RAW 264.7 macrophages.
Media: cells incubated with media only; LPS: cells incubated with LPS; LPS+TAK 242: cells with LPS and the TLR-4 antagonist TAK 242; other treatment conditions include the bean derived SDF (black or pinto) cooked or raw, with or without treatment with the TLR-4 inhibitor. Data are presented as means ± SD. LSD: least significant difference ($p < 0.05$). Differences from cells treated with media only are marked with an asterisk (*). Values differing more than LSD are significantly different from each other.

4.4.2.3. Effect of SDF-rich fractions on IL-6 production

Interleukin 6 (IL-6) is a pro-inflammatory cytokine that stimulates immune responses and helps in fighting infections, for instance, by elevating the body's temperature setpoint (Luo & Zheng, 2016). In this sense, IL-6 is a cytokine produced by activated macrophages to participate in the host defense responses (Ren et al., 2017). In the present study, the IL-6 levels observed from macrophages treated with SDF-rich fractions were significantly ($p < 0.05$) higher than those from untreated macrophages (Figure 8). Furthermore, within each SDF-rich fraction treatment, IL-6 levels were significantly ($p < 0.05$) reduced in the presence of TAK-242, suggesting that the secretion of IL-6 was the result of SDF-rich fractions recognition by TLR-4. Samarpita et al. (2020) reported that TAK-242 inhibited the expression of IL-6 in LPS-stimulated fibroblast-like synoviocytes cells, highlighting the role of TLR-4 in the production of IL-6.

IL-6 production from RAW 264.7 macrophages treated with extracted polysaccharides has been previously reported, in agreement with our current data (Ren et al., 2017; Tang et al., 2018; Yao et al., 2015).

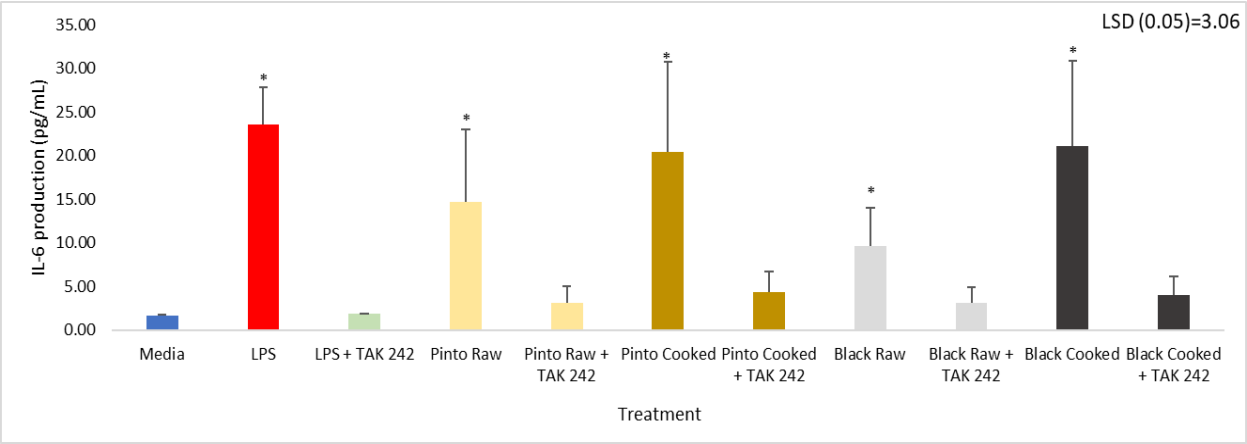

Figure 8. Immunomodulatory effect of SDF-rich fractions on IL-6 production in RAW 264.7 macrophages.
Media: cells incubated with media only; LPS: cells incubated with LPS; LPS+TAK 242: cells with LPS and the TLR-4 antagonist TAK 242; other treatment conditions include the bean derived SDF (black or pinto) cooked or raw, with or without treatment with the TLR-4 inhibitor. Data are presented as means ± SD. LSD: least significant difference ($p < 0.05$). Differences from cells treated with media only are marked with an asterisk (*). Values differing more than LSD are significantly different from each other.

However, when the IL-6 mRNA levels in treated macrophages were assessed (Figure 9), less than 2 fold changes in transcript levels were observed between the untreated macrophages and the SDF treated cells, except for the SDF extracted from black raw beans, where a 4 fold increase ($p<0.05$) in mRNA was observed. This discrepancy between IL-6 transcript and protein levels in treated macrophages deserves additional investigation. In addition, it has been described that mRNA expression does not always correlate with the final gene products due to variations in transcriptional and post-transcriptional processes (Young et al., 2020).

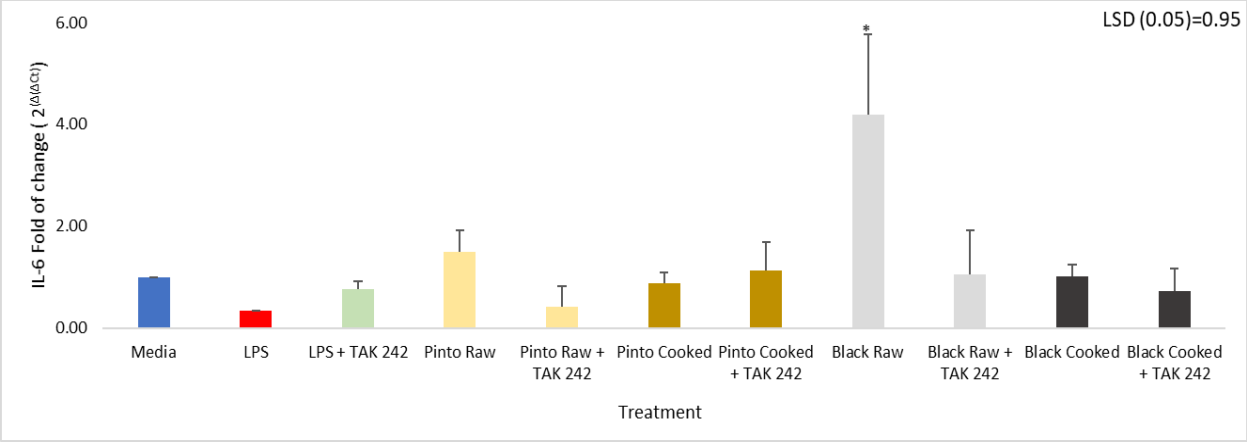

Figure 9. Immunomodulatory effect of SDF-rich fractions on IL-6 mRNA production in RAW 264.7 macrophages.
Media: cells incubated with media only; LPS: cells incubated with LPS; LPS+TAK 242: cells with LPS and the TLR-4 antagonist TAK 242; other treatment conditions include the bean derived SDF (black or pinto) cooked or raw, with or without treatment with the TLR-4 inhibitor. Data are presented as means ± SD. LSD: least significant difference ($p < 0.05$). Differences from cells treated with media only are marked with an asterisk (*). Values differing more than LSD are significantly different from each other.

4.4.2.4. Effect of SDF-rich fractions on IL-1β production

Interleukin 1β (IL-1β) is a pro-inflammatory cytokine implicated in pain, inflammation and autoimmune conditions (Ren & Torres, 2009). For instance, it is an inducer of several components of the inflammation acute phase response, such as cyclooxygenase type 2 (COX-2), prostaglandin-E2 (PGE2), iNOS and NO (Dinarello, 2009; Kaneko et al., 2019). Thus, IL-1β has been associated with fever mediation, lower pain threshold, vasodilation and hypotension (Dinarello, 2009). Therefore, the IL-1β levels from macrophages treated with the bean extracted SDF-rich fractions were determined and are shown in Figure 10. Although the IL-1β levels appear to be slightly higher in SDF treated macrophages than in control cells, the differences were not statistically significant. When the TLR-4 antagonist was used, the IL-1β levels also appeared to be reduced, but the differences were not statistically significant. Compared to the IL-1β levels produced by macrophages stimulated by LPS, the levels of IL-1β produced by SDF-treated macrophages were about 10 fold lower. This difference in response is in marked

contracdiction with the response observed with the other cytokines: the levels of IL-6 observed with the SDF treated macrophages appeared to be at the same order of magnitude as those observed with LPS treated macrophages; with TNF-a, the levels observed following stimulation of macrophages with SDF were 1.5 to 2 fold larger than the levels observed from LPS stimulated macrophages. Our results are in agreement with those of Xie et al. (2016). They observed lower levels of IL-1β when RAW 264.7 macrophages were stimulated with *Dendrobium huoshanense* polysaccharides (DHP) (consisting of glucose, mannose and galactose) compared to macrophages treated with LPS. Furthermore, Xie et al. (2016) performed antibody neutralization experiments with an anti-TLR-4 monoclonal antibody that showed effective blocking of the DHP, resulting in decreased secretion of IL-1β from macrophages.

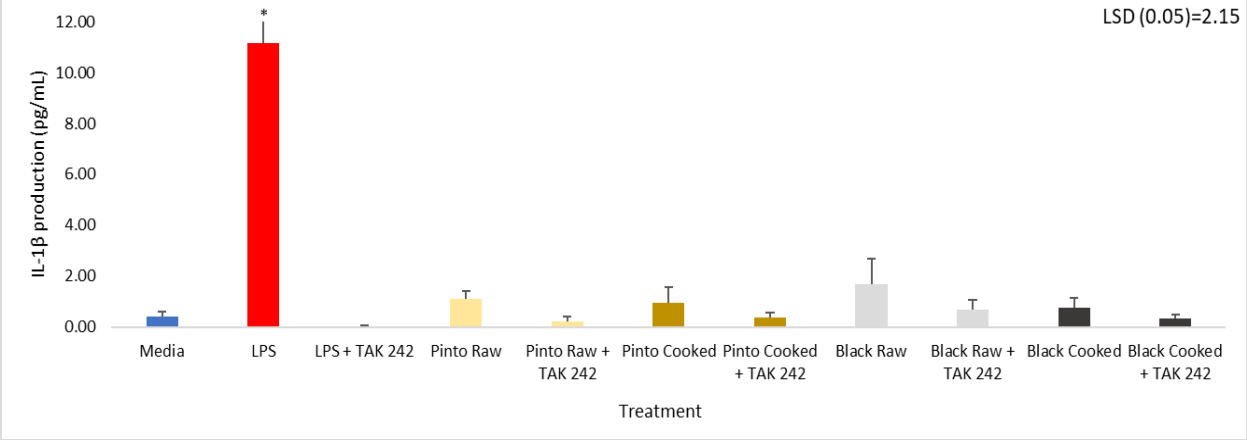

Figure 10. Immunomodulatory effect of SDF-rich fractions on IL-1β production in RAW 264.7 macrophages.
Media: cells incubated with media only; LPS: cells incubated with LPS; LPS+TAK 242: cells with LPS and the TLR-4 antagonist TAK 242; other treatment conditions include the bean derived SDF (black or pinto) cooked or raw, with or without treatment with the TLR-4 inhibitor. Data are presented as means ± SD. LSD: least significant difference ($p < 0.05$). Differences from cells treated with media only are marked with an asterisk (*). Values differing more than LSD are significantly different from each other.

The changes in IL-1β mRNA levels were also investigated and are shown in Figure 11. The SDF-rich fractions elicited significantly ($p < 0.05$) higher IL-1β mRNA levels than the untreated macrophages. The high levels in IL-1β mRNA appear to contrast with the relatively

low IL-1β protein levels measured from the macrophages supernatant. This difference in results could be due to the low half-life of IL-1β in macrophages. Dinarello (2018) described that IL-1β mRNA levels rise within 15 minutes, reach peak levels at 4 hours, and then start to decline due to the half-life of mRNA. However, the transcript levels were significantly reduced when the macrophages were treated with the bean derived SDF and the TLR-4 inhibitors as compared to the macrophages treated with the SDF alone, in agreement with the results observed with the other cytokines.

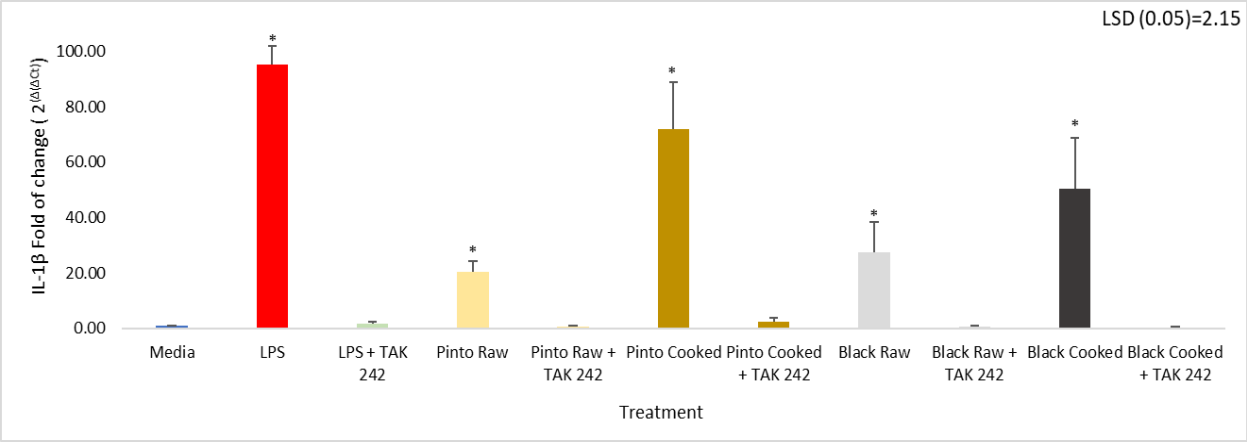

Figure 11. Immunomodulatory effect of SDF-rich fractions on IL-1β mRNA production in RAW 264.7 macrophages.
Media: cells incubated with media only; LPS: cells incubated with LPS; LPS+TAK 242: cells with LPS and the TLR-4 antagonist TAK 242; other treatment conditions include the bean derived SDF (black or pinto) cooked or raw, with or without treatment with the TLR-4 inhibitor. Data are presented as means ± SD. LSD: least significant difference ($p < 0.05$). Differences from cells treated with media only are marked with an asterisk (*). Values differing more than LSD are significantly different from each other.

