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The Economic Burden of Gluten-Free Products and the Potential of Dietary Inhibitors of Transglutaminase-2

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THE ECONOMIC BURDEN OF GLUTEN-FREE PRODUCTS AND THE POTENTIAL OF DIETARY INHIBITORS OF TRANSGLUTAMINASE-2

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ABSTRACT

Celiac disease (CD) is a chronic immune-mediated disease of the small intestine caused by the ingestion of gluten. Gluten presents to the intestine largely intact where it is deamidated by Transglutaminase-2 (TG2), increasing affinity for Human Leukocyte Antigen DQ2 (HLA-DQ2) and forming a complex that elicits an inflammatory response ultimately leading to villous atrophy. The only current treatment is strict adherence to a gluten-free diet, though TG2 inhibition is an attractive therapy due its central role in CD pathogenesis. Cocoa contains procyanidin-B2, theobromine and caffeine and may be capable of inhibiting TG2-induced intestinal inflammation and reduce CD symptoms. Procyanidin-B2 rich cocoa extracts reduced TG2 levels by up to 77% *in vitro* using Caco-2 cells. Significant TG2 inhibition was seen when cocoa extracts contained at least 8.5 μM procyanidin-B2 ($p<0.05$). Other CD inflammatory biomarkers including COX-2 and IL-15 were also significantly decreased in the presence of cocoa extracts. Serum cytokines IL-6, IL-8 and IL-1β are commonly used to monitor CD and were analyzed using ELISA to confirm the inhibition of inflammatory biomarkers. This study shows promising results for use of a bioactive-rich cocoa product as a dietary inhibitor of TG2 that can be used with wheat-based products as an alternative therapy in CD.

CHAPTER 1: INTRODUCTION

Celiac Disease (CD) is a chronic immune-mediated disease of the small intestine precipitated by the ingestion of gluten in genetically susceptible individuals. Gluten is the protein component of wheat, rye and barley and formed by the combination of glutenin and gliadin in an aqueous mixture. It provides functionality to wheat-based baked goods by trapping air bubbles and giving the desirable lift and height found in these products. In CD patients deamidated gliadin, rich in glutamine and proline peptides, is responsible for the inflammation. The only current treatment of CD is a strict, lifelong adherence to a gluten-free diet that includes avoidance of all types of wheat. However, the low availability and high costs of a gluten-free diet may affect CD patient compliance, which stresses the need for additional therapeutic options. In addition, the consumer-perceived health advantages of gluten-free foods pose a challenge to the wheat-based products industry.

Food products or beverages capable of inhibiting immunotoxic gluten peptide-induced inflammation in small intestinal epithelium could mitigate gluten toxicity. A safe treatment like a cocoa product to reduce inflammation in individuals suffering from active or inactive CD would be welcome. Procyanidin trimers and tetramers have shown binding affinity for gliadin peptides and can be an effective therapy in CD, although no other studies investigate the inhibitory action of procyanidins on TG2 (Dias and others 2015). Caffeine and theobromine are also present in cocoa, and have been linked to reduced TG2 levels and expression of inflammatory cytokines (Cho and others 2012; Sarria and others 2015). This study will discuss these potential dietary inhibitors of TG2 that could be used with wheat-based products as therapy for CD.

This proof-of-concept study will determine whether cocoa standardized to its proanthocyanidin-B2 content can reduce TG2-induced inflammation in an established in vitro model of CD. Specifically, our aims were to:

1) Determine the concentration of procyanidin-B2 and other bioactive compounds (caffeine and theobromine) in cocoa by liquid chromatography with diode array detector.
2) Determine the proliferation of Caco-2 cells in the presence of procyanidin-B2 and bioactive compounds extracted from cocoa.
3) Determine the efficacy of cocoa extracts on inhibiting TG2 levels in Caco-2 cells.
4) Determine the effect of cocoa extracts on other biomarkers of inflammation in CD including IL-15 and COX-2, and the serum cytokines IL-6, IL-8 and IL-1β.

CHAPTER 2: LITERATURE REVIEW

2.1 Celiac Disease

Celiac Disease (CD) is a chronic immune-mediated disease of the small intestine precipitated by the ingestion of gluten in genetically susceptible individuals. Ingestion of gluten for those with CD causes flattening of the intestinal villi and results in variable degrees of intestinal damage, which leads to manifestation of gastrointestinal symptoms. Although epidemiological studies in the United States and Europe show the prevalence to be only 0.5-1% of the population there is evidence that the prevalence is increasing (Lohi and others 2007). Identification of the link between gluten exposure and CD was discovered by the Dutch physician Dicke in the 1940's and the first diagnostic criteria was published in 1969 in the European Society of Pediatric Gastroenterology and Nutrition (Evan and Sanders 2012), which involved the observance of improvement of intestinal villous structure while on a gluten-free diet (GFD) and relapse when gluten was introduced.

The gastrointestinal issues that occur from ingesting gluten may present in a variety of symptoms, including some extraintestinal manifestations. Both children and adults with CD are prone to these issues, and up to one-third of patients experience persistent symptoms (Evan and Sanders 2012). While many studies have found different prevalence of these symptoms, the common manifestations of the disease are the same (Table 2.1). The type and prevalence of the common symptoms may be explained by differences among populations and access to gluten-free foods. Extraintestinal symptoms such as peripheral neuropathy, migraines, gastroesophageal reflux and low bone density are also seen in CD patients (Lebwohl and others 2012). Those with positive serology for Human Leukocyte Antigen-DQ2 (HLA-DQ2) also have a genetic

predisposition to other autoimmune diseases including Type 1 diabetes, autoimmune thyroiditis and multiple sclerosis (MS) (Ventura and others 1999).

Table 2.1: Common symptoms seen in Celiac Disease

	% Children	% Adults
Diarrhea	9-55	47-76
Abdominal pain	12-33	28-83
Abdominal bloating	12-29	26
Anemia	2-19	38-51
Weight loss	13-26*	34-69

*Failure to thrive
Cranney and others (2007), Reilly and others (2011), Telega and others (2008), Vivas and others (2008), Schuppan and Zimmer (2013)

Today, biopsies of the duodenum are used to confirm a CD diagnosis, in which destruction of the villi is observed when gluten is ingested (Figure 2.1). Serological testing can also be done to confirm results from a biopsy, although variable serology without intestinal enteropathy excludes a CD diagnosis. Figure 2.2 is a summarization of the Mayo Clinic Celiac Disease Diagnostic Testing Algorithm (2015). Serological testing is typically done first because it is a less invasive test than the duodenal biopsy, and normal values practically rule out CD. The serology is tested for elevations of anti-gliadin antibodies (AGAs) such as Immunoglobin A (IgA), tissue Transglutaminase (tTG), and endomysial antibody (EMA).

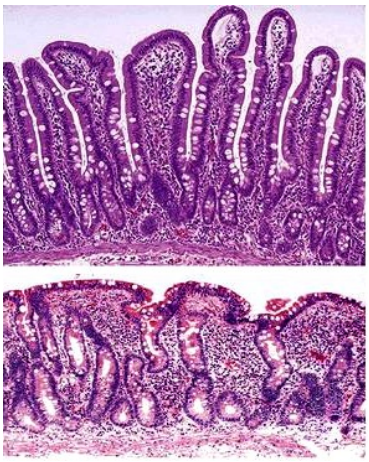

Figure 2.1: Normal duodenal biopsy (top) compared to Celiac Disease biopsy with flattening of the villi (bottom). Celiac Disease Foundation (2015)

Total IgA count was originally thought to be the best serological test for CD, but recent research points to tTG and EMA as better tests due to their high sensitivity and specificity (Lebwohl and others 2012; Evan and Sanders 2012; Ludvigsson and others 2013). If tTG and EMA levels are elevated (positive CD serology), a duodenal biopsy is used to confirm the diagnosis (Figure 2.2). The typical endoscopic findings include atrophy of the villi (flattening, as seen in Figure 2.1), scalloping and/or fissures and decreased folds in the duodenum (Lebwohl and others 2012). The procedure is invasive and some physicians recommend that four or more duodenal specimens should be taken before a CD diagnosis can be confirmed, which is why serology is normally done first. Negative values point towards a different disorder unless the patient is considered high risk (has a family history or is experiencing symptoms with gluten ingestion). If a duodenal biopsy comes back negative after positive serology, genetic testing for HLA-DQ2 is useful because 95% of those with CD carry this gene (Kaukinen and others 2002).

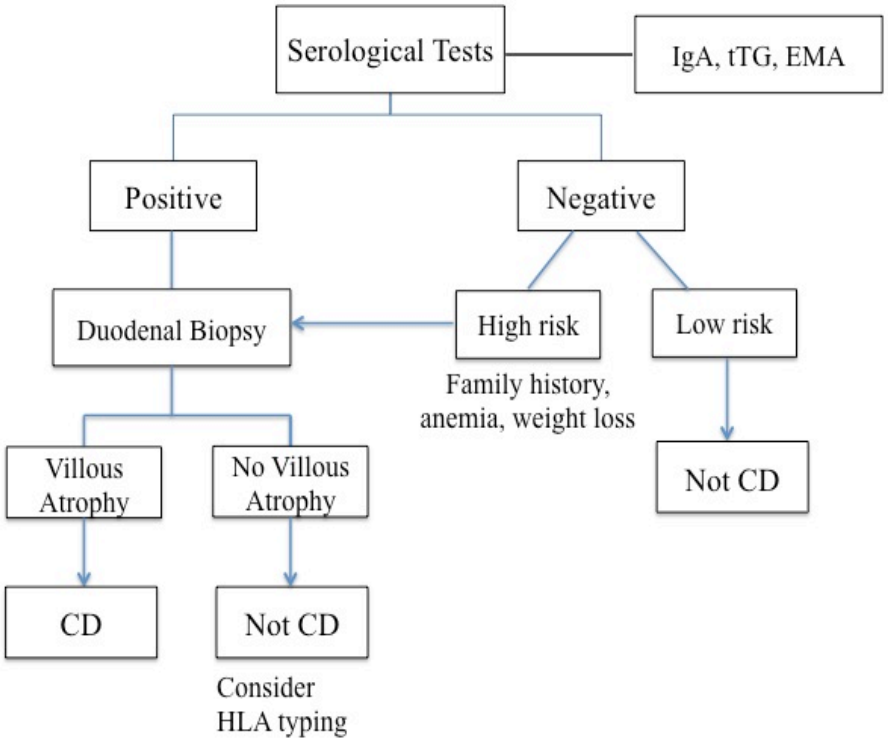

Figure 2.2: Serological testing for Celiac Disease

Because of the complexity surrounding this disease, experts in CD diagnosis have defined 15 terms known as the Oslo definitions (Ludvigsson and others 2013). With these definitions the results of serological testing, duodenal biopsies, and symptoms manifested can be used to categorize a patient's CD into many definitions including Classical, Asymptomatic CD, Non-classical, Potential, Subclinical, Symptomatic, Genetically at risk, etc. Although absence of intestinal enteropathy (negative biopsy) excludes a CD diagnosis, there are diagnoses such as non-celiac gluten sensitivity and gluten-related disorder. While this diagnosis may not be as severe as CD, adoption of a gluten-free diet is suggested to prevent any symptoms from occurring.

2.2 Gluten-Induced Inflammation in Celiac Disease

Gluten is the protein component of wheat, rye and barley and it is formed by the combination of glutenin and gliadin in an aqueous solution. It provides functionality to baked goods by trapping air bubbles and giving the desirable lift and height found in these products. In CD patients deamidated gliadin, rich in glutamine and proline peptides, is responsible for the inflammation. Gluten is quite resistant to digestion in the stomach even in those without CD, therefore it presents to the intestine largely intact. The digestion of gluten and following inflammatory response is demonstrated in Figure 2.3.

Gastrointestinal hydrolysis of gluten produces the gliadin peptide sequences 31-43 and 57-68 (referred to as p31-43, p57-68), which are responsible for the synergism of innate and adaptive immunological response seen in CD (Caputo and others 2012). The innate immune response to gliadin is marked by an increase production of IL-15, Cylcooxygenase-2 (COX-2) and Transglutaminase-2 (TG2) in enterocytes within three hours of contact. TG2 is a calcium-dependent enzyme and is activated by an increase in intracellular calcium, which occurs when

gliadin peptides present to intestinal cells. Wheat gliadin is a preferred substrate for TG2 because it contains many glutamine residues capable for modification (Ciccocioppo and others 2005).

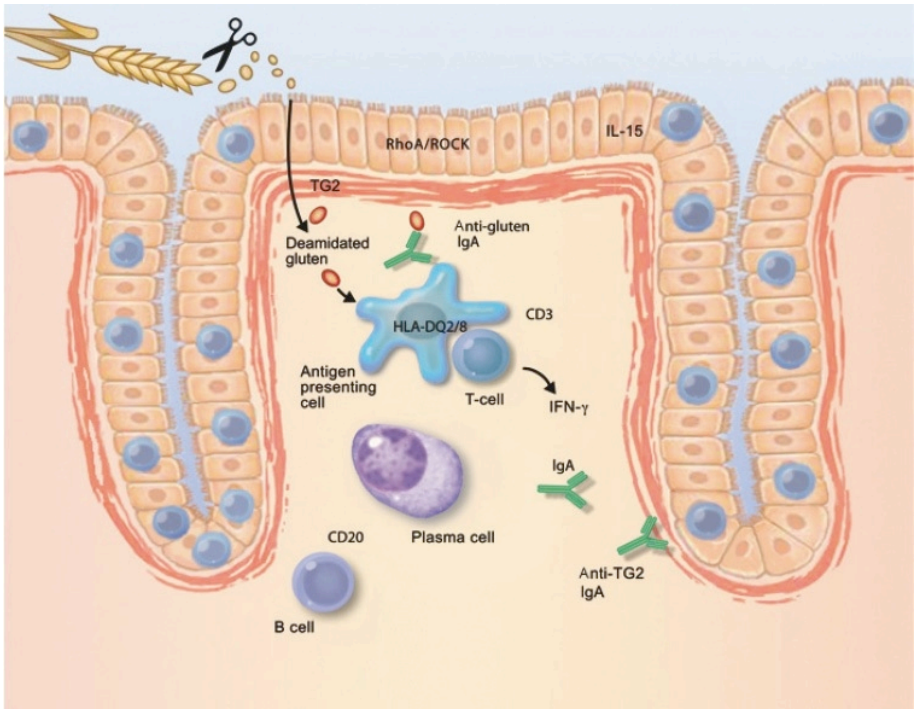

Figure 2.3: Gluten digestion in individuals with Celiac Disease (Sollid and Khosla 2011)

TG2 deamidates gliadin peptides and forms immunotoxic peptides that have a high affinity for HLA-DQ2. This post-translational modification of gliadin by TG2 therefore plays a central role in the pathogenesis of CD, because the formation of the HLA-DQ2 and deamidated gliadin complex is largely responsible for the inflammation in CD. The binding of HLA-DQ2 and deamidated gliadin elicits a response from T-cells causing increased production of Interferon-gamma (IFN-γ), which triggers immunoregulatory activities including production of Tumor Necrosis Factor (TNF-α) and interleukin-15 (IL-15) (Ludvigsson and others 2013). Nuclear Factor κβ (NF-κβ) and cyclooxygenase (COX-2) are also up-regulated in CD (Fernandez-Jimenez and others 2013; Vincentini and others 2015). Elevated levels of the inflammatory cytokines IL-6, IL-1β and IL-8 have been found in the serum of CD patients

(Cinova and others 2007). Production of these inflammatory biomarkers stems from the action of TG2 and is primarily responsible for the destruction of the intestinal mucosa, causing the major symptoms of CD.

2.3 Treatments of Celiac Disease

Celiac Disease can be diagnosed at any age, and early diagnosis is crucial because untreated CD worsens over time and will significantly impact quality of life. Left untreated, the risk for other long-term health disorders increases. These disorders include anemia, osteoporosis, lactose intolerance, gastrointestinal disorders, nutrient deficiency and other autoimmune disorders previously mentioned (Type I diabetes, thyroiditis, MS). The later in life a CD diagnosis occurs, the increased chance a patient has of developing another one of these disorders (Ventura and others 1999). For some patients even 50 mg of gluten a day can cause symptoms to occur, and with the wide range of applications of gluten in food, beverages and cosmetics accidental ingestion is not uncommon (Catassi and others 2007). This makes treatment extremely vital once a diagnosis is confirmed, although a gluten-free diet is the only current effective treatment. Other experimental treatments are being investigated including oral enzyme therapy, tight junction enhancement, and transglutaminase inhibition, which have shown promising effects in early stages of research (Sollid and Khosla 2011).

2.3.1 Gluten-Free Diet

The only current treatment of CD is a strict, lifelong adherence to a gluten-free diet (GFD) that includes avoidance of all types of wheat, rye, and barley. Following this diet involves consuming naturally gluten-free foods such as fruit, vegetables, meat/poultry, beans/legumes, and dairy (if not lactose intolerant). There are also naturally gluten-free grains that can be incorporated into a GFD including rice, potato, buckwheat, cassava, and others. The GF market

is growing and as a result commercially GF substitutes for common items like bread, cereals, soup/sauces and frozen foods are easier to find than in previous decades. Seventy percent of patients with the classic symptoms of CD improve within two weeks after initiation of a gluten-free diet, and patient serology can normalize within 3-12 months (Schuppan and Zimmer 2013). This diet can also protect against the extraintestinal manifestations previously described, so although it is the only treatment option for this disease it is effective.

In addition to accidental ingestion of gluten, compliance to a GFD is a major issue for those with CD. Especially in children, who may not enjoy the taste of GF foods and are more tempted to try gluten-containing foods, compliance is highly variable at around 45% to 81% (Hill and others 2005). Even in adults compliance is low and ranges from 40-60% (Sugai and others 2010; Vahedi and others 2003). GF foods generally have poor palatability and availability compared to gluten containing foods, which is why transgression from a GFD commonly occurs. In most categories for GF foods (cereal, breads, pasta, etc.) CD patients reported being little or only moderately satisfied with taste (do Nascimento and others 2015).

Strictly following a GFD can have significant impacts on general well being for those with CD. Food choice is restricted and with fear of accidental gluten consumption, daily activities are affected and social events like dining out are restricted as well. Thirty percent of CD patients reported that they sometimes consume gluten to feel “normal” or to satisfy cravings (do Nascimento and others 2015). This diet does show improvement to quality of life and is protective against the major symptoms but the issues of taste, compliance and lower availability decrease its effectiveness as a treatment for CD.