4.4.2.5. Effect of SDF-rich fractions on TLR-4 production

Macrophage activation by extracted plant polysaccharides is assumed to be mediated by the recognition of polysaccharide polymers by specific receptors (Schepetkin & Quinn, 2006) since polysaccharides cannot enter cells directly due to their large molecular mass and their hydrophilicity (Xie et al., 2016). One of the receptors associated with the polysaccharides recognition in macrophages is TLR-4. Previous studies suggest that upon TLR-4 recognition, the

polysaccharides activate the myeloid differentiation primary response protein 88 (MyD88) dependent pathway, leading to IL-1 receptor-associated kinase (IRAK) recruitment. (Gupta et al., 2017; Park et al., 2010; Xie et al., 2016; Zhang et al., 2016). Activated IRAK then binds to and activates TNF receptor-associated factor 6 (TRAF6). These associations stimulate the activation of nuclear factor kappa B (NF-κB) and mitogen-activated protein kinase (MAPK) signaling pathways. Activated NF-κB then translocates to the nucleus, where it activates pro-inflammatory mediators such as iNOS, and stimulates the expression and release of inflammatory cytokines such as TNF-α, IL-6 and IL-1β. At the same time, MAPK extracellular signal-regulated kinase 1/2 (ERK1/2), p38, and Jun N-terminal kinases (JNK) pathways modulate the activation of additional transcription factors, like activator protein-1 (AP-1), resulting in signal amplification (Park et al., 2010; Rahimifard et al., 2017; Takeda & Akira, 2004; Xie et al., 2016).

Because our data indicate that the analyzed SDF-rich fractions were recognized by TLR-4, resulting in a pro-inflammatory response, the expression levels of TLR-4 mRNA on macrophages were analyzed (Figure 12). Significantly ($p < 0.05$) higher levels of TLR-4 transcripts were observed from SDF-rich fractions treated macrophages than from non-treated cells. In addition, macrophages treated with SDF-rich fractions denoted a significantly ($p < 0.05$) higher expression of TLR-4 mRNA when compared to the LPS control. These results suggest that SDF-rich fractions could modulate TLR-4 signaling by enhancing the TLR-4 expression on the cells, thus amplifying the immune response. Furthermore, in the treatments where macrophages were cultured with TAK-242 and the SDF-rich extracted fractions, significantly ($p < 0.05$) lower levels of TLR-4 transcripts were observed when compared to cells treated with SDF fractions alone. In this sense, upon recognition, the SDF-rich fractions upregulate the expression of TLR-4 in macrophages, exhibiting an enhanced immune response.

In addition, positive and highly significant ($p < 0.01$) correlations were found between the transcript levels of TLR-4, iNOS, TNF-α, and IL-6 (Table 10). In this sense, the transcriptional upregulation of the different pro-inflammatory molecules that were studied appears to be associated with the activation of TLR-4 receptors following the interaction with SDF-rich fractions. However, the lack of correlation between the TLR-4 and IL-1β transcripts suggests that an increase in IL-1β transcripts could result from the recognition of another receptor by the SDF-rich fractions.

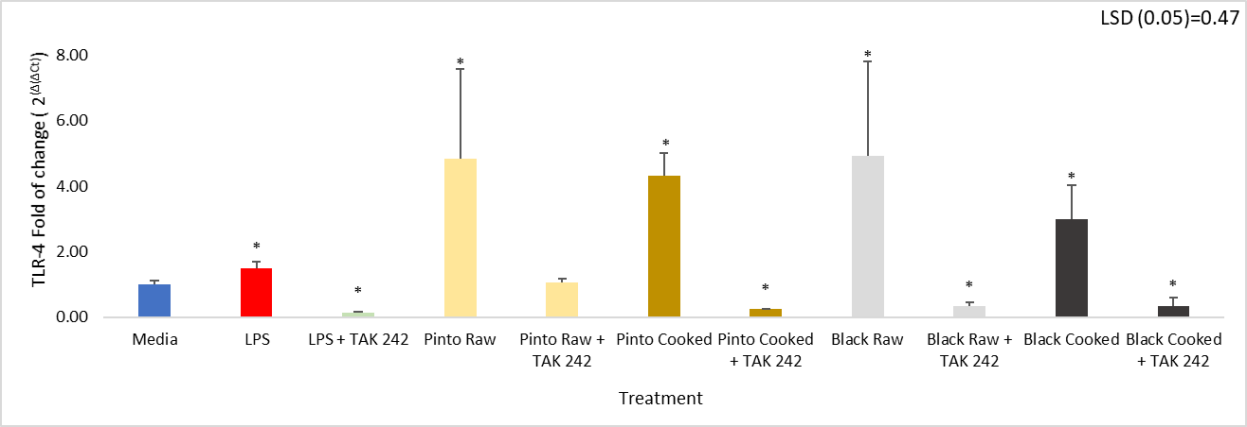

Figure 12. Immunomodulatory effect of SDF-rich fractions on TLR-4 mRNA production in RAW 264.7 macrophages.
Media: cells incubated with media only; LPS: cells incubated with LPS; LPS+TAK 242: cells with LPS and the TLR-4 antagonist TAK 242; other treatment conditions include the bean derived SDF (black or pinto) cooked or raw, with or without treatment with the TLR-4 inhibitor. Data are presented as means ± SD. LSD: least significant difference ($p < 0.05$). Differences from cells treated with media only are marked with an asterisk (*). Values differing more than LSD are significantly different from each other.

Table 10. Simple linear correlation coefficients between immunomodulatory properties (using qRT-PCR).

Sample	iNOS ($2^{\Delta(\Delta Ct)}$)	TNF-α ($2^{\Delta(\Delta Ct)}$)	IL-6 ($2^{\Delta(\Delta Ct)}$)	IL-1β ($2^{\Delta(\Delta Ct)}$)	TLR4 ($2^{\Delta(\Delta Ct)}$)
TLR4 ($2^{\Delta(\Delta Ct)}$)	0.643***	0.588**	0.945***	NS	1.00

NS: nonsignificant; *: $p > 0.05$; **: $p \leq 0.01$; and ***: $p \leq 0.001$; n= 24.

In addition, the immunomodulatory activity of the SDF-rich fractions on LPS-stimulated macrophages (Figure 13) was investigated to study if the SDF-rich fractions could enhance or reduce the LPS-dependent immune effects. Surprisingly, additive effects or in other words combined effects, where the LPS-stimulation and the SDF-rich fraction stimulation equal the sum of each individual effect on macrophages, were not found for any of the pro-inflammatory cytokines. In contrast, enhanced productions of NO, TNF-α, IL-6 and IL-1β were observed in the presence of the bean-derived rich fiber extracts (Figure 13). The NO, TNF-α, and IL-6 produced when the LPS-stimulated macrophages were treated with SDF-rich extracts were significantly ($p < 0.05$) higher than the LPS-stimulated macrophages control. The obtained results are explained by the observed upregulation of TLR-4 mRNA when macrophages were cultured with SDF-rich fractions. As more TLR-4 was expressed on the cells, the bean-derived fiber fractions did not have to compete with the LPS for binding sites. This was reflected in an amplified cytokine response, resulting in a stronger inflammation response. Furthermore, the stronger inflammation can be conceived as a protective inflammation that could boost the defense against infection at localized sites.

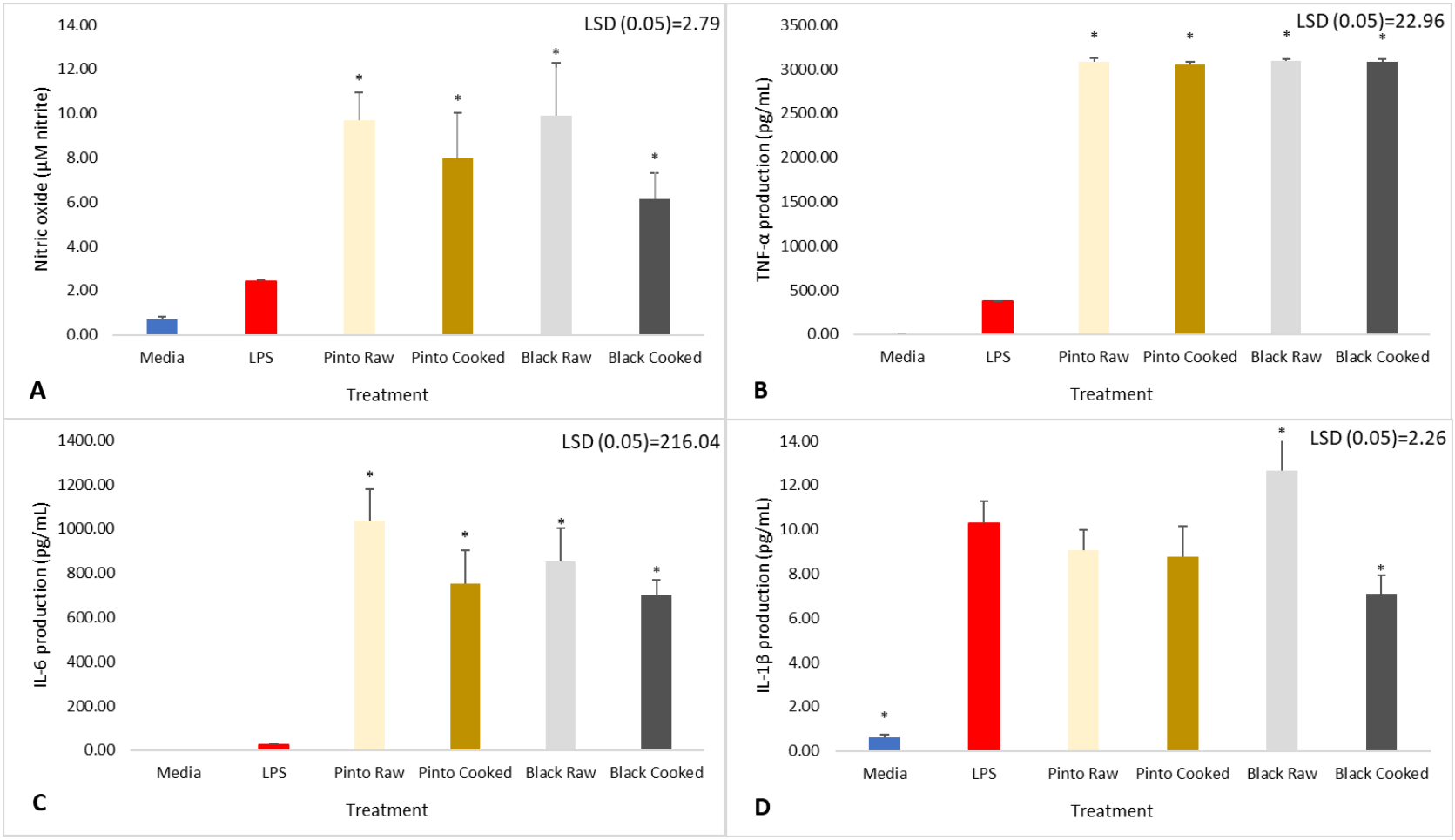

Figure 13. Immunomodulatory effect of SDF-rich fractions on LPS induced (A) nitric oxide, (B) TNF-α, (C) IL-6, and (D) IL-1β in LPS-stimulated macrophages.

LPS-stimulated macrophages were incubated with 1 µg/mL of LPS for 2 hours. Media: cells incubated with media only; LPS: cells incubated with LPS; other treatment conditions include the bean derived SDF (black or pinto) cooked or raw. Data are presented as means ± SD. LSD: least significant difference ($p < 0.05$). Differences from cells treated with LPS are marked with an asterisk (*). Values differing more than LSD are significantly different from each other.

4.4.2.6. Relationship of SDF-rich fractions with immunomodulatory properties

Growing evidence regarding plant polysaccharides' capacity to activate macrophages leading to the secretion of pro-inflammatory cytokines and enhanced levels of NO has been reported (Dai et al., 2014; Ramberg et al., 2010; Razali et al., 2014; Z. Ren et al., 2017; Yao et al., 2015; Yu et al., 2018). However, some previous publications reporting polysaccharides' immunostimulatory activity on macrophages have failed to report the relationship between the polysaccharides' composition or structure and the observed immune response (Gupta et al., 2017; Li & Xu, 2011; Park et al., 2010; Xie et al., 2016; Xu et al., 2020). In this regard, the monosaccharide composition (Chapter 2) of the SDF-rich fractions correlations with the measured immunomodulatory properties was performed (Table 11). Few significant ($p < 0.05$) correlations were found, including the positive and significant ($p < 0.05$) correlation between NO levels with xylose (0.402) and mannose (0.410), the negative and significant ($p < 0.05$) correlation between TLR-4 mRNA expression with arabinose (-0.430) and glucose (-0.493), the positive and highly significant ($p < 0.01$) correlation between IL-1β mRNA expression with arabinose (0.552), glucose (0.883) and galacturonic acid (0.528), the positive and significant ($p < 0.05$) correlation between IL-6 mRNA expression with arabinose (0.433) and the negative and highly significant ($p < 0.01$) correlation between IL-6 mRNA expression with glucose (-0.533), as well as the negative and significant ($p < 0.05$) correlation between TNF-α mRNA expression with glucose (-0.476). In other words, to a greater extent, glucose and arabinose, and to a lesser extent, xylose, mannose and galacturonic acid seemed to relate with the elicited immune activity. Furthermore, Zhang et al. (2016) reported that the monosaccharide composition is a critical factor determining polysaccharide activity through TLR-4, highlighting the relevance of glucose and mannose as their homoglycan forms, glucan and mannan, are known TLR-4

ligands. As described in Chapter 2, the monosaccharide composition of the SDF-rich fractions suggests the presence of pectic polysaccharides, hemicelluloses (xyloglucans), and the raffinose family oligosaccharides (RFOs). In this sense, Dai et al. (2014) reported that verbascose (RFOs member) from mung beans (*Phaseolus aureus*) elicited pro-inflammatory activity due to the enhanced levels of NO, IL-1β, IL-6, interferon (IFN)-α and IFN-γ released by RAW 264.7 macrophages. Additionally, Razali et al. (2014) suggested that their studied polysaccharide fractions from *Solanum nigrum*, containing pectic polysaccharides, stimulated a pro-inflammatory response from RAW 264.7 macrophages. However, the lack of structural data of the SDF-rich fractions, such as degree of substitution and linkage types, limits the study of further significant correlations with the immunomodulatory results.

Table 11. Simple linear correlation coefficients between immunomodulatory properties and dry bean soluble fiber-rich fraction composition.

Sample	NO [a] (μm NO_2^-)	iNOS [b] ($2^{\Delta(\Delta Ct)}$)	TNF-α ($2^{\Delta(\Delta Ct)}$)	IL-6 ($2^{\Delta(\Delta Ct)}$)	IL-1β ($2^{\Delta(\Delta Ct)}$)	TLR4 ($2^{\Delta(\Delta Ct)}$)
Arabinose (%)	NS	NS	NS	0.433*	0.552**	-0.430*
Galactose (%)	NS	NS	NS	NS	NS	NS
Glucose (%)	NS	NS	-0.476*	-0.533**	0.883***	-0.493*
Xylose (%)	0.402*	NS	NS	NS	NS	NS
Mannose (%)	0.410*	NS	NS	NS	NS	NS
Fructose (%)	NS	NS	NS	NS	NS	NS
Galacturonic acid (%)	NS	NS	NS	NS	0.528**	NS
Total sugars (%)	NS	NS	NS	-0.417*	0.797***	NS

NS: nonsignificant; *: $p > 0.05$; **: $p \leq 0.01$; and ***: $p \leq 0.001$; n= 24.
[a] NO: Nitric oxide.
[b] iNOS: Inducible nitric oxide synthase.

4.5. Conclusions

The study showed that SDF-rich fractions extracted from dry beans promoted the secretion of pro-inflammatory molecules NO, TNF-α, IL-6, and IL-1β by RAW 264.7 murine macrophages. The results suggest that the SDF-rich fraction elicited the pro-inflammatory response of macrophages through TLR-4 mediated activation. In addition, SDF-rich fractions promoted TLR-4 mRNA expression, suggesting the enhancement of the macrophages' immune response. The obtained results show the possibility of considering SDF-rich fractions extracted from dry beans as immunostimulators to aid in the prevention and fight of infections.

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5. DRY BEAN SOLUBLE DIETARY FIBER INHIBITORY EFFECTS ON 3T3-L1 ADIPOCYTE DIFFERENTIATION

5.1. Abstract

Adipocyte differentiation, or adipogenesis, plays a vital role in the expansion of adipose (fat) tissue, causing adipocyte enlargement by the accumulation of lipid droplets. Potential health benefits have been associated with dry beans-derived dietary fiber, but its impact on adipogenesis has not been explored yet. Therefore, the present study assessed the impact of conditioned media obtained from macrophages treated with different dry bean soluble dietary fiber (SDF) rich fractions on the differentiation of 3T3-L1 adipocytes. Adipogenic differentiation was evaluated by examining oil red O (ORO) staining of lipid droplets, triglycerides (TGA) content, and the expression of adipogenesis markers. We observed that the adipogenic differentiation of 3T3-L1 cells was impaired in the presence of macrophage conditioned media (MCM) obtained from SDF-rich fractions treated macrophages. In contrast, MCM obtained from macrophages treated with SDF-rich fractions and a TLR-4 inhibitor (TAK-242) did not impair 3T3-L1 cells' adipogenic differentiation. Our results suggest that secreted factors secreted by macrophages upon stimulation by SDF-rich fractions inhibit the formation of mature adipocytes. Therefore, SDF-rich fractions extracted from dry beans are good candidates for the development of functional ingredients that could prevent obesity by limiting adipose tissue expansion.

5.2. Introduction

Overweight and obesity are defined as excessive fat accumulation, which increases the risk of metabolic diseases, cardiovascular diseases, diabetes, and various cancers (World Health Organization, 2021). Obesity is a complex health disorder resulting from an imbalance between energy-rich food consumption and energy spent, and leads to adipose tissue accumulation after

incremental changes in adipocyte (fat cell) number (hyperplasia) and volume (hypertrophy) (Ambele et al., 2020; Gesta et al., 2007; Teng et al., 2020). Furthermore, the process from which pre-adipocytes, which originate from stem cells committed to adipocyte lineage, differentiate and develop into functioning adipocytes is called adipogenesis (Warne, 2003; Zhu et al., 2016). In this sense, inhibiting adipogenesis may limit hyperplasia and hypertrophy, hence aiding in obesity reduction (Liu et al., 2018). Thus, finding food ingredients that display anti-obesity activity would be beneficial for reducing the obesity epidemic (Teng et al., 2020).

Legumes' nutritional components, such as protein, starch, flavonoids, phytoestrogens, and dietary fibers, have been associated with weight control and obesity management (Zeron-Rugerio & Izquierdo-Pulido, 2019). Within legumes, dry beans (*Phaseolus vulgaris* L.) are excellent sources of proteins, starch, phytochemicals, and both soluble (SDF) and insoluble dietary fibers (IDF) (Ramírez-Jiménez et al., 2015; Rondini et al., 2013). Previous research has suggested the efficacies of dry bean components for obesity attenuation (Lu et al., 2018; Monk et al., 2019, 2021). For instance, Lu et al. (2018) studied how fecal fermentation of dry bean-derived dietary fiber products, predominantly producing acetate, was counteracting adipogenesis by suppressing the transcription of key factors (peroxisome proliferator-activated receptor γ (PPARγ) and CCAAT/enhancer-binding proteins α (C/EBPα)) that signal adipocytes differentiation and maturation. In addition, Monk et al. (2021) conducted an *in vivo* study in obese mice by supplementing their diets with cooked whole dry bean powder, which resulted in increased the fermentation of carbohydrates, presumably dietary fibers, that improved intestinal health and alleviated adipose tissue inflammation. Besides fermentation, dietary fiber has been associated with other properties that affect obesity management. For instance, while IDF is fermented and contributes to fecal bulk, thus promoting laxation, SDF forms viscous solutions that delay gastric

emptying, control appetite by inducing satiety, slow intestinal transit, and control postprandial glucose and lipid levels by slowing the absorption rate of nutrients (Kristensen & Jensen, 2011; Rebello et al., 2014). However, the impact of dry bean extracted SDFs on adipogenesis has not been explored yet.

Polysaccharides extracted from different plant sources have been reported to inhibit pre-adipocyte differentiation (Liu et al., 2018; Teng et al., 2020; Zhu et al., 2016). For instance, Zhu et al. (2016) reported that extracted barley β-glucans inhibited adipocyte differentiation and lipid accumulation by downregulating the principal adipogenesis regulators, PPARγ and C/EBPα. In addition, Liu et al. (2018) observed the inhibition of PPARγ, C/EBPα, and sterol regulatory element-binding protein-1c (SREBP-1c) when pine needles polysaccharides along with kudzu flavonoids were cultured with pre-adipocytes. These studies agree with Teng et al. (2020) findings, who reported that extracted polysaccharides from quinoa suppressed PPARγ, C/EBPα, SREBP-1c, and adipocyte protein 2 (AP2) expression when cultured with pre-adipocytes. The studies mentioned above utilized the 3T3-L1 pre-adipocyte cell line, which is an established model of adipose cell differentiation that mirrors *in vivo* processes (Gagnon & Sorisky, 1998). However, from a physiological perspective, in fat tissue, pre-adipocytes and adipocytes are not in direct contact with food-derived polysaccharides and dietary fiber. However, these pre-adipocytes and adipocytes can be indirectly modulated by cytokines secreted from activated macrophages that are in contact with the dietary fiber. Therefore, in our study, we treated pre-adipocytes with the conditioned media of macrophages that had been stimulated with bean-derived dietary fiber. Thus, the aim of the present study was to assess the impact of the conditioned media of macrophages, treated with the soluble dietary fiber (SDF) rich fraction extracted from dry beans, on pre-adipocytes (3T3-L1 cell line) differentiation.

5.3. Materials and Methods

5.3.1. Materials

The RAW 264.7 murine macrophages and the 3T3-L1 embryonic fibroblast mouse cells were purchased from the American Type Culture Collection (ATCC) (Manassas, VA). Dulbecco's Modified Eagle's Medium (DMEM) (ATCC®. 30-2002), Fetal Bovine Serum (FBS) (ATCC®. 30-2020) and Calf Bovine Serum (CBS) (ATCC®. 30-2030) were purchased from ATCC (Manassas, VA). Trypsin (0.25%) EDTA (1X) (VWR, 02-0154-0100) was purchased from VWR (Solon, OH). Lipopolysaccharides (LPS) from *E. coli*, Serotype O111:B4 (1 mg/mL, ALX-581-012-L002) were obtained from Enzo Life Sciences (Farmingdale, NY). TAK-242 (TLR-4 signaling inhibitor, A3850) was purchased from APExBIO (Houston, TX). Dexamethasone (DEX, D2915), 3-Isobutyl-1-methylxanthine (IBMX, I5879), insulin solution (I9278), and Oil Red O solution (O1391) were purchased from Sigma-Aldrich (St. Louis, MO). Pierce™ BCA Protein Assay Kit (23225) was purchased from Thermo Scientific (Waltham, MA). Triglyceride Colorimetric Assay Kit (10010303) was obtained from Cayman Chemical (Ann Arbor, MI). Adipogenesis Marker Antibody Sampler Kit (12589) was purchased from Cell Signaling Technology (Danvers, MA). Clarity Western ECL substrate (1705061) was obtained from Bio-Rad Laboratories, Inc. (Hercules, CA).

5.3.2. Preparation of Conditioned Media

RAW 264.7 cells were plated at 1×10^6 cells per well in 6 well-plates and incubated overnight. The treatments described in Chapter 3 were followed; thus, the final working concentration of each compound was: SDF-rich fractions (500 µg/mL), LPS (1 µg/mL), TAK-242 (10 µM). The macrophage-conditioned media (MCM) was collected, centrifuged at 13000 rpm for two minutes, aliquoted and stored frozen at -20°C until use.

5.3.3. Culture and Differentiation of 3T3-L1 Pre-adipocytes

3T3-L1 murine pre-adipocytes cells were grown in DMEM supplemented with 10% CBS, penicillin (100 U/mL) and streptomycin (100 µg/mL) at 37°C under a humidified atmosphere of 95% air and 5% CO_2. The cells were cultured in the media described above until they reached 80% confluency. To induce differentiation, an adipogenic medium consisting of 90% DMEM, 10% FBS, 1.0 µM dexamethasone, 0.5 mM IBMX, and 1.0 µg/mL insulin was utilized for 48 hours. Then, the media was replenished every two days with the same adipogenic medium without dexamethasone and IBMX. To assess the effect of the MCM in the pre-adipocytes differentiation, the pre-adipocytes were incubated with media consisting of 85% of the macrophage conditioned media (MCM) and 15% of 6X concentration of 1.0 µM dexamethasone, 0.5 mM IBMX, and 1.0 µg/mL insulin (working concentration of dexamethasone, IBMX and insulin was 1X), for a total of eight days.

5.3.4. Quantification of Lipid Accumulation in Adipocytes

3T3-L1 pre- adipocytes cells were plated at 2×10^3 cells per well in 96 well-plates and induced into adipocytes as described in section 4.3.3. To determine the lipid accumulation, the cells were stained with oil red O (ORO) at differentiation days 5 and 8. Briefly, the media was removed from the cells, and the cells were washed twice with PBS. Then, the cells were fixed with 4% paraformaldehyde and incubated at room temperature for 30 minutes. Next, the paraformaldehyde was discarded, and the cells were washed twice with PBS. Then, 60% isopropyl alcohol was incubated with the cells for 5 minutes. Next, the isopropyl alcohol was removed, and the ORO working solution (60 % ORO : 40 % dH_2O) was added and incubated for 1 hour protected from light. After incubation, the cells were washed five times with dH_2O. The stained cells were kept in PBS for microscopic image analysis (Leica DM500 Fluorescence

Microscope). Finally, to quantify the ORO stain, PBS was discarded, and 100% isopropanol (100 µL) was added to each well, and then quantified at 510 nm using a SpectraMax M5 microplate reader (Molecular Devices, Sunnyvale, CA).

5.3.5. Triglyceride Content

3T3-L1 pre-adipocyte cells were plated at 8×10^4 cells per well in 6 well-plates and differentiated into adipocytes as described in section 4.3.3. Triglyceride content was analyzed at differentiation day 8 using a triglyceride colorimetric assay kit (Cayman Chemical) according to the manufacturer's instructions. Results were expressed as mg/dL.

5.3.6. Western Blot Analysis

3T3-L1 pre- adipocytes cells were plated at 8×10^4 cells per well in 6 well-plates and differentiated into adipocytes as described in section 4.3.3. After eight days of differentiation, adipocytes were collected and lysed in ice-cold RIPA lysis buffer containing 1 mM DTT, 1 mM PMSF, 1% Nonidet P-40 (NP-40), 0.5 % w/v Na Deoxycholate and 0.1% SDS. The plates were swirled with the lysis buffer every 10 min for 30 min, then the lysates were collected and frozen at -80°C for 30 min. The lysate was recovered in Eppendorf tubes and centrifuged. The supernatant was recovered, aliquoted and kept frozen at -20°C until use. The protein concentration was determined using the Pierce™ BCA Protein Assay Kit.

Proteins from cell lysates (30 µg/lane) were separated using 12% or 15% SDS-PAGE and transferred onto PVDF membranes (Millipore). Membranes were blocked with 5% BSA in 1X TBS solution for 1 hour, incubated with different antibodies (Adiponectin; fatty acid-binding protein 4 (FABP4) peroxisome proliferator-activated receptor γ (PPARγ)) from the Adipogenesis Marker Antibody Sampler Kit (Cell Signaling) at the recommended dilution (1/1000) in 5% BSA-TBS solution overnight at 4°C, and with HRP-linked secondary antibodies for 1 hour at

room temperature. The blots were washed with TBS-tween 0.1% between incubation with the different antibodies. The bands on the blot were visualized using Clarity Western ECL HRP substrate (Bio-Rad), and the images were captured using a C-DiGit Blot Scanner (LI-COR Biosciences).

5.3.7. Statistical Analysis

All the cell culture experiments were done in duplicate. The analysis of variance (ANOVA) was conducted using the SAS software (version 9.4, SAS Institute, Cary, NC, USA). The least significant difference (LSD) value was used to separate means, and the significance was accepted at $p < 0.05$.

5.4. Results and Discussion

5.4.1. Macrophage-Conditioned Media Effect on 3T3-L1 Differentiation

As described in Chapter 3, SDF-rich fractions extracted from raw and cooked pinto and black dry beans elicited a pro-inflammatory response on RAW 264.7 macrophages. The pro-inflammatory response was characterized by the production of nitric oxide (NO) and the pro-inflammatory cytokines tumor necrosis factor α (TNF-α), interleukin 6 (IL-6) and IL-1β. Furthermore, the results suggested that the SDF-rich fractions had affinity for toll-like receptor 4 (TLR-4), implying that TLR-4 was responsible for macrophage activation because when a TLR-4 antagonist (TAK-242) was used, the inflammatory response, thus the secretion of pro-inflammatory molecules, was inhibited. For this reason, the macrophage conditioned media (MCM) cultured with the SDF-rich fractions with and without TAK-242 was collected to assess its effect on adipocyte (3T3-L1) differentiation. The oil red O (ORO) lipid accumulation quantification and microscopic images of 3T3-L1 adipocytes at day 5 (middle-stage) and day 8 (mature adipocytes) of differentiation are presented in Figures 14-17.

At day 5 of differentiation, the lipid accumulation was significantly ($p < 0.05$) lower in 3T3-L1 cells cultured with MCM treated with LPS than in the untreated differentiated 3T3-L1 adipocytes (positive control without MCM) (Figure 14). In this experiment, the MCM obtained from LPS-stimulated macrophages was used as a negative control (adipogenesis inhibitor). Similar levels of lipid accumulation were observed with the untreated adipocytes, and with adipocytes treated with MCM from LPS-stimulated macrophages and TLR-4 inhibitor (TAK-242), as well as conditioned media from TAK-242 treated macrophages. In addition, the adipocytes treated with MCM from SDF-rich fractions stimulated macrophages without TLR-4 inhibitor showed significantly ($p < 0.05$) lower lipid accumulation than adipocytes treated with the MCM from SDF and TLR-4 inhibitor treatments. Overall, the lipid accumulation was significantly ($p < 0.05$) reduced in the absence of TAK-242 in the MCM. The change in lipid accumulation could also be observed by microscopy, where the dark red spots represent the formed lipid vacuoles surrounding the nucleus of the 3T3-L1 cells (Figure 15).