2.3.2 Experimental Treatments

Although a GFD is proven to be an effective therapy to CD, according to research 5-10% of CD patients do not respond to a GFD (Silvester and Rashid 2007). The other issues associated with a GFD of compliance and decreased quality of life (related to general well being) increase the need for additional therapeutic options. Furthermore, there is a large economic burden associated with a GFD (discussed later). This literature review presents several of the alternative therapies to a GFD that have reached clinical trials including oral enzyme therapy and tight junction enhancers as well as TG2 inhibition. It is important to note that other research into treatment exists, including HLA-DQ2-blockers, gluten-sequestering polymers, gluten tolerization and anti-CD antibodies (Sollid and Khosla 2011). For those suffering from CD, these therapies could significantly improve quality of life. In some cases several of these therapies could work together to ensure that if gluten is consumed (whether intentionally or not) it will not produce the inflammatory response that causes symptoms to manifest and destruction of the intestinal villi.

2.3.2.1 Oral Enzyme Therapy

Oral enzyme therapy in the form of glutenases has been the first alternative therapy in CD to reach clinical trials (Table 2.2). The theory is that these glutenases are able to digest gluten into non-toxic peptides in the stomach, which will prevent the gliadin peptides from reaching the intestine and triggering the inflammatory response typically seen in CD. Glutenases are protease enzymes and are derived from bacteria, fungi or cereals. The fungi *Aspergillus niger*, derived from germinating barley produces a prolyl endoprotease and has been investigated in several studies as AN-PEP and AnP2. ALV003 is a combination of a cysteine endoprotease (EP-B2) and a *Sphingomonas* capsulate bacterium.

Glutenases appear to be safe in CD patients and several promising results in clinical trials have been shown (Table 2.2). The main issue that these clinical trials encounter is the dose dependency of the glutenases and how much gluten it is able to breakdown. In order for this to be an effective therapy there must be clear guidelines on how they can be used, but in any case the results show that they can prevent enteropathy from occurring if a small amount of gluten (~50-900 mg) is ingested.

Table 2.2: Studies on the effects of glutenases as therapy in Celiac Disease

Enzyme investigated	Results from studies and clinical trials
ALV003	No significant deterioration of intestinal villi using 900 mg ALV003. N=20 (Lahdeaho and others 2014)
	300 mg ALV003 eliminated 88% of digested gluten. N=8, P=.0009 (Siegel and others 2012)
AN-PEP	AN-PEP with 50 g gluten led to complete disappearance of T-cell stimulatory peptides in vitro (Mitea and others 2008)
	No overall changes in degree of mucosal damages or serology in CD patients fed AN-PEP containing topping vs. control. N=16 (Tack and others 2013)
AnP2	A protease:substrate ratio 1:64 was effective at completely degrading gluten in vitro (Toft-Hansen and others 2014)

2.3.2.2 Tight Junction Enhancers

Tight junctions (TJs) control intestinal permeability and are regulated by both endogenous and exogenous stimuli. CD patients have altered TJ morphology and increased permeability, as gluten triggers the opening of TJs resulting in the increased presence of immunotoxic peptides to intestinal lamina propia. TJ enhancers would reduce transport of gluten through the intestine thus preserving tight junction structure and preventing symptoms from occurring. Larazotide acetate is a TJ regulator peptide that inhibits disassembly and dysfunction of TJ epithelial cells. This peptide has been investigated with success in clinical trials, showing

no difference in serology between those with CD on a gluten challenge with larazotide acetate and the control group (Leffler and others 2012; Kelly and others 2013).

Symptom severity was also shown to be significantly lower, as determined by a GSRS score (Gastrointestinal Symptom Rating Scale), a widely used questionnaire in CD research. In a 2015 phase II clinical trial by Leffler and others, CD patients given 0.5 mg larazotide acetate (referred to as AT-001) three times per day improved the symptoms of CD patients who experience persistent symptoms while following a GFD (n=340). The extraintestinal symptoms such as migraines and tiredness also improved, which shows that this treatment could significantly improve quality of life for CD patients. Overall efficacy and risk/benefit ratio need to be fully assessed for larazotide acetate before it can be an approved therapy, but these results show great potential.

2.3.2.3 Transglutaminase-2 Inhibition

As previously described TG2 plays a crucial role in the pathogenesis of CD, therefore preventing TG2 activation may reduce the effects of gliadin-induced inflammation. TG enzymes are implicated in several disorders and not just CD, including neurodegenerative disorders such as Alzheimer's, Huntington's and Parkinson's diseases as well as pathogenesis for cancer metastasis, liver injury and immune system damage (Ozaki and others 2010). The wide variety of disorders implicated by TG enzymes makes the discovery of TG inhibitors extremely important to disease prevention and/or treatment. Inhibitors of TG2 function at the active site and are either reversible or irreversible.

Transglutaminase-2 inhibitors have not yet reached clinical trials and are still in the discovery phase, though proof-of-concept studies have shown promising results *in vitro* and *ex vivo*. Several identified TG2 inhibitors include disulfide compounds such as cystamine and

cysteamine, α,β unsaturated amides found in the piperine family (Marrano and others 2001; Ozaki and others; Pardin and others 2008). TG2 active site inhibitors R281 and R283 reduced gliadin-induced inflammation *ex vivo* as evidenced by the number of proliferating enterocytes in crypts and decreased levels of the biomarker IL-15 (Rauhvirta and others 2013). It is important to note that TG2 does have function in apoptosis and wound healing (through activity in blood clotting pathway) and before a TG2 inhibition therapy in CD could be approved it would have to be localized to the small intestine.

2.4 Economic Burden of a Gluten-Free Diet

In the United States (US), the availability of gluten-free foods is limited because not all grocery stores carry these products. In a survey about the perception of GF foods, the majority (54%) of CD patients stated having some difficulty in finding GF foods. Multiple studies have found that GF foods are more expensive on average than similar products that contain gluten. In the US and Canada, GF foods were found to be 2-3 times more expensive, whereas in the United Kingdom (UK) prices of GF foods were 5 times more expensive than their gluten counterparts (Lee and others 2007; do Nascimento and others 2015; Burden and others 2015). While GF are more available on-line and in health food stores, these options are also more expensive than regular supermarkets (Singh and Whelan 2011). The economic burden is therefore not only associated with higher cost of GF foods compared to gluten-containing foods, but the fact that availability is low in typical supermarkets and require CD patients to shop at more expensive markets.

Besides the actual price and availability of GF foods, there are other factors that contribute to the economic burden of a GF diet. As stated earlier, following a GFD means consuming more naturally gluten-free products such as fruits, vegetables and meats, which are

also foods that are associated with a high cost diet due to their perishability (Drewnowski and others 2004). Additionally, a GFD can be nutritionally deficient in terms of certain vitamins and fiber, which may require supplementation of nutrients for CD patients and contribute to the high cost of this diet. For example iron, calcium and vitamin D supplements are commonly prescribed for children with CD (Kapur and others 2003, Malterre 2009). Gluten free products are low in folate and other B-vitamins, and CD patients have shown to be nutritionally deficient in these vitamins, putting them at risk for other health disorders to arise, especially cardiovascular events, which may also contribute to the burden associated with a GFD due to increased health costs (Bituh and others 2011; Hallert and others 2002).

A GFD includes great dietary restriction, which as stated earlier affects quality of life and general well being, and because of this CD patients on a GFD are more likely to suffer from depression and anxiety (Addolorato and others 1996; Fera and others 2003). While psychological support counseling has shown to reduce depression and improve GFD compliance, this can add to the costs of treating this disease (Addolorato and others 2004). Family therapy has often been suggested for parents with children who suffer from CD due to the financial stressors of a GFD and the need for parents to monitor compliance by choosing specific foods (Flamez and others 2014). Nutritional counseling at diagnosis is common as well, since changing to a GFD requires many adjustments and monitoring of dietary intake. The counseling and therapy associated with CD is therefore an often-unforeseen burden that affects an individual both financially and mentally.

The U.S. gluten-free market is growing and was expected to reach $15.6 billion in 2013 (Mintel Group 2013). The increase in this market is detrimental to the wheat-based products industry and consumers who cannot afford this diet. In 2014 a new US Food and Drug

Administration (FDA) rule took effect for foods labeled "gluten-free" and under this new rule a gluten-free claim can be applied to products that do not contain grains with gluten and products that do not inherently contain gluten (such as meats or dairy) (Grossman 2014). While this is useful to prevent accidental ingestion of gluten, the increased labeling may affect consumer perceptions of gluten-containing foods. Consumers who do not have CD believe that avoiding gluten products can be a way to achieve weight loss and that gluten-free foods are healthier than their gluten counterparts (Dunn and others 2014). These health claims are unsubstantiated, but this perception could greatly affect the wheat industry.

In a recent survey of 365 individuals with CD, 66% were interested in medication to treat their disease rather than a GFD, especially those with a lower quality of life and those who stated they frequently dine out (Tennyson and others 2013). The large economic burden of a GFD decreases its effectiveness as a therapy for CD, and research into alternative treatments is growing in recent years. Food products or beverages capable of inhibiting immunotoxic gluten-induced inflammation in small intestinal epithelium could mitigate gluten toxicity. A safe treatment to reduce inflammation in individuals suffering from active or inactive CD would be welcome and could have significant impacts on quality of life and general well being for those with the disease.

2.5 Potential of Cocoa as an Alternative Therapy in CD

Recent research has shown that cocoa and chocolate consumption is linked to several health benefits (Buijsse and others 2010; Monagas and others 2009). The benefits involve reduction of inflammatory responses due to the presence of bioactive polyphenols (including flavanols like catechins and procyanidins). Studies have shown that cocoa products are capable of reducing levels of IL-1β, IL-6 and IL-8 whose role in CD disease has previously been

described (Sarria and others 2014). While the investigated health benefits have been linked to primarily cardiovascular events (lowering blood pressure and increasing HDL cholesterol), there is potential for these bioactive compounds to reduce the immunological response in CD.