At day 8 of differentiation, the lipid accumulation of non-treated differentiated 3T3-L1 adipocytes was significantly ($p < 0.05$) higher than that from adipocytes treated with conditioned media from LPS-stimulated macrophages (Figure 16). Furthermore, adipocytes differentiated with conditioned media from LPS-stimulated macrophages in the presence of TAK-242 had a similar lipid content compared to the non-treated adipocytes. Similar results were found for the 3T3-L1 adipocytes cultured with the TAK-242 MCM treatment, suggesting that the TLR-4 inhibitor did not affect lipid accumulation. In addition, the adipocytes treated with MCM from SDF-rich fractions without TAK-242 had significantly ($p < 0.05$) lower lipid accumulation when compared to the non-treated adipocytes control. Moreover, the lipid accumulation in adipocytes cultured with MCM from SDF-rich fractions with TAK-242 was significantly ($p < 0.05$) higher

when compared with MCM from fibers without TLR-4 inhibitor treatments. These contrasts in lipid accumulation are illustrated in Figure 17. The 3T3-L1 cells cultured with MCM in the presence of TAK-242 have enlarged adipocytes and distinguishable round clustered lipid droplets around the cell nucleus. These round lipid droplets were not visible when adipocytes were treated with MCM without TLR-4 inhibitor.

These results suggest that the differentiation of 3T3-L1 adipocytes was carried out successfully when the cells were cultured with the conditioned media of non-treated macrophages, and that the products secreted from SDF-stimulated macrophages decreased the differentiation ability of 3T3-L1 cells. Similar results were obtained by Lacasa et al. (2007) and Ma et al. (2020); in both studies, researchers assessed the impact of conditioned media from macrophages eliciting an inflammatory response, thus secreting inflammatory cytokines. They also reported that macrophage-secreted factors inhibited adipogenic differentiation, identifying TNF-α as the key regulator of the inhibitory effect. In addition, other previously published studies (Cawthorn & Sethi, 2008; Heilbronn & Campbell, 2008; Jiang et al., 2019; Makki et al., 2013; Warne, 2003) have also pointed out TNF-α as a potent inhibitor of adipogenesis, supporting the obtained results.

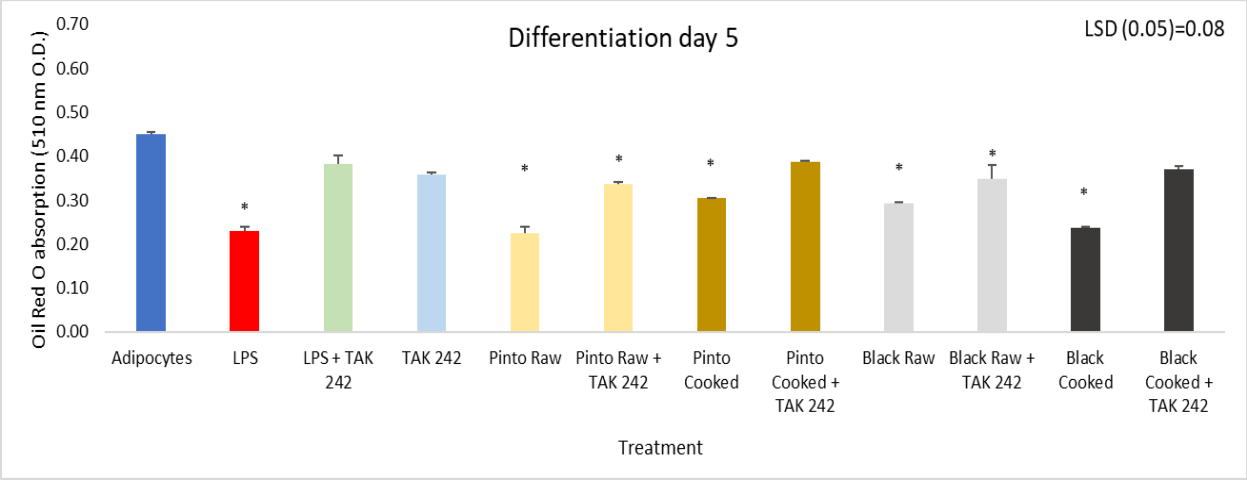

Figure 14. Effect of macrophage conditioned media (MCM) treated with SDF-rich fractions on lipid accumulation in 3T3-L1 cells at differentiation day 5.
Adipocytes: cells treated with differentiation media only; The rest of the treatment labels indicate the MCM conditions as follows. LPS: cells incubated with LPS; LPS+TAK 242: cells with LPS and the TLR-4 antagonist TAK 242; TAK 242: cells with TLR-4 antagonist TAK 242; other treatment conditions include the bean derived SDF (black or pinto) cooked or raw, with or without treatment with the TLR-4 inhibitor. Data are presented as means ± SD. LSD: least significant difference ($p < 0.05$). Differences from adipocytes are marked with an asterisk (*). Values differing more than LSD are significantly different from each other.

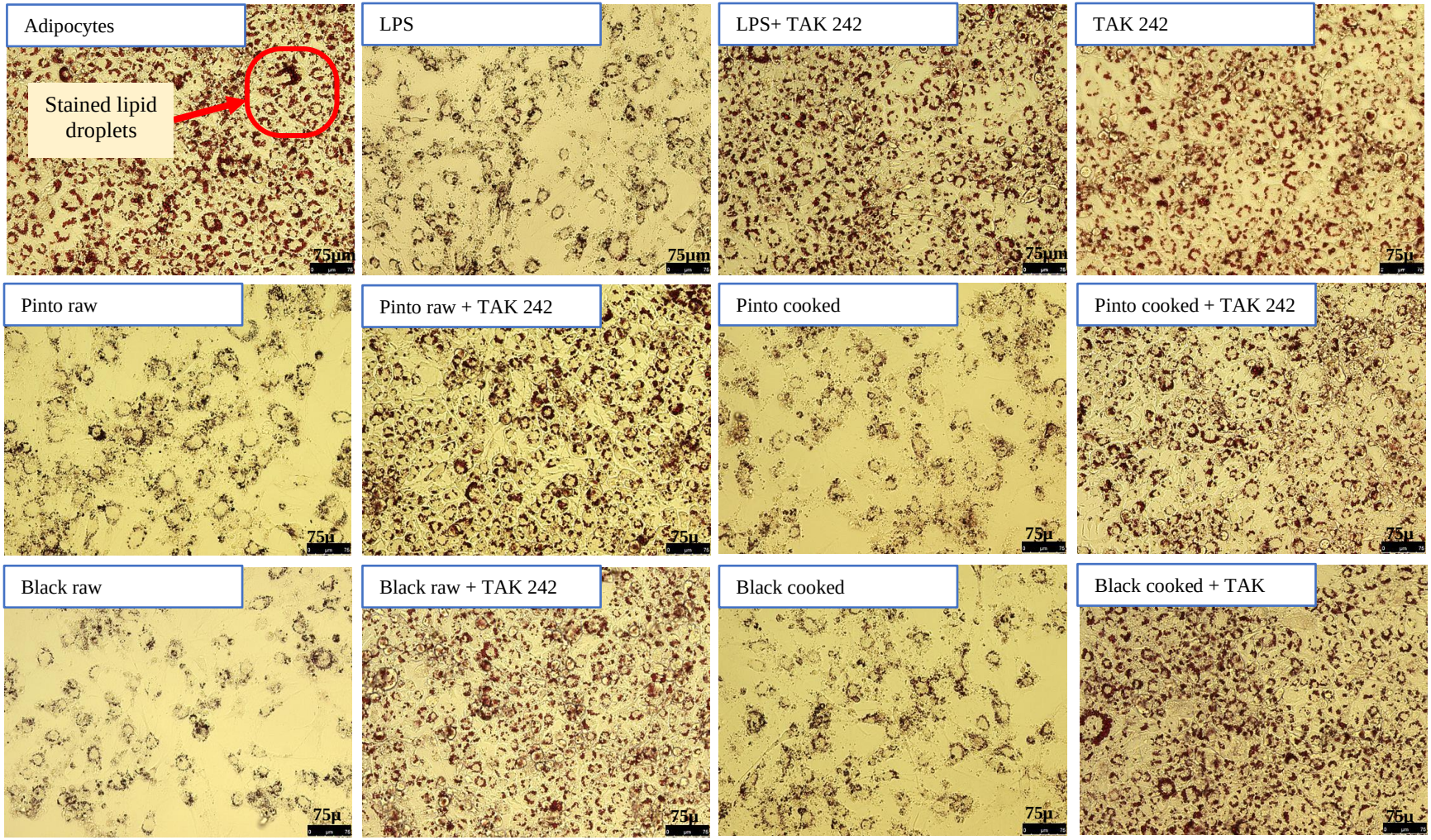

Figure 15. Effect of macrophage conditioned media (MCM) treated with SDF-rich fractions on lipid accumulation in 3T3-L1 cells at differentiation day 5 assessed by oil red O (ORO) staining. 20X objective photographs. Scale bar: 75 µm.

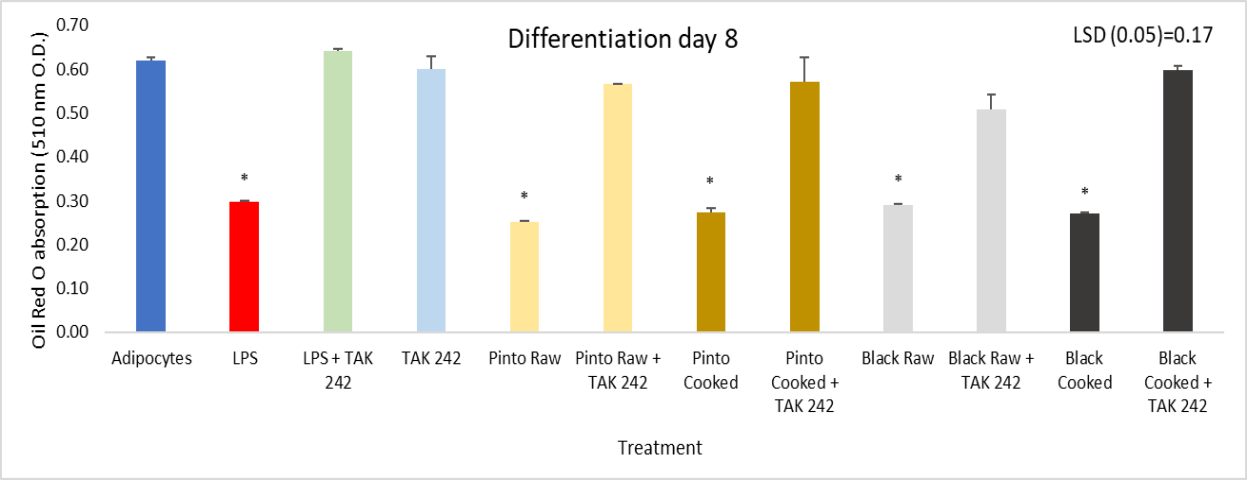

Figure 16. Effect of macrophage conditioned media (MCM) treated with SDF-rich fractions on lipid accumulation in 3T3-L1 cells at differentiation day 8.
Adipocytes: cells treated with differentiation media only; The rest of the treatment labels indicate the MCM conditions as follows. LPS: cells incubated with LPS; LPS+TAK 242: cells with LPS and the TLR-4 antagonist TAK 242; TAK 242: cells with TLR-4 antagonist TAK 242; other treatment conditions include the bean derived SDF (black or pinto) cooked or raw, with or without treatment with the TLR-4 inhibitor. Data are presented as means ± SD. LSD: least significant difference ($p < 0.05$). Differences from adipocytes are marked with an asterisk (*). Values differing more than LSD are significantly different from each other.

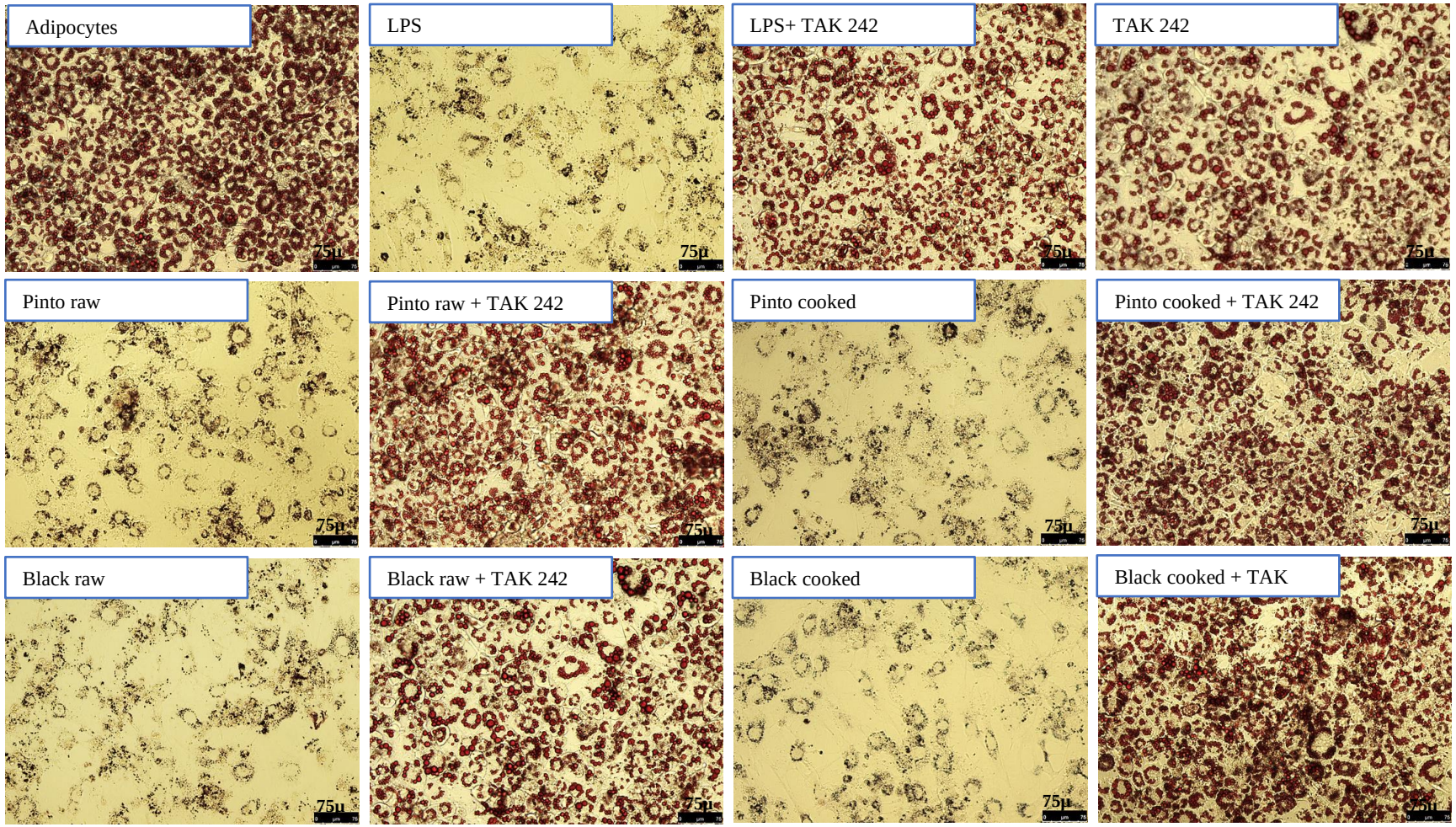

Figure 17. Effect of macrophage conditioned media (MCM) treated with SDF-rich fractions on lipid accumulation in 3T3-L1 cells at differentiation day 8 assessed by oil red O (ORO) staining. 20X objective photographs. Scale bar: 75 µm.