Cocoa contains procyanidins, caffeine and theobromine, all of which are capable of reducing TG2-induced inflammation. Concentrating and extracting these compounds from cocoa powder may exhibit a synergistic effect on the inhibition. Theobromine typically has the highest concentration in cocoa, followed by procyanidins and caffeine, although it is not as water-soluble (Ortega and others 2010; Sarria and others 2015). This study will discuss these dietary inhibitors of inflammation in CD, including TG2 inhibition as well as other CD biomarkers. The result is a potential alternative to a GFD that can be used with wheat-based products as therapy for CD. The gain to consumers and the wheat-based and cocoa industry will be significant.

2.5.1 Procyanidins

Procyanidin trimers and tetramers have shown binding affinity for gliadin peptides and can be an effective therapy in CD (Dias and others 2015). This quenching of gliadin peptides forms a stable complex that could block the immunological response that occurs in the intestine in CD. Procyanidin dimers are stable during gastric transit and reach the small intestine unchanged. In humans, these dimers appear in plasma 2-3 hours after digestion and intestinal bioavailability is not thought to be a limiting factor of their potential benefits (Rios and others 2002). This compound is not toxic and cross Caco-2 cells (an *in vitro* model of the intestine) by paracellular transport (Deprez and others 2001; Kosinska and Andlauer 2012) and therefore reaches the intestine unmodified (Sano and others 2003).

The presence of 10 hydroxyl groups on procyanidin molecules (Figure 2.5) confers substantial chelating activity to compete with TG2 for available calcium. Increased intracellular

calcium levels is a key event in the inflammatory pathway, not only activating TG2 but also up-regulating inflammatory cytokines produced by T-cells (Parekh and Putney Jr. 2005). Concentrations of procyanidin-B2 as low as 10 nM have been shown to modulate calcium levels, and this inhibition of calcium levels increases in a concentration-dependent manner (Verstraeten and others 2008). In a model of CD, procyanidin-B2 may penetrate Caco-2 cells before gliadin peptides and inhibit TG2 levels.

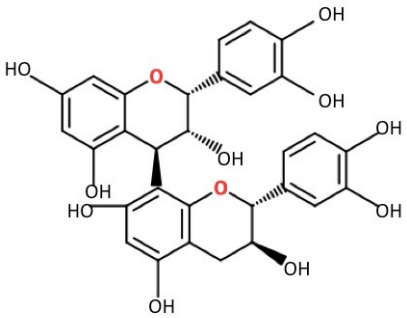

Figure 2.4: Structure of procyanidin-B2

Procyanidins in cocoa are responsible for inhibition of digestive enzymes (Gu and others 2011), though their effects on TG2 have not been investigated. If effective, by reducing the levels of TG2, gliadin will not be deamidated into immunotoxic peptides that bind with HLA-DQ2. Additionally, the affinity that procyanidin trimers and tetramers (which are also present in cocoa) have for gliadin give products rich in procyanidins great potential as an alternative therapy to a GF diet. Cocoa is an example of a product that is rich in procyanidins and could protect against the inflammatory response in CD. To the date of this research, the efficacy of procyanidin dimers from cocoa as inhibitors of TG2 and other biomarkers in CD has not yet been investigated.

2.5.2 Caffeine and Theobromine

Caffeine, which is present in cocoa, has also been shown to reduce TG2 activity when studied *in vitro* (Cho and others 2012; Sarria and others 2015). The mechanism of TG2 inhibition is caffeine's role in regulation of intracellular calcium and inhibition of phosphodiesterases (Johnson and others 2012). Phosphodiesterases are part of a pathway that induces TG2 activation; therefore inhibiting these molecules may have potential to inhibit TG2 in CD. Structurally, theobromine and caffeine are very similar (Figure 2.6). Like caffeine, theobromine is a phosphodiesterase inhibitor and is also involved in the regulation of NF-κβ (Sugimoto and others 2014). Despite these similarities theobromine is shown to be more stable during gastric transit and is excreted in its unmodified form, while caffeine is more likely to be metabolized to other constituents (Martinez-Pinilla and others 2015). Theobromine is also the compound in cocoa that has been associated with reduction of several inflammatory cytokines, including IL-1β, IL-6, and IL-8 (Sarria and others 2015). Together with procyanidins, there is great potential for these compounds to inhibit inflammation in CD.

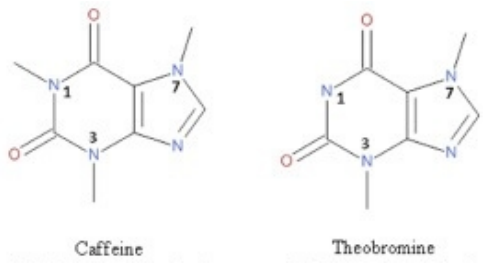

Figure 2.5: Structure of caffeine and theobromine (Martinez-Pinilla and others 2015)

2.6 The Caco-2 Cell Line in Celiac Disease Research

The Caco-2 cell line (Cancer coli-2) is derived from colon carcinoma though upon reaching confluence, differentiates spontaneously (Fogh and others 1977). The differentiated cells express functional and morphological properties of intestinal enterocytes, including a brush border with microvilli and small intestinal enzymes. Because of this, Caco-2 cell line is the most

extensively used *in vitro* model in CD research because of its consistency of the epithelial layer of the intestine in those affected with the disease. Studying effects of a food bioactive through *in vivo* models can be difficult, especially when dealing with a disease that has such severe side effects. For early stages of CD research there are several proposed methods in addition to Caco-2 cells including animal models, *ex vivo* and other *in vitro* cultures, each with its own advantages and limitations depending on the purpose of the research.

Investigating novel therapies in CD requires a reliable model of gluten-induced inflammation leading to the villous atrophy that is characteristic of the disease. Creating an animal model is difficult because the animals typically used in research (mice for example) do not express the HLA-DQ2 gene and do not produce celiac-type antibodies (de Kauwe and others 2009; Smart and others 1992). Animal models are typically an expensive option, and while researchers can introduce these genes into an animal model, it is not a guarantee that it will produce all the elements seen in CD. Because of this *ex vivo* and *in vitro* options may be more appropriate models to research.

Ex vivo cultures come from small-intestinal mucosal biopsies from CD patients. This method enables researchers to study the variety of inflammatory biomarkers that elevate from gluten ingestion, and for this reason is mainly used to investigate the pathogenesis of CD (Lindfors and others 2012). While this model has potential for studying the efficacy of novel treatments, most of the research using *ex vivo* cultures has been geared towards analyzing pathogenesis. CD patient-derived T-cell lines can also be used, although intestinal T-cells do not always recognize gluten peptides, which is a limiting factor when investigating gluten-induced inflammation. Another limitation to *ex vivo* cultures is the low throughput, since this cell line depends on biopsies from patients and can be hard to obtain.

Two *in vitro* epithelial cell culture models are commonly used in CD research are T84 cells and Caco-2 cells. Like the *ex vivo* models, T84 cells are used to study disease pathogenesis. While they are derived from colonic origin, T84 cells exhibit similar intracellular junctions of intestinal cells and are therefore useful to study things like gluten permeability and toxicity (Madara and others 1987). Either gliadin, IFN-γ or TNF-α can be used in Caco-2 cells to induce expression of other biomarkers seen in CD (Perry and others 1999; Ciccocioppo and others 2006). It is used more extensively than T84 cells in CD research, notably for testing of novel treatments; therefore its use is more appropriate to proof-of-concept studies (Lindfors and others 2012).

The disadvantages of the Caco-2 cell line are due to the fact that it cannot reproduce all the characteristics of a human intestinal epithelium, which is not unexpected for *in vitro* models. Human intestinal cells contain multiple cell types while Caco-2 cells only express enterocytes. Transport of lipophilic molecules may be decreased since Caco-2 cells do not contain a mucus layer with bile acids as human intestinal cells do (Lea 2015). While Caco-2 cells are appropriate for CD research it is important to acknowledge these limitations, since data from *in vitro* studies does not equal a direct correlation to what will occur *in vivo*.

CHAPTER 3: MATERIALS AND METHODS

3.1 Natural Cocoa

To carry out this research, natural cocoa powder was generously donated from Mars Chocolate North America, Inc. (Hackettstown, NJ). This was a natural process cocoa powder, meaning that its acids had not been stripped as is done in Dutch-processed cocoa (alkaline treated).

3.2 Extraction of Procyanidin-B2

Procyanidin-B2-rich cocoa extracts were obtained by dissolving the natural cocoa powder in deionized water in a 1:4 ratio. The extracts were centrifuged and the supernatants were collected, followed by removing the water through lyophilization. To get a more concentrated stock this process was repeated.

To remove impurities such as sugars and organic acids that may be present in cocoa powder, procyanidins were extracted using a method outlined by Counet and others (2004), using an acetone:water:acetic acid solvent (70:28:2). The solvent was removed via rotary evaporation and the stock was lyophilized again.