Lipid droplets in adipocytes play an important role in lipid metabolism; for example, lipid accumulation regulates triglycerides (TGA) synthesis (Lee et al., 2018). In addition, a major function of adipose tissue is to store energy as TGA under conditions of nutritional excess (Rangwala & Lazar, 2000). In this sense, the TGA content was measured on differentiation day 8 (Figure 18) to confirm the adipogenic inhibitory effect of the MCM cultured with SDF-rich fractions. As expected, the TGA content from cells treated with MCM cultured with TAK-242 and SDF-rich fractions had similar TGA content as the adipocytes control, except for the SDF-rich fractions extracted from raw beans, which had a modest but significantly ($p < 0.05$) lower TGA accumulation. Although this reduced accumulation was statistically significant, it does not necessarily mean that this lower accumulation is physiologically relevant. Furthermore, significantly ($p < 0.05$) lower TGA accumulation was measured in each adipocyte treatment differentiated with MCM from bean-derived fibers when compared with its counterpart in the presence of TAK-242. These results are further evidence of the inhibitory effect of intracellular lipid accumulation in 3T3-L1 adipocytes due to SDF-rich fractions macrophage stimulation.

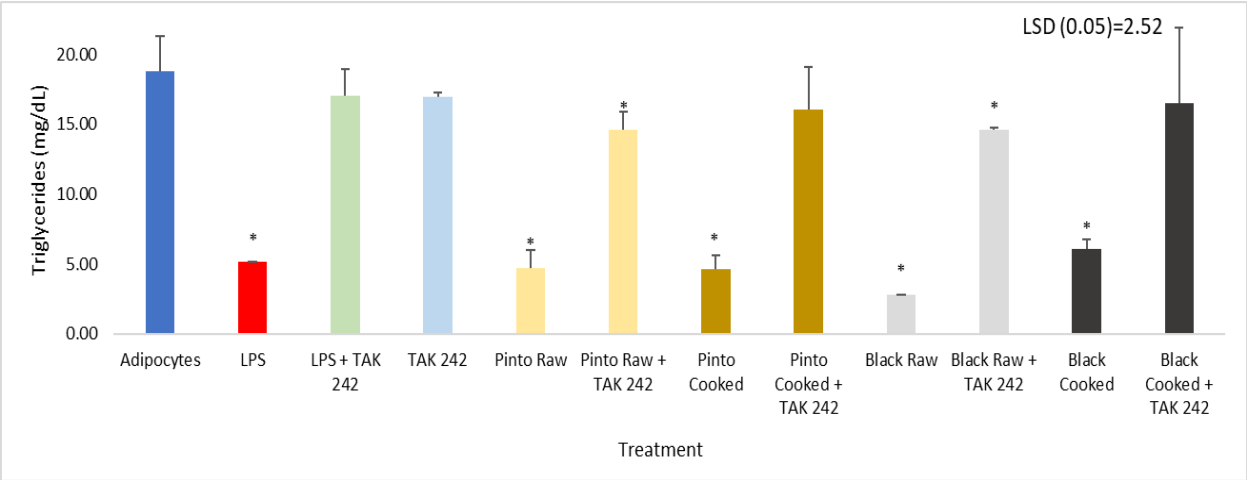

Figure 18. Effect of macrophage conditioned media (MCM) treated with SDF-rich fractions on triglycerides (TGA) in 3T3-L1 cells at differentiation day 8.
Adipocytes: cells treated with differentiation media only; The rest of the treatment labels indicate the MCM conditions as follows. LPS: cells incubated with LPS; LPS+TAK 242: cells with LPS and the TLR-4 antagonist TAK 242; TAK 242: cells with TLR-4 antagonist TAK 242; other treatment conditions include the bean derived SDF (black or pinto) cooked or raw, with or without treatment with the TLR-4 inhibitor. Data are presented as means ± SD. LSD: least significant difference ($p < 0.05$). Differences from adipocytes are marked with an asterisk (*). Values differing more than LSD are significantly different from each other.

5.4.2. Macrophage-Conditioned Media Effect on Adipogenesis Markers

Differentiation of pre-adipocytes into mature adipocytes (lipid-filled adipocytes) relies on transcriptional regulatory factors, such as peroxisome proliferator-activated receptor γ (PPAR-γ) and CCAAT/ enhancer-binding proteins (C/EBPs), that are key transcriptional regulators of adipose tissue (Jiang et al., 2019; Kim et al., 2015; Sarantopoulos et al., 2018). During the early stage of adipogenesis, PPAR-γ is expressed and upregulates C/EBPα expression, consequently creating a feedback loop that commits pre-adipocytes to differentiate into mature adipocytes (Lefterova & Lazar, 2009). Therefore, PPAR-γ is considered the master regulator of adipogenesis (Otto & Lane, 2005). Additionally, Imai et al. (2004) reported that PPAR-γ is essential for mature adipocytes' survival since its ablation leads to cell death. When PPAR-γ is activated, besides promoting adipocyte differentiation, it stimulates the secretion of adipokines

such as adiponectin and fatty acid-binding proteins 4 (FABP4) (Dou et al., 2020; Ouchi et al., 2011). Functional adipocytes exclusively synthesize adiponectin, and adiponectin expression is downregulated in dysfunctional adipocytes (Fasshauer & Blüher, 2015; Ouchi et al., 2011). Also, FABP4 expression is highly induced during adipogenesis as it is recognized as the main cytosolic fatty acid transporter, thus playing a key role in lipogenesis (Samulin et al., 2008). Therefore, to further investigate the suppressive effect of macrophage-derived pro-inflammatory cytokines, secreted by macrophages activated by SDF-rich fractions, on adipogenesis and lipid accumulation, PPAR-γ, adiponectin and FABP4 protein expression levels were examined by Western Blot analysis (Figure 19-21).

Regarding PPAR-γ, the bands corresponding to the PPAR-γ protein were more intense in the 3T3-L1 adipocytes incubated with conditioned media from untreated macrophages than from macrophages treated with SDF-rich fractions (Figure 19). When the adipocytes were treated with SDF and the TLR-4 inhibitor, the intensity of the bands was similar to those of untreated adipocytes. Similarly, when the adipokines adiponectin and FABP4 were assessed, clear differences in protein band levels were seen between the treatments with MCM cultured with SDF-rich fractions in the presence of TAK-242 and their counterparts without TAK-242 (Figures 20, 21). The results suggest the inhibition of the adipokines expression, supporting the hypothesis that SDF-rich fractions can affect adipocyte differentiation through the macrophage pro-inflammatory secreted molecules. As described in Chapter 3, one of the cytokines found in the analyzed macrophage conditioned media was TNF-α. Previous research has described TNF-α as a potent inhibitory agent of adipogenesis via the activation of the extracellular regulated kinase (ERK) 1/2 pathway through the phosphorylation and consequent functional inhibition of PPAR-γ activity (Cawthorn & Sethi, 2008; Makki et al., 2013; Warne, 2003). In addition, the

obtained results agree with Ma et al. (2020) findings of a dramatic decrease in the expression of adipogenic differentiation-related markers, such as PPAR-γ, due to the TNF-α and IL-1β cytokines found in macrophage supernatants (Ma et al., 2020).

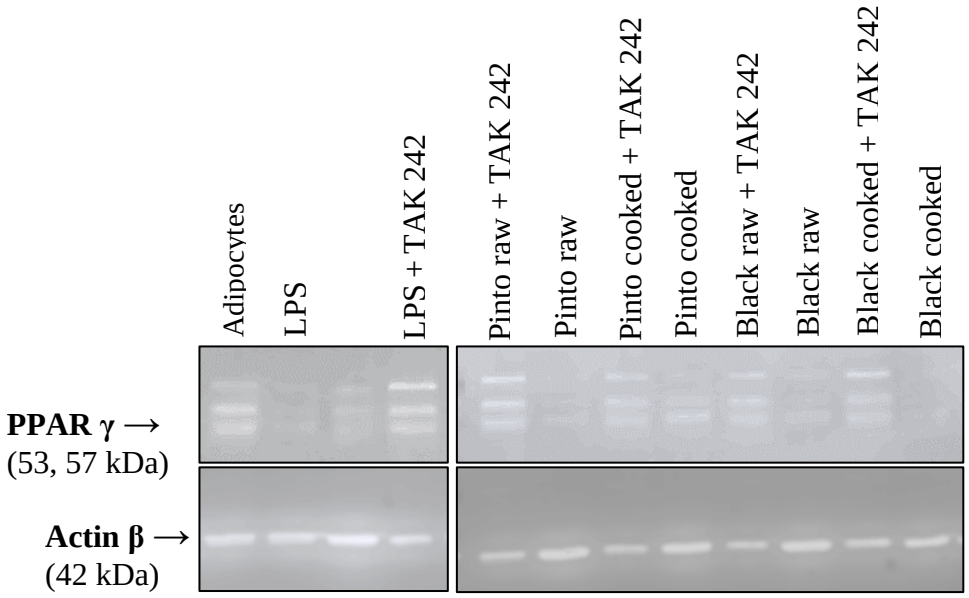

Figure 19. Effect of macrophage conditioned media (MCM) treated with SDF-rich fractions on PPAR γ protein expression in 3T3-L1 cells differentiation at day 8 by Western Blot analysis. Adipocytes: cells treated with differentiation media only; The rest of the lane labels indicate the MCM conditions as follows. LPS: cells incubated with LPS; LPS+TAK 242: cells with LPS and the TLR-4 antagonist TAK 242; other treatment conditions include the bean derived SDF (black or pinto) cooked or raw, with or without treatment with the TLR-4 inhibitor.

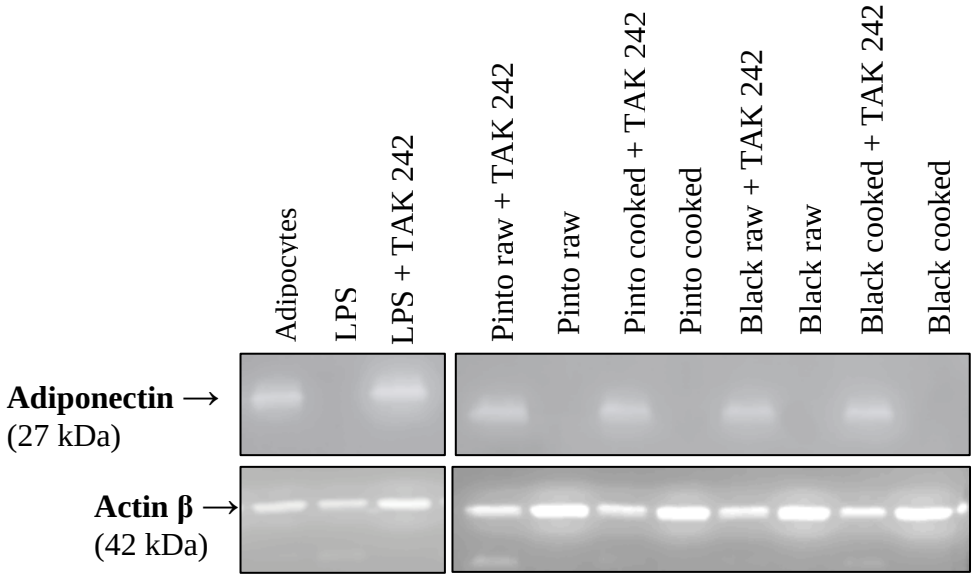

Figure 20. Effect of macrophage conditioned media (MCM) treated with SDF-rich fractions on adiponectin protein expression in 3T3-L1 cells differentiation at day 8 by Western Blot analysis. Adipocytes: cells treated with differentiation media only; The rest of the lane labels indicate the MCM conditions as follows. LPS: cells incubated with LPS; LPS+TAK 242: cells with LPS and the TLR-4 antagonist TAK 242; other treatment conditions include the bean derived SDF (black or pinto) cooked or raw, with or without treatment with the TLR-4 inhibitor.

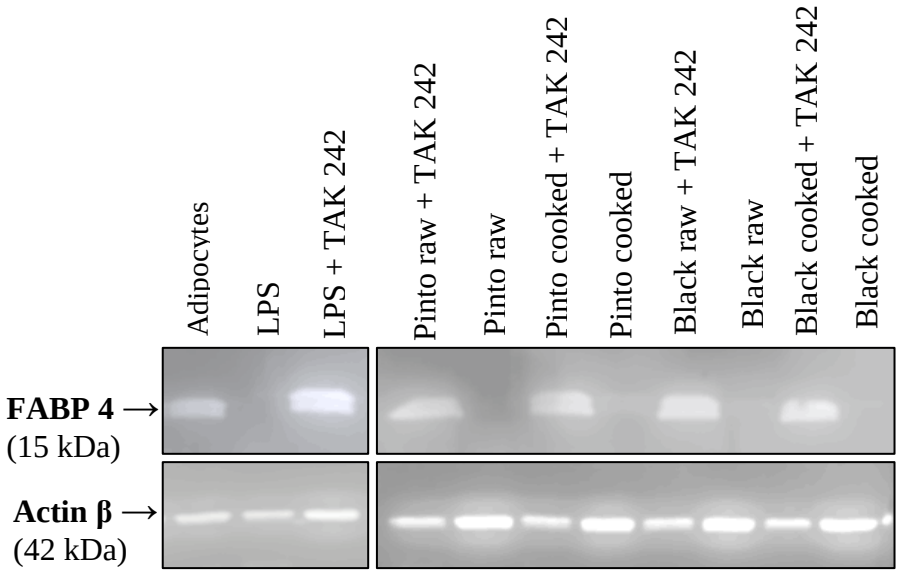

Figure 21. Effect of macrophage conditioned media (MCM) treated with SDF-rich fractions on FABP 4 protein expression in 3T3-L1 cells differentiation at day 8 by Western Blot analysis Adipocytes: cells treated with differentiation media only; The rest of the lane labels indicate the MCM conditions as follows. LPS: cells incubated with LPS; LPS+TAK 242: cells with LPS and the TLR-4 antagonist TAK 242; other treatment conditions include the bean derived SDF (black or pinto) cooked or raw, with or without treatment with the TLR-4 inhibitor.