3.3 UHPLC Analysis of Cocoa Extracts

Ultra High Performance Liquid Chromatography (UHPLC) was performed with a ThermoScientific Ultimate 3000 (Waltham, MA), which is equipped with a binary gradient pump, sample injector, a column oven and a photodiode array detector. The equipment was run under the following conditions: HSS C18 Column (100Å, 1.8 µm, 2.1 mm X 50 mm), HSS C18 SB VanGuard Pre-column (100Å, 1.8 µm, 2.1 mm X 5 mm) (Waters Corporation, Milford, MA) was used with a flow rate of 0.3 mL/min. Chromeleon™ 7.2 Chromatography Data System (CDS) software (ThermoScientific, Waltham, MA) was used to measure and identify each peak

by its specific retention time. Procyanidin extracts were diluted 1:20 with water. Injections of 2.5 μL were used and the oven was held at a temperature of 25°C.

The protocol was adapted from Cooper and others (2007) that involved UPLC analysis of major cocoa polyphenols in chocolate. The binary system phases were (A) water/THF/TFA (98:2:0.1 v/v/v) and (B) acetonitrile with 0.1% TFA. The two minute gradient was as follows: 0.0-0.2 min, 90-87% A; 0.2-0.75 min, 87-85% A; 0.75-0.775 min, 85-0% A; 0.775-1.25 min, 0% A linear; 1.25-1.275, 0-90% A; 1.275-2 min, 90% A re-equilibration time. The standards and cocoa extracts were analyzed at 280 nm wavelength.

Procyanidin-B2 standard was purchased from ChromaDex (Irvine, CA) and the standards of theobromine and caffeine from Sigma-Aldrich (Bellefonte, PA), were used to construct standard curves for analysis. The concentrations of procyanidin-B2, caffeine and theobromine in the cocoa extracts were identified by comparing the retention times of their peak with that of standards. A linear response was obtained for procyanidin-B2 in the concentration ranges of 25-1000 μg/mL and caffeine and theobromine in the concentration ranges of 50-500 and 50-1000 μg/mL, respectively. The results report the standard curve analysis and chromatogram of the cocoa extracts.

3.4 Cell Proliferation Assay

Caco-2 cells (10^3 per well) were seeded on 96-well plates and incubated with culture medium combined with cocoa extracts containing different concentrations of procyanidin-B2 between 10 nM and 500 μM. The highest concentrations of nontoxic procyanidin-B2 were used for subsequent experiments.

3.4.1 MTS Cell Proliferation Assay

Cells were incubated for 2, 6 and 24 h at 37°C in an incubator with 5% CO_2. After incubation cells were washed and incubated for 4 h with a solution of PMS (phenazine methosulfate) and a tetrazolium compound (MTS) according to the protocol provided by the supplier CellTiter 96 Aqueous One solution (Promega, Madison, WI). Cells bioreduce MTS into a formazan product and the absorbance was read at 490 nm using a BioRad Model 680 microplate reader (Hercules, CA). The quantity of formazan product is directly proportional to the number of living cells in culture and was used to compare cell proliferation versus concentrations of procyanidin-B2. The cell proliferation assay was performed in triplicate and results were presented as a percentage of the control.

3.5 Cell Cultures

The Caco-2 cell line was used as a model of human intestinal epithelial cell. Caco-2 cells (ATCC, Manassas, VA) were grown in 75 cm^2 Dulbecco's Modified Eagle's Medium (DMEM) (GIBCO, Grand Island, NY), 10% fetal calf serum (GIBCO), 100 units/mL penicillin streptomycin (GIBCO), and 2 mM L-glutamine (GIBCO). Caco-2 cells (2 x 10^5 per well) were incubated at 37°C in a humidified atmosphere containing 5% CO_2. Upon reaching 80% confluence, cells in the logarithmic phase were subcultured weekly at a split ratio of 1:3 by trypsinization.

To determine the efficacy of procyanidin-B2's ability to inhibit TG2 along with the synergism between procyanidin-B2, caffeine and theobromine Caco-2 cells were seeded into cell culture plates for 72 h and incubated with the nontoxic concentrations of procyanidin-B2 standardized cocoa extracts and either 10 ng/mL Interferon-γ (IFN) or 20 μg/mL α-gliadin (31-43) to stimulate the inflammatory process. IFN and gliadin treated Caco-2 cells were also

incubated with caffeine (50 and 500 μM) and theobromine (50 and 500 μM) to better observe the effect of these compounds alone.

Various concentrations of cysteamine (Sigma-Aldrich, Bellefonte, PA) were included in cell cultures to compare the effects of the cocoa extracts versus a known inhibitor of TG2. IFN-γ was purchased from ThermoScientific (Waltham, MA) and α-gliadin (p31-43) from AnaSpec (Fremont, CA). After 72 h, supernatants were separated from the adhering Caco-2 cells for analysis of inflammatory cytokines through ELISA. Cell homogenates (cytoplasmic protein fraction) for analysis of TG2 and COX-2 through Western Blot were prepared through cell lysis.

3.6 Western Blot Analysis of TG2 and COX-2

Cells were washed with Phosphate Buffered Saline (PBS) and collected by scraping in 50 μL of hypotonic buffer (10 mmol/L HEPES pH 7.9, 1.5 mmol/L $MgCl_2$, 10 mmol/L KCl, 0.2 mmol/L PMSF, 0.5 mmol/L DTT, 5 mmol/L NaF, 1 mmol/L Na_3VO_4). The mixture was vortexed for 10 s and incubated for 30 min on ice, then centrifuged at 14,000 x g for 15 min and the supernatant was retained as cytoplasmic extracts.

The pellet was re-suspended in 50 μL of high-salt buffer (20 mmol/L HEPES pH 7.9, 25% glycerol, 1.5 mmol/L $MgCl_2$, 1.2 mol/L KCl, 0.1 mmol/L EDTA, 420 mmol/L NaCl, 0.5 mmol/L DTT, and 0.2 mmol/L PMSF) by pipetting up and down and vortexing 10 s on high setting. The mixture was incubated for 30 s on ice and centrifuged at 14,000 x g for 10 min (pre-cooled at 4°C). The supernatant was retained as nuclear fractions and stored at −80°C until use. Total protein in cytoplasmic and nuclear fractions was determined by the Bicinchoninic Acid (BCA) protein assay (ThermoFisher Scientific, Waltham, MA).

Equal amounts of protein (725 μg) of cytoplasmic protein fractions from control and procyanidin-B2 treated cells were mixed with LDS sample buffer (Invitrogen, Carlsbad, CA)

then boiled for 5 min (to denature proteins) and vortexed at a high setting. Thirty microliters of each sample were added in each lane of a 4-12% Bis-Tris SDS polyacrylamide gel (Invitrogen, Carlsbad, CA). Proteins of interest were transferred to a polyvinylidene fluoride (PVDF) membrane (0.4 μm pore size) (ThermoFisher Scientific, Waltham, MA), then blocked in 5% bovine serum albumin (BSA) in PBST (PBS with 0.1% Tween-20) for 1 h. The primary antibody was prepared in 5% BSA and incubated with the membrane overnight at 4°C on a shaker. The membrane was washed three times for 10 minutes using PBST, then incubated for one hour with secondary antibody and the washes were repeated.

Visualization of the bound antibody was done in a dark room using West Pico Substrate, an enhanced chemiluminescent HRP-substrate (ThermoFisher Scientific, Waltham, MA) and a BioRad ChemiDoc MP System (Hercules, CA). After analyzing band density, membranes were stripped and re-probed with β-actin to serve as a loading control. Results were reported as a ratio of the density of each band to its β-actin for cells incubated with either gliadin or IFN and cocoa extracts. Caco-2 cells that were treated with Caffeine, Theobromine and Cysteamine were compared using this ratio and comparing it to either gliadin or IFN (relative level, %) to measure the percent decrease in Transglutaminase-2 after 72 h incubation.

The primary antibodies β-actin, COX-2, and Transglutaminase-2 were purchased from Cell Signaling Technology (Danver, MA) and the conjugated secondary antibodies (anti-rabbit and anti-mouse) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

3.7 ELISA Analysis of Inflammatory Cytokines

The supernatants of Caco-2 cells were collected after 72 h for analysis of inflammatory cytokines including Interleukin-8 (IL-8), Interleukin-6 (IL-6), Interleukin-1β (IL-1β) and Interleukin-15 (IL-15). Analysis of human IL-8, IL-6 and IL-1β was done using commercial kits

from Peprotech (Rocky Hill, NJ). Human IL-15 ELISA kit was purchased from Affymetrix eBioscience (San Diego, CA).

The absorbance of each cytokine (pg/mL) was read using a BioRad Model 680 microplate reader (Hercules, CA). Each kit was provided with a protocol that was followed. For IL-15, which is not typically seen in cell supernatants, total cell lysates (50 μL) were tested. Samples were normalized to contain 1 mg/mL protein and incubation time was increased from 2 h to overnight at 4°C to maximize the sensitivity. Testing each sample was done in triplicate, and the results are expressed as mean ± SD of the concentration of interleukin.

3.8 Statistical Analysis

Each experiment was performed at least three times. The data was expressed as the mean ± SD and analyzed using one-way analysis of variance (ANOVA) followed with a Tukey test for multiple comparisons. The differences were considered significant at the $P < 0.05$ level. Statistical analysis was conducted using the Statistical Analysis Software (SAS) (version 9.4).