5.5. Conclusions

The present study assessed the impact of the macrophages conditioned media (MCM) treated with the soluble dietary fiber (SDF) rich fractions extracted from dry beans on 3T3-L1 cells differentiation. The MCM from SDF-rich fractions exhibited a marked reduction of adipogenesis as assessed by decreased lipid accumulation and reduced protein expression of adipogenic markers. Furthermore, adipogenesis was not affected when the 3T3-L1 cells were treated with MCM from SDF-rich fractions with TLR-4 inhibitor (TAK-242). Overall, our results suggest that the SDF extracted from dry beans has the potential to reduce adipogenesis and could be used as food supplements to reduce or prevent obesity.

5.6. References

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6. OVERALL CONCLUSION AND FUTURE RESEARCH

This dissertation aimed to understand how potential food ingredients can be extracted from dry beans to enhance human health. The ingredient extracted and characterized in the present research was soluble dietary fiber (SDF). Therefore, the assessment of the composition and physical properties of the dietary fiber was essential for understanding its potential usage in the food industry. And the further study of immunomodulatory properties provided an insightful perspective of how soluble dietary fiber can be utilized as a therapeutic agent. In this sense, the first study demonstrated that SDF-rich fraction was successfully extracted from dry bean flours with low residual protein content (<20%). The extracted SDF-rich fractions sugar composition indicated the presence of pectic polysaccharides, hemicelluloses and RFOs. Overall, SDF-rich fraction solutions exhibited a shear-thinning behavior. The second study showed that SDF-rich fractions extracted from dry beans promoted the secretion of pro-inflammatory molecules NO, TNF-α, IL-6, and IL-1β by RAW 264.7 murine macrophages. The results suggest that the SDF-rich fraction elicited the pro-inflammatory response of macrophages through TLR-4 mediated activation. Finally, the third study assessed the impact of the macrophages conditioned media (MCM) treated with the soluble dietary fiber (SDF) rich fractions extracted from dry beans on 3T3-L1 cells differentiation. The MCM from SDF-rich fractions exhibited a marked reduction of adipogenesis as assessed by decreased lipid accumulation and reduced protein expression of adipogenic markers. Overall, the results suggest how the human health can be enhanced with bean derived SDF, while the dry bean market value is increased.

The present research successfully extracted and characterized the soluble dietary fiber (SDF) rich fraction from dry beans. However, it will be worth to investigate a series of aspects, such as the interaction of bean-derived fiber extracts with different macrophage surface receptors

(e.g., TLR-2). Also, investigate the impact of SDF in the production of other inflammation indicators, like the cyclooxygenase 2 (COX-2) enzyme and the consequent prostaglandins (PG). These studies will provide a better understanding of the observed pro-inflammatory effect. Therefore, better suggestions on the potential therapeutic induced inflammation by SDF-rich extracts can be elaborated.

Additionally, the study of the SDF *in vitro* fermentation, as well as the analysis of its fermentation products, short-chain fatty acids (SCFA), will be worth exploring. Then, the fermentation products analysis could be followed by the assessment of SCFA impact on *in vitro* models using the immune system-derived cells, such as macrophages. This type of experiment will help elucidate if SCFA elicit an immunostimulatory effect.

In addition, it will be valuable to study the co-culture of SDF-stimulated macrophages with adipocytes. This experimental approach is used to study paracrine interactions (cross talk) between the cultured cell types to understand better the role of macrophage secreted products on adipogenesis in a physiologically relevant microenvironment. The question relies on whether the same anti-adipogenic effect will be observed. Also, this experiment will elucidate if the SDF affects macrophage migration towards the adipose tissue. In this sense, further research exploring the collective effect of fiber and immune cells is much encouraged.

Also, the fractionation of materials, either raw or cooked, is a decisive step in the development of the food industry. However, since limited research has been conducted on the isolation of dietary fiber from dry beans, future research directions should be focused on the development of extraction methods with the highest yields and purities of SDF. Optimizing SDF extractability from different dry bean market classes with a laboratory-scale method will be the first step of a long-term research project. The scale-up fractionation of dry bean SDF should be

the second phase of the project, where pilot plant scale equipment should be considered. For instance, the type of vessel in which the enzymatic digestion with controlled temperature steps should be carried. The scaling-up stage will also raise challenges regarding how to process or take advantage of the generated by-products, such as the residual water with the concentrated insoluble dietary fiber fraction with potential protein contamination. Therefore, elucidating a laboratory-scale stepwise protocol for extracting SDF fraction with high purity will be a prerequisite for developing further scale-up protocols. The project offers the potential enhancement of dry bean economic value as novel bean-derived ingredients with potential health benefits will be of great interest to the food sector.

APPENDIX A. MOLECULAR WEIGHT DETERMINATION BY SEC-MALS DETECTOR

The combination of size exclusion chromatography (SEC) with multi-angle light scattering (MALS) provides reliable distributions of molar mass and size without relying on calibration standards (WYATT TECHNOLOGY, 2016). In static light scattering (SLS, or MALS), the intensity of the scattered light is measured as a function of the angle between the detector and the incident beam direction (Figure A1). Therefore, the molar mass of a sample is determined by the intensity of the scattered light.

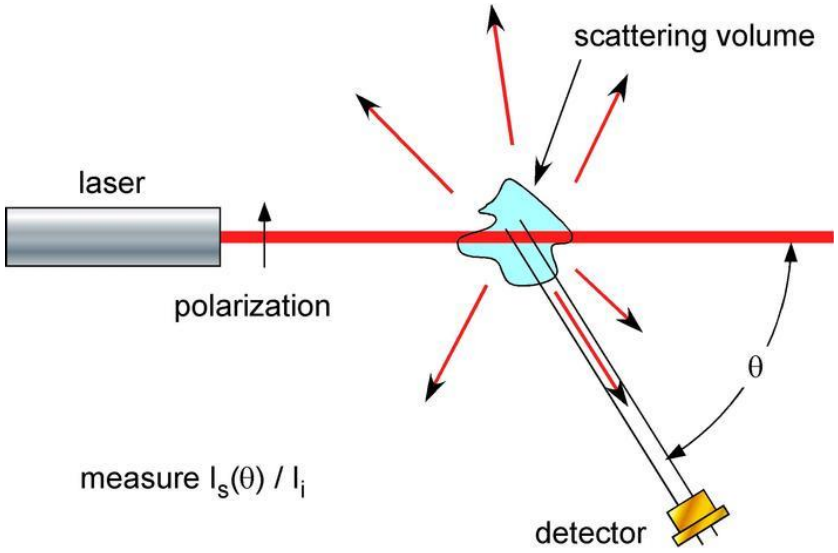

Figure A1. Light scattering experimental parameters.
I_i: intensity of the incident light; I_s: scattered light; θ: angle of scattered light from detector center.

First, a laser beam passes through the sample. Then, the sample scatters light at all angles, while a number of detectors placed at different angular positions around the sample will provide a response directly proportional to the intensity of the scattered light they received. The light scattering signals are digitized and sent to a computer for processing. The ASTRA

computer software performs the analyses to extract the absolute molar mass based on the following formula:

$$\frac{K^* c}{R(\theta)} = \frac{1}{MP(\theta)} + 2A_2 c$$

Figure A2. Zimm's equation for ASTRA online analysis.
R(θ): Rayleigh ratio is the relative amount of light scattered by the solute at an angle θ; K*: is a constant; c: is the solute's mass/volume concentration (mg/mL); M: is the weight-average molar mass; and P(θ) is the form factor, which describes the scattered light's angular dependence and depends on the size and structure of the scattering molecules.

A Debye plot can assess the quality of the fit to the light scattering equation. The Debye plot (Figure A3) shows the angular fit of the light scattering data. The control graph shows the chromatogram and indicates the slice for which the Debye Plot is displayed. For a good fit, the points overlay the fit line within their error bars, and there are no systematic deviations, as seen from our results in Figure A3. Conversely, a poor Debye plot can indicate poor normalization, dirty flow cell, or an inappropriate fit model or fit degree.

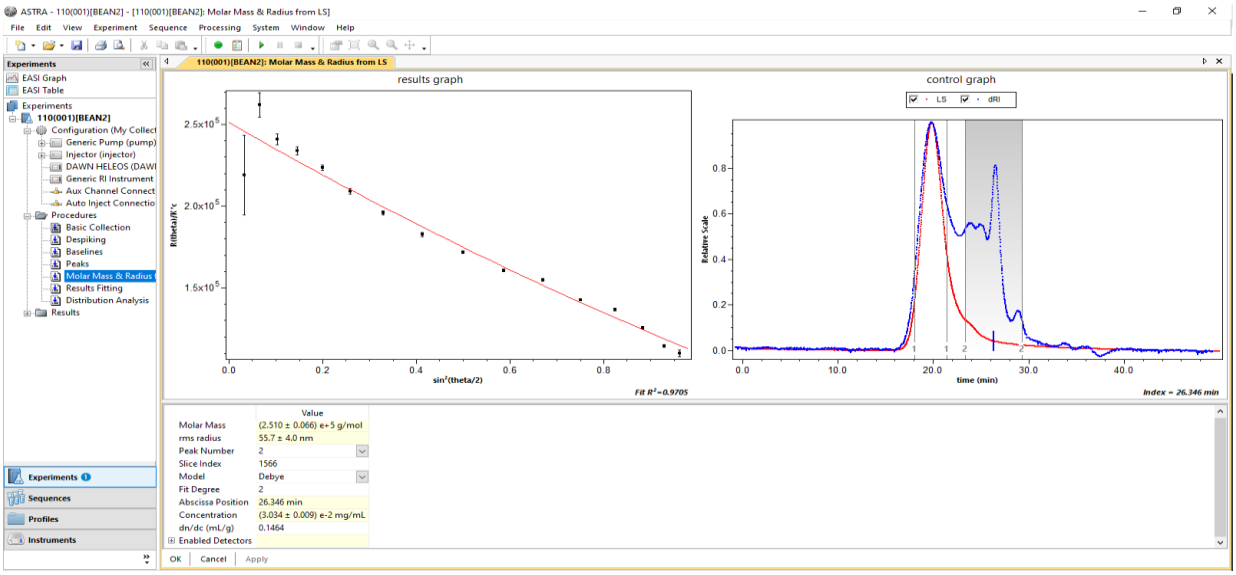

Figure A3. SDF-rich fraction light scattering data and Debye plot.

To calculate the molar mass moments, such as weight-average molar mass, the concentration (c_i) for each slice of the chromatogram is measured by the concentration detector (usually refractive index detector), while the molar mass (M_i) for each slice in the chromatogram is calculated from the intensity of the scattered light (Figure A4).

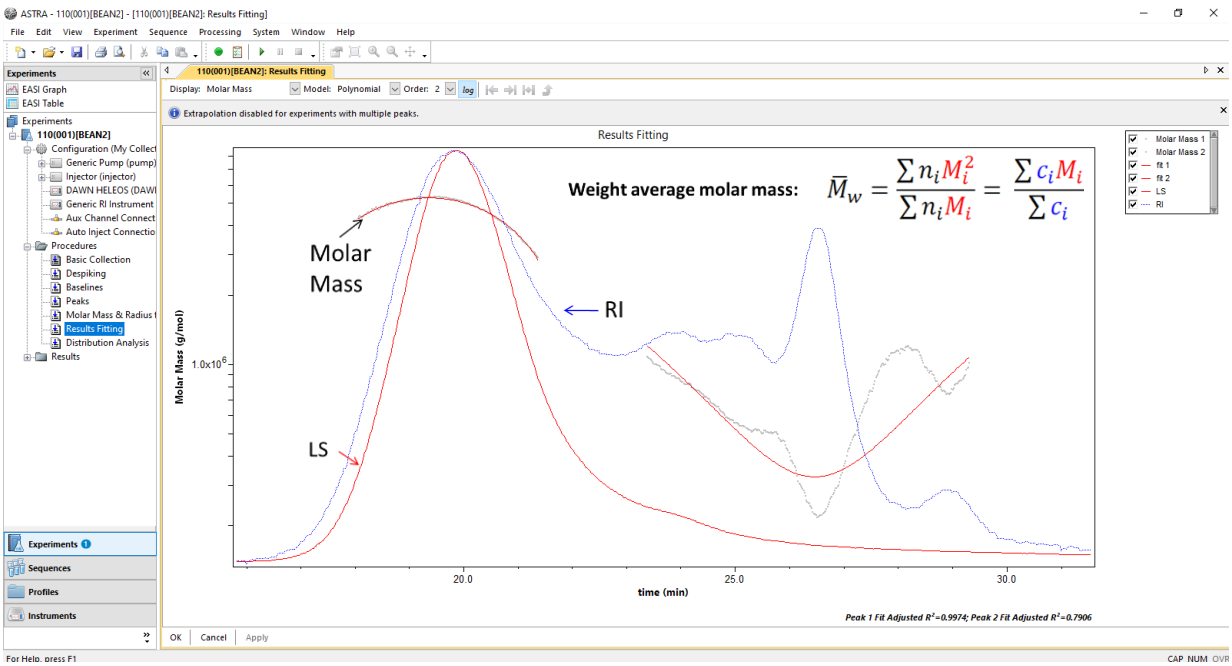

Figure A4. SDF-rich fraction molar mass, light scattering (LS) and reflective index (RI) results.

A.1. References

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APPENDIX B. SDF RHEOLOGICAL PROPERTIES STUDIED BY TEMPERATURE RAMPS

Temperature ramps (Figures B1 – B4) were studied by preparing a 1% (w/v) SDF-rich fraction solution and 50 mM $CaCl_2$ at 90°C with magnetic stirring. The hot solution was poured on the rheometer immediately after preparation. Gel formation was studied under controlled heating and cooling from 25°C to 90°C and 90°C to 25°C at a rate of 5°C/min under a constant strain of 10 %.

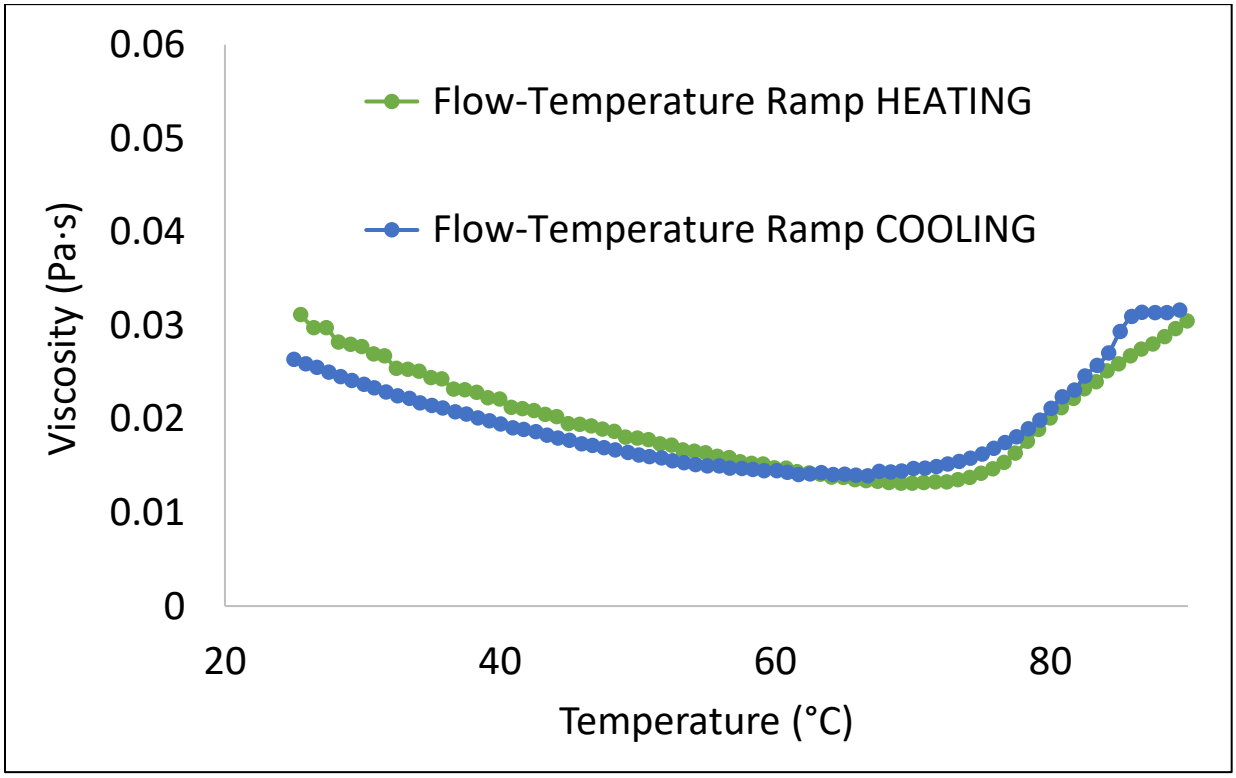

Figure B1. Flow behavior of soluble dietary extracted from raw pinto dry bean.

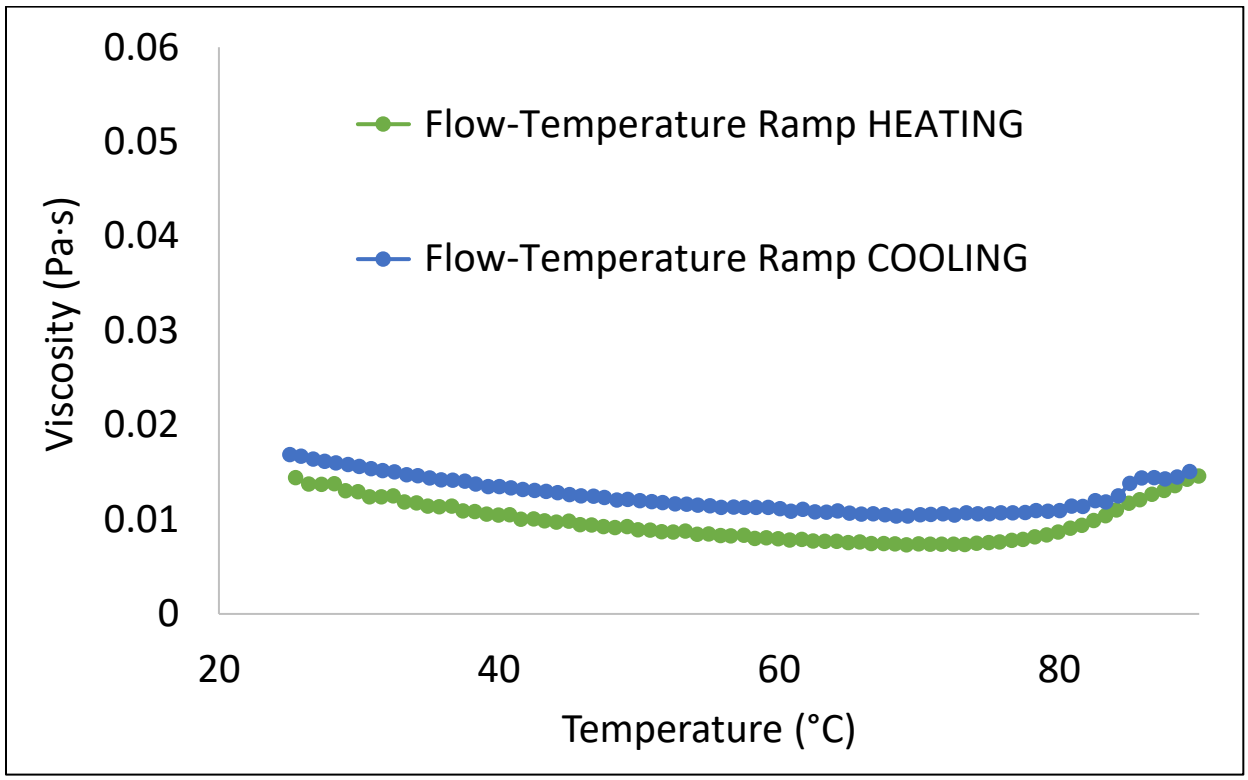

Figure B2. Flow behavior of soluble dietary extracted from cooked pinto dry bean.

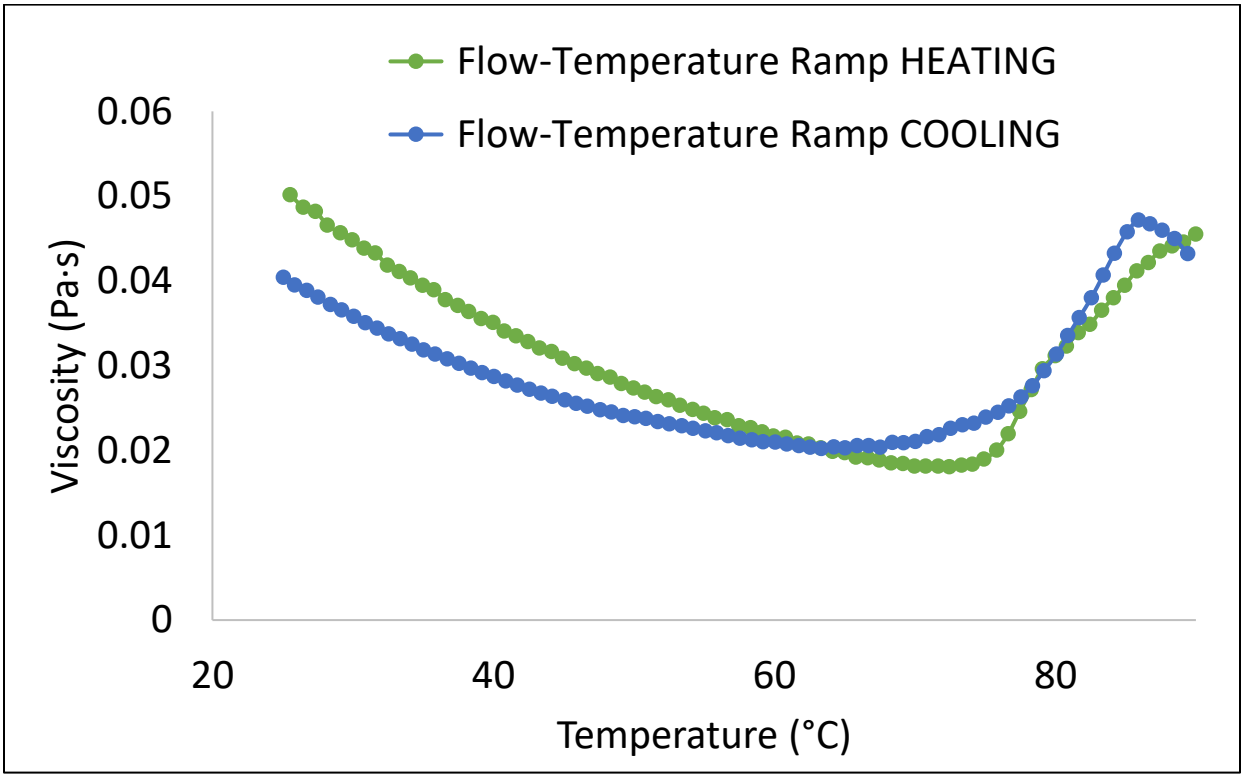

Figure B3. Flow behavior of soluble dietary extracted from raw black dry bean.

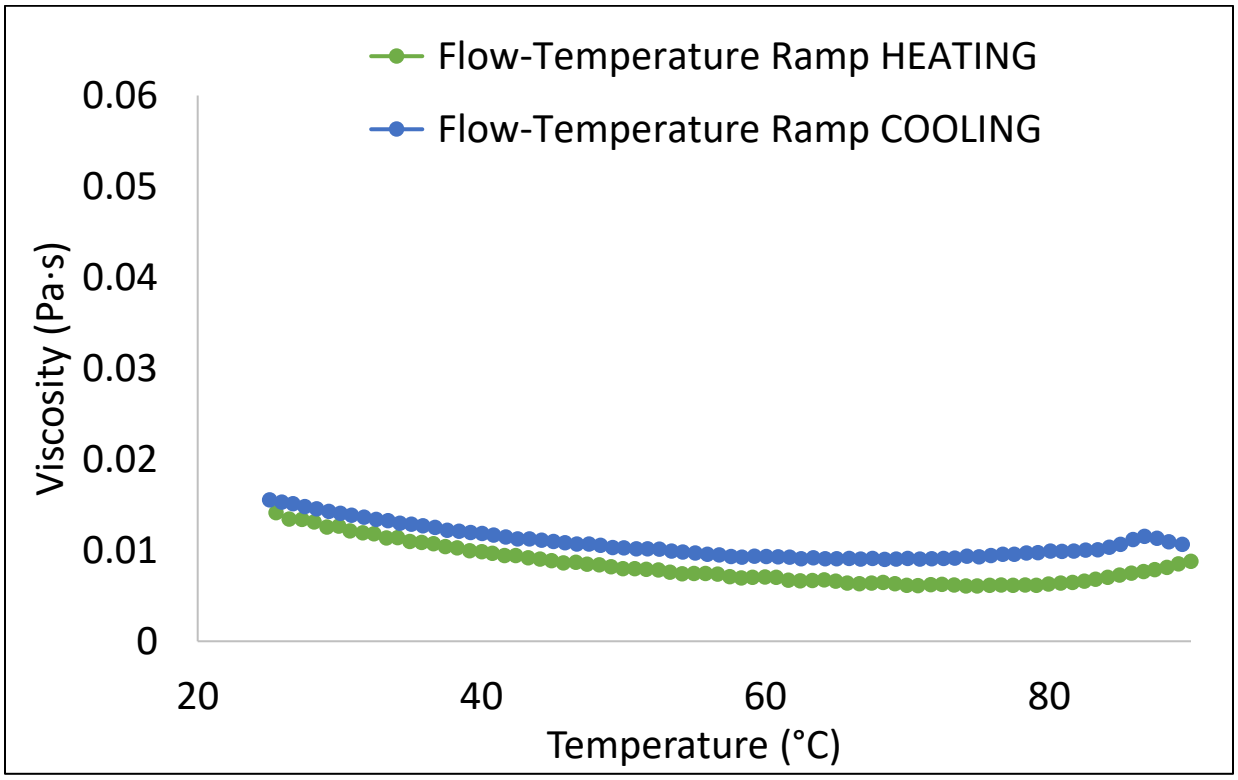

Figure B4. Flow behavior of soluble dietary extracted from cooked black dry bean.

APPENDIX C. ANOVA TABLES

Table C1. Analysis of variance for dry bean flour proximate analysis and phytic acid content.

Parameter	Sources of variation	DF	Type III SS	MS	*F* value	
Moisture	LOC	3	1.16508	0.58254	0.0044	**
	VTY	9	1.81916	1.81916	<.0001	**
	TRT	9	179.807	179.807	<.0001	**
	LOC*VTY	9	0.2172	0.1086	0.0095	**
	LOC*TRT	9	1.81542	0.90771	<.0001	**
	VTY*TRT	9	0.02795	0.02795	0.1811	
	LOC*VTY*TRT	9	0.82322	0.41161	<.0001	**
Ash	LOC	3	0.3141	0.15705	0.0005	**
	VTY	9	0.10709	0.10709	0.0002	**
	TRT	9	7.18088	7.18088	<.0001	**
	LOC*VTY	9	0.30361	0.1518	<.0001	**
	LOC*TRT	9	0.06195	0.03098	0.0037	**
	VTY*TRT	9	0.07711	0.07711	0.0005	**
	LOC*VTY*TRT	9	0.00192	0.00096	0.7185	
Protein	LOC	3	5.00236	2.50118	0.319	
	VTY	9	21.441	21.441	0.0018	**
	TRT	9	25.545	25.545	0.001	**
	LOC*VTY	9	5.49755	2.74877	0.1403	
	LOC*TRT	9	3.69532	1.84766	0.2443	
	VTY*TRT	9	0.01001	0.01001	0.9266	
	LOC*VTY*TRT	9	2.03458	1.01729	0.4361	
Lipid	LOC	3	0.73583	0.36792	0.0625	
	VTY	9	0.00167	0.00167	0.8894	
	TRT	9	0.54	0.54	0.0299	**
	LOC*VTY	9	0.23083	0.11542	0.2915	
	LOC*TRT	9	0.0325	0.01625	0.8225	
	VTY*TRT	9	0.06	0.06	0.4128	
	LOC*VTY*TRT	9	1.1875	0.59375	0.0131	**
Starch	LOC	3	1.08533	0.54267	0.0892	
	VTY	9	25.5788	25.5788	<.0001	**
	TRT	9	2.95203	2.95203	0.022	**
	LOC*VTY	9	0.76325	0.38162	0.4098	
	LOC*TRT	9	4.13594	2.06797	0.0295	**
	VTY*TRT	9	0.53927	0.53927	0.268	
	LOC*VTY*TRT	9	1.1147	0.55735	0.2865	
Phytic acid	LOC	3	0.86791	0.43395	0.024	**
	VTY	9	34.1339	34.1339	<.0001	**
	TRT	9	173.765	173.765	<.0001	**
	LOC*VTY	9	13.6077	6.80386	<.0001	**
	LOC*TRT	9	0.77777	0.38888	0.0084	**
	VTY*TRT	9	34.0898	34.0898	<.0001	**
	LOC*VTY*TRT	9	1.75626	0.87813	0.0006	**

DF: Degrees of freedom; SS: Sum of square; MS: Mean square; **: significant at $p < 0.05$.