CHAPTER 4: RESULTS AND DISCUSSION

4.1 UHPLC Analysis

UHPLC has known advantages over other chromatographic methods, including shorter analysis time, higher peak efficiency and resolution. While HPLC requires between 50 and 80 min per sample, the separation of cocoa compounds was successfully achieved in a short 2-min gradient. The method was similar to Cooper and others (2007) in that a C18 column with a short length (50 mm) was used as well as the same binary system phases (described in Chapter 3). However, the 8-min gradient outlined in this study was not adaptable to the cocoa extracts in our study, as all compounds eluted at once. Using the 2-min method not only greatly decreased analysis time, but it also improved separation and peak efficiency. The elution of the standards in the two-minute gradient can be seen in Figure 4.1.

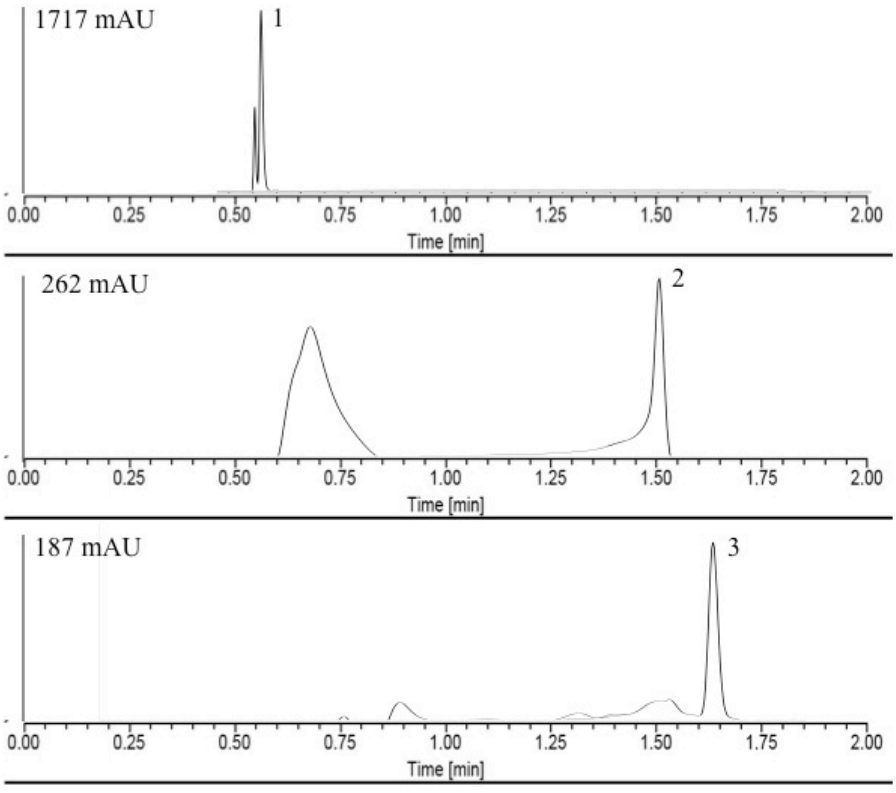

Figure 4.1: Chromatograms of the standards (1) theobromine (2) caffeine and (3) procyanidin-B2 at 280 nm. The concentration of each analyte was 100 μg/mL.

4.1.1 Standard Solutions

External standardization was performed to create standard curves for analysis (Figure 4.2). In a study by Ortega and others (2010) analyzing polyphenols and alkaloids in cocoa, theobromine eluted first followed by caffeine and procyanidin-B2, which is in agreement with our research (Figure 4.1). Theobromine eluted at 0.590 min, while caffeine eluted at 1.507 min and procyanidin-B2 at 1.65 min.

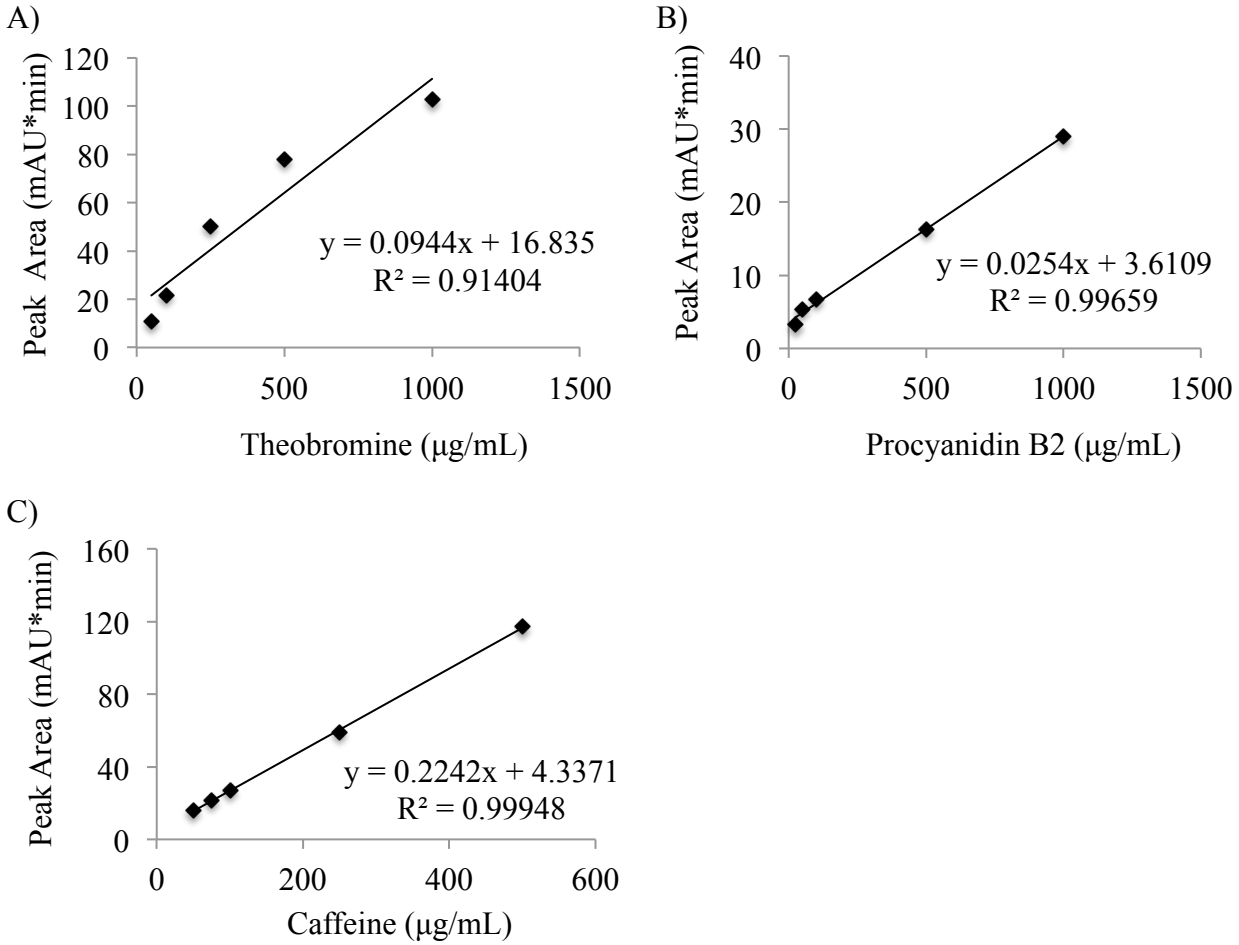

Figure 4.2: Standard curves of A) theobromine B) procyanidin-B2 and C) caffeine

Each standard displayed multiple peaks, which may speak to the purity of the compounds purchased and show that standards for UHPLC require compounds with higher purity. The peaks were used to construct the standard curves seen in Figure 4.2. In order to determine which peak

should be used for analysis, cocoa extracts were spiked with 500 µg of the standard to show where the compound eluted in our sample and the specific retention time using peak identification software (Chromeleon™ CDS software, ThermoScientific).

4.1.2 Analysis of Cocoa Extracts

Five distinct peaks were found in the extracts, and the compounds of interest were identified by spiking and comparing retention times to the standards (Figure 4.3). While catechin and epicatechin analysis was not included, according to other research it is likely that the large peak eluting after theobromine is epicatechin (Cooper and others 2007; Ortega and others 2010); the small peak eluting around the same time as procyanidin-B2 is likely catechin. Catechin and epicatechin have mostly been linked to coronary disease research by reducing LDL cholesterol and recycling antioxidants; therefore we did not consider these compounds relevant to our research in CD (Zhu and others 1999; Rice-Evans and others 1996).

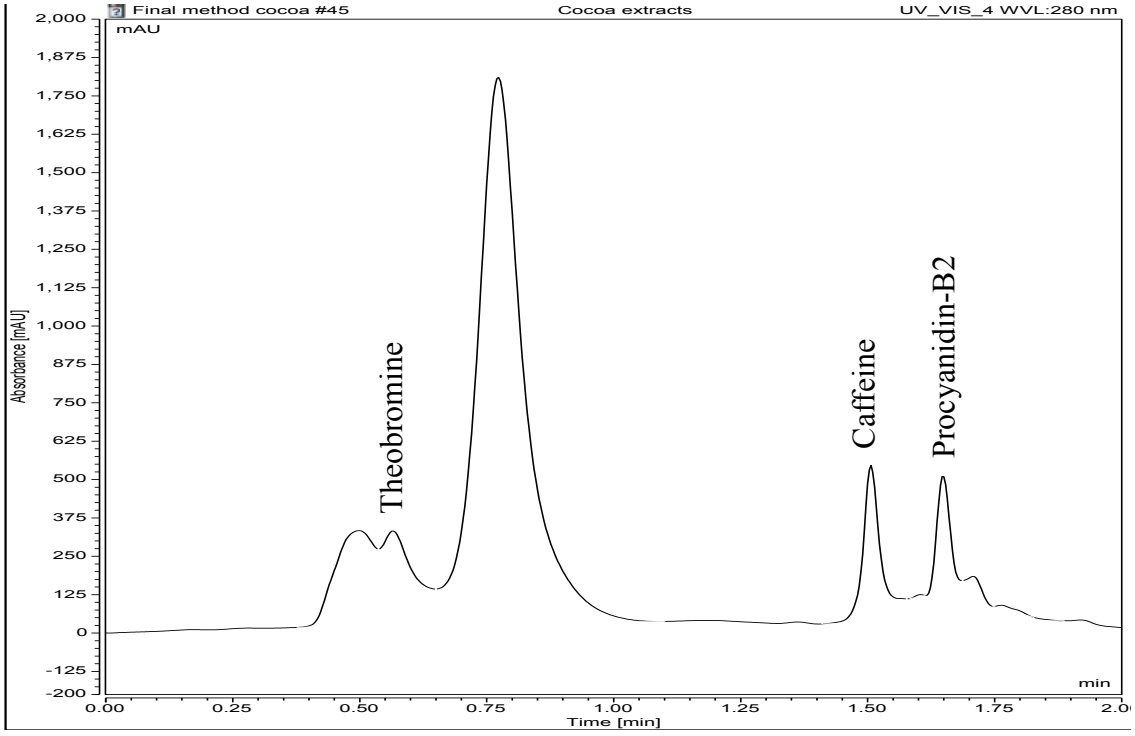

Figure 4.3: UHPLC analysis of cocoa extracts at 280 nm

The concentrations of the identified compounds, in milligram per gram of cocoa extract are listed in Table 4.1. As evidenced by other research, theobromine is found in higher concentrations in cocoa than both procyanidin-B2 and caffeine (Smit and Blackburn 2005; Ortega and others 2010; Sarria and others 2015). Typically caffeine and procyanidin-B2 are found in very low levels in cocoa (below 4 mg), although the efforts to concentrate these compounds through extraction and lyophilization were effective in substantially increasing their concentrations.

Table 4.1: Concentration of bioactive compound expressed as mg per g of cocoa extract found through UHPLC

Compound of interest	Concentration (mg/g)	% (w/w)
Theobromine	22.61	40.3
Caffeine	11.20	20
Procyanidin-B2	21.39	38.2

4.2 Effect of Cocoa Extracts on Cell Proliferation

The cocoa extracts were standardized to their concentration of procyanidin-B2 for use in all further analysis, since it is this compound that we were primarily interested in studying. Viable cells were tested using CellTiter 96 Aqueous One solution (Promega, Madison, WI). Cells bioreduce the MTS reagent into a formazan product and the absorbance was read at 490 nm. The quantity of formazan product is directly proportional to the number of living cells in the culture. Caco-2 cells were incubated with cocoa extracts ranging from 1-50 μM for 2, 6 and 24 h.

Cocoa extracts varying in concentration of procyanidin-B2 did not have adverse effects on Caco-2 cell proliferation (Figure 4.4 and Figure 4.5). Preliminary studies included concentrations below 1 μM but did not exhibit a decrease in TG2 levels or other CD biomarkers; therefore the rest of the data only reports results from concentrations of 8.5 μM and above. No significant differences were observed in cell proliferation for any concentration of procyanidin-

B2 ($p<0.05$), which has previously been reported for concentrations up to 50 μM in Caco-2 cells (Ramos and others 2011).

A significant increase in cell proliferation is seen over time when Caco-2 cells were incubated with cocoa extracts for 24 h (Figure 4.4). This effect of cocoa extracts on Caco-2 cells is likely the action of procyanidin-B2 and other bioactive compounds on inhibiting apoptotic pathways. Procyanidin-B2 and epicatechin are capable of reducing oxidative stress that leads to apoptosis, which explains the increase in proliferation with higher concentrations of B2.

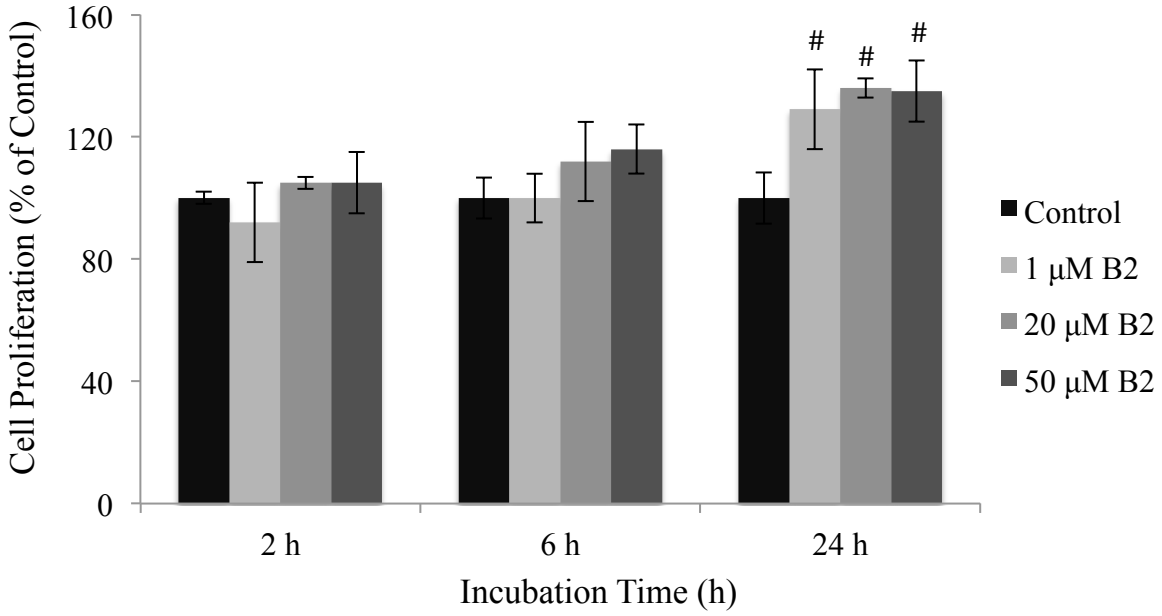

Figure 4.4: Caco-2 cell proliferation during incubation with cocoa extracts standardized to procyanidin-B2 content. No significant differences found between concentrations. # denotes a significant difference over time ($p<0.05$)

Their antioxidant properties have been shown to prevent cytotoxicity and have anti-apoptopic effects in Caco-2 cells (Rodriguez-Ramiro and others 2011). As stated in the literature review, TG2 also plays a role in cell death, and inhibition of this enzyme from the cocoa extracts may also explain the increase in the number of living cells. After these low concentrations were studied for their inhibitory effects on TG2, an additional cell proliferation assay was conducted

to analyze whether higher concentrations of procyanidin-B2 exhibit greater inhibition of inflammatory biomarkers (Figure 4.5).

No significant differences were observed in cell proliferation for the concentrations of 100-500 μM. Cocoa extracts with higher B2 concentrations of 750 μM and 1 mM were tested *in vitro* without performing a cell proliferation assay, though after the 72 h incubation appeared toxic to cells causing most cells to die and were thus excluded from analysis.

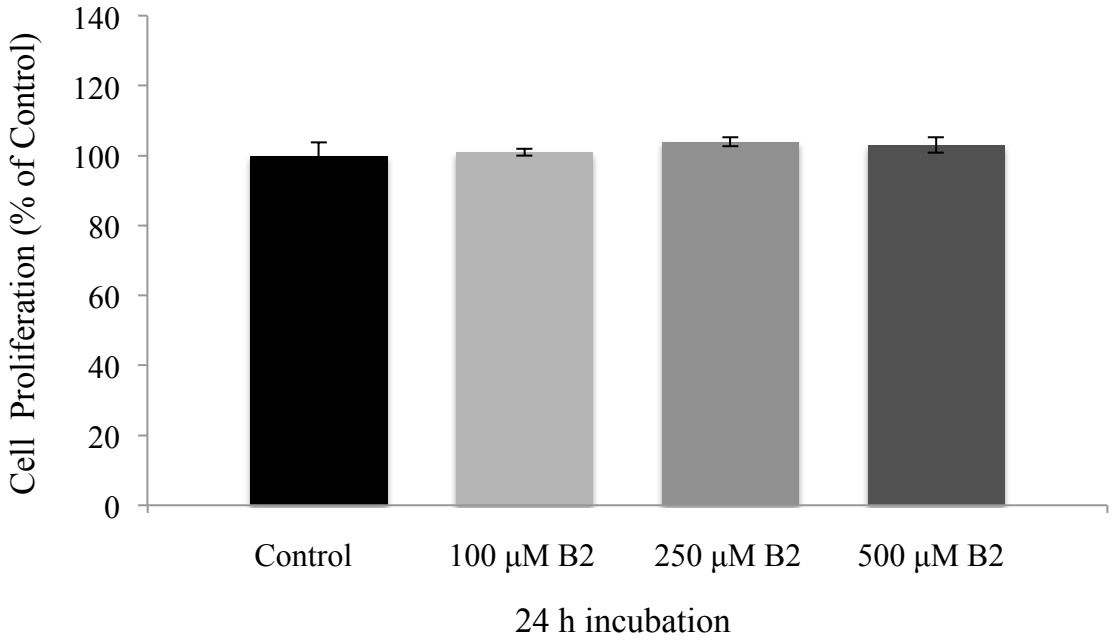

Figure 4.5: Caco-2 cell proliferation during incubation with cocoa extracts of higher concentration procyanidin-B2. No significant differences found between concentrations ($p<0.05$)

4.3 Effect of Cocoa Extracts on Transglutaminase-2 (TG2) Levels

Transglutaminase-2 (TG2) is involved in the pathogenesis of several autoimmune and inflammatory disorders, making TG2 inhibition an attractive target for therapy for diseases including CD. CD is a combination of the innate and adaptive immune response, though the action of TG2 plays a larger role in the latter. TG2 deamidates gliadin peptides at specific glutamine residues, resulting in formation of peptides that bind with HLA-DQ2 and eliciting an inflammatory response from T-cells. Additionally when high levels of TG2 are present, T-cells

increase their reactivity to gliadin (Caccamo and others 2010). IFN-γ is the most potent inducer of TG2 and when released by T-cells in CD, activates the pathway that causes destruction of intestinal mucosa (Petersen and others 2014). The cellular level of TG2 in Caco-2 cells was studied by Western blotting (WB). As shown in Figure 4.6, the exposure of the cells to IFN resulted in a significant increase of TG2.