Table C2. Analysis of variance for dry bean flour content of insoluble (IDF), soluble (SDF) and total dietary fiber (TDF).

Parameter	Sources of variation	DF	Type III SS	MS	*F* value	
IDF	LOC	3	0.77583	0.38792	0.7707	
	VTY	9	25.0104	25.0104	0.0001	**
	TRT	9	11.3438	11.3438	0.0019	**
	LOC*VTY	9	2.98083	1.49042	0.1414	
	LOC*TRT	9	4.7475	2.37375	0.0602	
	VTY*TRT	9	0.77042	0.77042	0.2895	
	LOC*VTY*TRT	9	1.96583	0.98292	0.2514	
SDF	LOC	3	2.66083	1.33042	0.1009	
	VTY	9	0.30375	0.30375	0.3772	
	TRT	9	61.7604	61.7604	<.0001	**
	LOC*VTY	9	2.4825	1.24125	0.074	
	LOC*TRT	9	0.06083	0.03042	0.918	
	VTY*TRT	9	2.87042	2.87042	0.0189	**
	LOC*VTY*TRT	9	0.50583	0.25292	0.5135	
TDF	LOC	3	2.28583	1.14292	0.3691	
	VTY	9	29.9267	29.9267	<.0001	**
	TRT	9	127.882	127.882	<.0001	**
	LOC*VTY	9	1.77583	0.88792	0.2109	
	LOC*TRT	9	4.28583	2.14292	0.0445	**
	VTY*TRT	9	0.735	0.735	0.2461	
	LOC*VTY*TRT	9	0.7225	0.36125	0.4969	

DF: Degrees of freedom; SS: Sum of square; MS: Mean square; **: significant at $p < 0.05$.

Table C3. Analysis of variance for dry bean flour extractable phenols content and antioxidant properties.

Parameter	Sources of variation	DF	Type III SS	MS	*F* value	
Total phenols	LOC	3	0.47622	0.23811	0.4058	
	VTY	9	0.07936	0.07936	0.4817	
	TRT	9	170.953	170.953	<.0001	**
	LOC*VTY	9	0.51478	0.25739	0.2285	
	LOC*TRT	9	0.31923	0.15962	0.3788	
	VTY*TRT	9	0.49097	0.49097	0.1012	
	LOC*VTY*TRT	9	0.79325	0.39663	0.1212	
Metal chellating	LOC	3	14.1275	7.06375	0.1497	
	VTY	9	14.3177	14.3177	0.0069	**
	TRT	9	41.7936	41.7936	0.0002	**
	LOC*VTY	9	2.19558	1.09779	0.4294	
	LOC*TRT	9	3.57398	1.78699	0.2712	
	VTY*TRT	9	16.3646	16.3646	0.0047	**
	LOC*VTY*TRT	9	3.53474	1.76737	0.2746	
ABTS	LOC	3	0.3701	0.18505	0.8116	
	VTY	9	16.73649	16.7365	0.0001	**
	TRT	9	568.5743	568.574	<.0001	**
	LOC*VTY	9	7.520696	3.76035	0.0073	**
	LOC*TRT	9	0.156153	0.07808	0.8337	
	VTY*TRT	9	4.284632	4.28463	0.011	**
	LOC*VTY*TRT	9	7.189815	3.59491	0.0083	**
FRAP	LOC	3	1.708774	0.85439	0.0409	**
	VTY	9	10.7465	10.7465	<.0001	**
	TRT	9	294.3706	294.371	<.0001	**
	LOC*VTY	9	0.711924	0.35596	0.1262	
	LOC*TRT	9	1.970157	0.98508	0.0132	**
	VTY*TRT	9	4.139884	4.13988	0.0004	**
	LOC*VTY*TRT	9	0.990416	0.49521	0.0688	

DF: Degrees of freedom; SS: Sum of square; MS: Mean square; **: significant at $p < 0.05$.

Table C4. Analysis of variance for dry bean flour hydrolyzable phenols content and antioxidant properties.

Parameter	Sources of variation	DF	Type III SS	MS	*F* value	
Total phenols	LOC	3	1.642114	0.82106	0.2132	
	VTY	9	0.000597	0.0006	0.9711	
	TRT	9	97.4898	97.4898	<.0001	**
	LOC*VTY	9	1.994715	0.99736	0.1536	
	LOC*TRT	9	2.349933	1.17497	0.1178	
	VTY*TRT	9	0.365887	0.36589	0.3799	
	LOC*VTY*TRT	9	0.775409	0.38771	0.439	
Metal chellating	LOC	3	1480.788	740.394	0.2296	
	VTY	9	160.5573	160.557	0.5591	
	TRT	9	357334	357334	<.0001	**
	LOC*VTY	9	360.4184	180.209	0.6736	
	LOC*TRT	9	2409.874	1204.94	0.1161	
	VTY*TRT	9	1295.033	1295.03	0.119	
	LOC*VTY*TRT	9	757.697	378.849	0.4521	
ABTS	LOC	3	0.619546	0.30977	0.0349	
	VTY	9	9.549618	9.54962	0.0012	
	TRT	9	56.95418	56.9542	<.0001	**
	LOC*VTY	9	1.93334	0.96667	0.1688	
	LOC*TRT	9	2.301329	1.15067	0.1287	
	VTY*TRT	9	23.80233	23.8023	<.0001	**
	LOC*VTY*TRT	9	3.763005	1.8815	0.0502	
FRAP	LOC	3	0.41842	0.20921	0.6079	
	VTY	9	19.50518	19.5052	0.0001	**
	TRT	9	388.2208	388.221	<.0001	**
	LOC*VTY	9	3.701612	1.85081	0.0582	
	LOC*TRT	9	0.906858	0.45343	0.4149	
	VTY*TRT	9	1.99985	1.99985	0.0684	
	LOC*VTY*TRT	9	1.328066	0.66403	0.2905	

DF: Degrees of freedom; SS: Sum of square; MS: Mean square; **: significant at $p < 0.05$.

Table C5. Analysis of variance for dry bean soluble fiber-rich fraction composition.

Parameter	Sources of variation	DF	Type III SS	MS	*F* value	
Arabinose	LOC	3	16.7343	8.36715	0.0395	**
	VTY	9	6.513571	6.51357	0.0372	**
	TRT	9	32.18606	32.1861	0.0004	**
	LOC*VTY	9	4.5657	2.28285	0.1797	
	LOC*TRT	9	0.006642	0.00332	0.997	
	VTY*TRT	9	0.031928	0.03193	0.868	
	LOC*VTY*TRT	9	16.5581	8.27905	0.0118	**
Galactose	LOC	3	3.248568	1.62428	0.0066	**
	VTY	9	17.22496	17.225	<.0001	**
	TRT	9	0.180921	0.18092	0.4015	
	LOC*VTY	9	0.383676	0.19184	0.4701	
	LOC*TRT	9	3.702747	1.85137	0.0103	**
	VTY*TRT	9	6.050325	6.05033	0.0007	**
	LOC*VTY*TRT	9	0.166171	0.08309	0.7099	
Glucose	LOC	3	0.094493	0.04725	0.4743	
	VTY	9	0.597063	0.59706	0.0008	**
	TRT	9	8.392258	8.39226	<.0001	**
	LOC*VTY	9	1.00304	0.50152	0.0004	**
	LOC*TRT	9	0.230242	0.11512	0.0382	**
	VTY*TRT	9	0.022157	0.02216	0.3619	
	LOC*VTY*TRT	9	0.823688	0.41184	0.0009	**
Xylose	LOC	3	7.024254	3.51213	0.0007	**
	VTY	9	6.919881	6.91988	0.0002	**
	TRT	9	1.20584	1.20584	0.0353	**
	LOC*VTY	9	0.818355	0.40918	0.1812	
	LOC*TRT	9	3.419368	1.70968	0.0079	**
	VTY*TRT	9	8.011332	8.01133	0.0001	**
	LOC*VTY*TRT	9	0.350573	0.17529	0.4439	
Mannose	LOC	3	1.446214	0.72311	0.0173	**
	VTY	9	32.95536	32.9554	<.0001	**
	TRT	9	6.634924	6.63492	0.0002	**
	LOC*VTY	9	0.664698	0.33235	0.1998	
	LOC*TRT	9	6.833802	3.4169	0.0005	**
	VTY*TRT	9	7.127598	7.1276	0.0001	**
	LOC*VTY*TRT	9	1.123187	0.56159	0.0855	
Fructose	LOC	3	111.8055	55.9027	0.0066	**
	VTY	9	314.1116	314.112	<.0001	**
	TRT	9	46.74761	46.7476	<.0001	**
	LOC*VTY	9	22.25939	11.1297	0.0001	**
	LOC*TRT	9	18.11499	9.05749	0.0003	**
	VTY*TRT	9	37.85044	37.8504	<.0001	**
	LOC*VTY*TRT	9	13.58375	6.79187	0.0008	**
Galacturonic acid	LOC	3	6.053566	3.02678	0.0008	**
	VTY	9	21.92354	21.9235	<.0001	**
	TRT	9	6.394273	6.39427	0.0005	**
	LOC*VTY	9	2.416125	1.20806	0.0303	**
	LOC*TRT	9	2.680733	1.34037	0.0234	**
	VTY*TRT	9	13.85353	13.8535	<.0001	**
	LOC*VTY*TRT	9	0.458518	0.22926	0.4042	

DF: Degrees of freedom; SS: Sum of square; MS: Mean square; **: significant at $p < 0.05$.

Table C6. Analysis of variance for dry bean soluble fiber-rich fraction high (HMW) and low molecular weight (LMW) distribution.

Parameter	Sources of variation	DF	Type III SS	MS	*F* value	
HMW (kDA)	LOC	3	2109897	1054949	0.001	**
	VTY	9	213948	213948	0.0005	**
	TRT	9	6048.375	6048.38	0.4005	
	LOC*VTY	9	192283	96142	0.0026	**
	LOC*TRT	9	150846	75423	0.0057	**
	VTY*TRT	9	4293.375	4293.38	0.4762	
	LOC*VTY*TRT	9	163141	81570	0.0044	**
LMW (kDA)	LOC	3	11765	5882.49	0.0595	
	VTY	9	6027.755	6027.76	0.0924	
	TRT	9	1439.176	1439.18	0.3816	
	LOC*VTY	9	2537.681	1268.84	0.5014	
	LOC*TRT	9	18344	9172.02	0.0289	**
	VTY*TRT	9	83963	83963	<.0001	**
	LOC*VTY*TRT	9	9411.331	4705.67	0.1157	
HMW (%)	LOC	3	29.78828	14.8941	0.0756	
	VTY	9	16.42191	16.4219	0.0429	**
	TRT	9	824.1082	824.108	<.0001	**
	LOC*VTY	9	36.73166	18.3658	0.0203	**
	LOC*TRT	9	28.18213	14.0911	0.0389	**
	VTY*TRT	9	51.78137	51.7814	0.0024	**
	LOC*VTY*TRT	9	3.162973	1.58149	0.6036	
LMW (%)	LOC	3	29.78828	14.8941	0.0756	
	VTY	9	16.42191	16.4219	0.0429	**
	TRT	9	824.1082	824.108	<.0001	**
	LOC*VTY	9	36.73166	18.3658	0.0203	**
	LOC*TRT	9	28.18213	14.0911	0.0389	**
	VTY*TRT	9	51.78137	51.7814	0.0024	**
	LOC*VTY*TRT	9	3.162973	1.58149	0.6036	

DF: Degrees of freedom; SS: Sum of square; MS: Mean square; **: significant at $p < 0.05$.

Table C7. Analysis of variance for dry bean soluble fiber-rich fraction consistency index (*K*) and flow behavior index (*n*).

Parameter	Sources of variation	DF	Type III SS	MS	*F* value	
2 % *K*	LOC	3	0.001205	0.0006	0.7645	
	VTY	9	0.072194	0.07219	<.0001	**
	TRT	9	0.631666	0.63167	<.0001	**
	LOC*VTY	9	0.03116	0.01558	0.0008	**
	LOC*TRT	9	0.002081	0.00104	0.3575	
	VTY*TRT	9	0.022814	0.02281	0.0007	**
	LOC*VTY*TRT	9	0.012429	0.00621	0.0152	**
2 % *n*	LOC	3	0.002979	0.00149	0.0429	**
	VTY	9	0.006198	0.0062	<.0001	**
	TRT	9	0.332996	0.333	<.0001	**
	LOC*VTY	9	0.004039	0.00202	0.0002	**
	LOC*TRT	9	0.001362	0.00068	0.007	**
	VTY*TRT	9	0.000227	0.00023	0.1161	
	LOC*VTY*TRT	9	1.17E-05	5.9E-06	0.9255	
4 % *K*	LOC	3	1.393473	0.69674	0.0468	**
	VTY	9	8.437312	8.43731	<.0001	**
	TRT	9	95.79347	95.7935	<.0001	**
	LOC*VTY	9	2.22457	1.11229	0.0008	**
	LOC*TRT	9	4.368995	2.1845	<.0001	**
	VTY*TRT	9	1.86136	1.86136	0.0004	**
	LOC*VTY*TRT	9	3.815861	1.90793	0.0001	**
4 % *n*	LOC	3	0.002981	0.00149	0.0035	**
	VTY	9	0.005367	0.00537	<.0001	**
	TRT	9	0.562529	0.56253	<.0001	**
	LOC*VTY	9	0.003809	0.0019	0.001	**
	LOC*TRT	9	0.007828	0.00391	<.0001	**
	VTY*TRT	9	0.002615	0.00262	0.001	**
	LOC*VTY*TRT	9	0.003314	0.00166	0.0015	**
6 % *K*	LOC	3	1.552807	0.7764	0.4864	
	VTY	9	253.7323	253.732	<.0001	**
	TRT	9	521.8039	521.804	<.0001	**
	LOC*VTY	9	0.235584	0.11779	0.6906	
	LOC*TRT	9	30.72893	15.3645	<.0001	**
	VTY*TRT	9	69.97258	69.9726	<.0001	**
	LOC*VTY*TRT	9	7.16314	3.58157	0.0031	**
6 % *n*	LOC	3	0.001075	0.00054	0.5378	
	VTY	9	0.024027	0.02403	<.0001	**
	TRT	9	0.292236	0.29224	<.0001	**
	LOC*VTY	9	0.001966	0.00098	0.008	**
	LOC*TRT	9	0.00547	0.00274	0.0002	**
	VTY*TRT	9	0.000117	0.00012	0.3376	
	LOC*VTY*TRT	9	0.002013	0.00101	0.0075	**

DF: Degrees of freedom; SS: Sum of square; MS: Mean square; **: significant at $p < 0.05$.