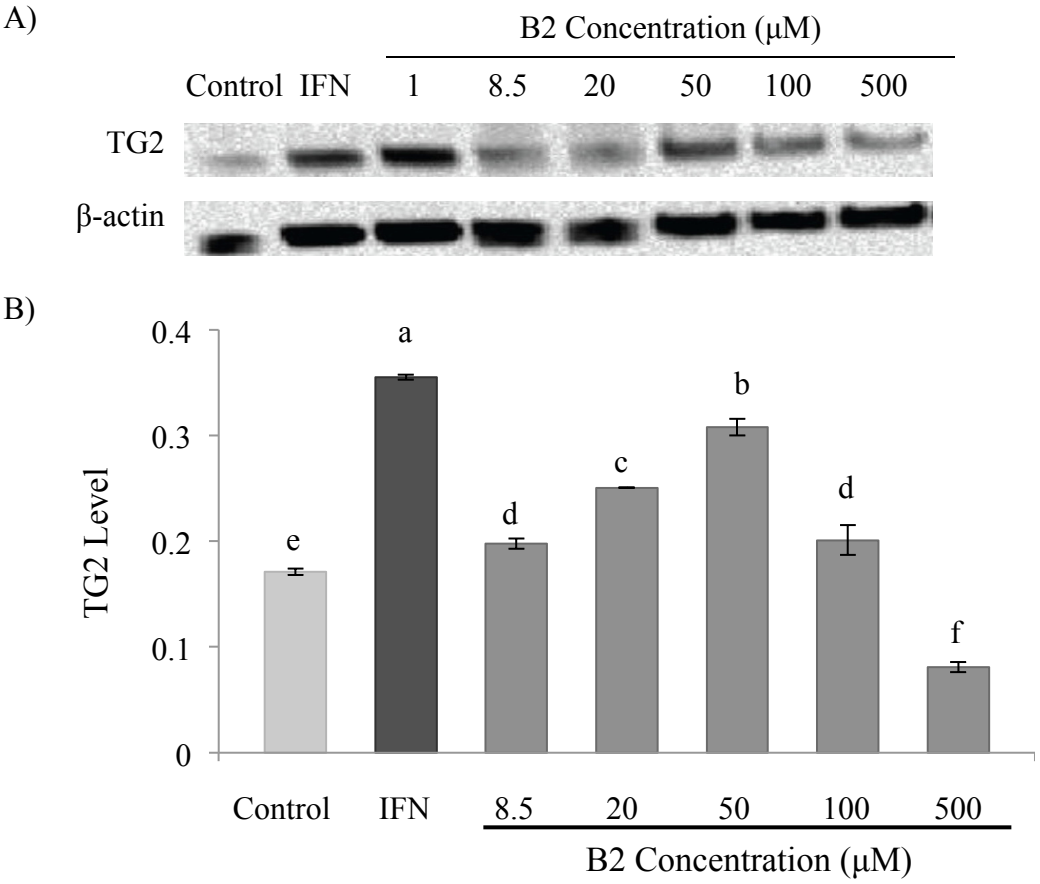

Figure 4.6: TG2 levels in Caco-2 cells exposed to IFN (10 ng/mL) or medium alone (Control) as resulted by Western blotting. A) Image from WB experiment from one of three exposures. B) TG2 levels were measured by mean densitometric value ± standard deviation of TG2 bands, normalized for corresponding actin density (ratio of TG2:actin). Letters abcdef are significantly different ($p<0.05$)

Cells were incubated with 10 ng/mL of IFN and treated with cocoa extracts standardized to their procyanidin-B2 content in concentrations ranging from 1-500 μM. As stated earlier, concentrations of 1 μM and below showed no decreases. Significant decreases in TG2 levels

were observed starting at 8.5 μM procyanidin-B2, and the greatest effects were seen at 500 μM. Introducing gliadin to Caco-2 cells led to an increase in TG2 levels (Figure 4.7). The gliadin peptide p31-43 is associated with innate immunity in CD (Jabri and others 2005). This peptide has a direct toxic effect on intestinal cells and has been shown to increase calcium mobilization leading to activation of TG2 (Caputo and others 2012).

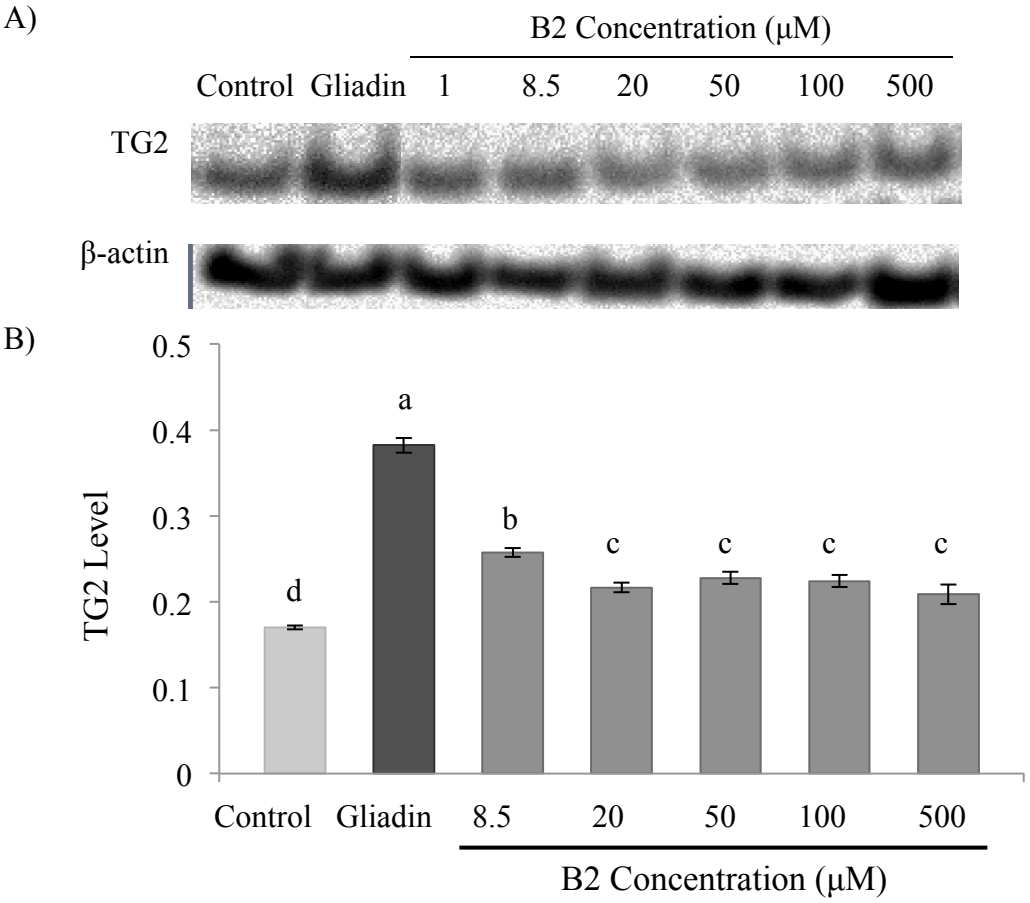

Figure 4.7: TG2 levels in Caco-2 cells exposed to gliadin (20 μg/mL) or medium alone (Control) as resulted by Western blotting. A) Image from WB experiment from one of three exposures. B) TG2 levels were measured by mean densitometric value ± standard deviation of TG2 bands, normalized for corresponding actin density (ratio of TG2:actin). Letters abcd are significantly different ($p<0.05$)

All cells were incubated with 20 μg/mL of gliadin (except for the control) and significant decreases in TG2 started as low as 8.5 μM procyanidin-B2. However, increasing the concentration of procyanidin-B2 did not show decreased TG2 levels after 20 μM. Innate and

adaptive immunity are thought to have a synergistic effect in the inflammatory response in CD, and TG2 is involved in both cases thus playing a crucial role in this disease. Cocoa extract standardized to procyanidin-B2 was effective at inhibiting TG2 levels as low as 8.5 μM in both IFN and gliadin (p31-43) treated cells. Higher concentrations of procyanidin-B2 were more effective in the IFN treated cells, and 500 μM exhibited the greatest TG2 inhibition. This effect was not seen in the gliadin treated cells, though all concentrations did significantly decrease TG2 levels.

4.3.1 TG2 Inhibition by Caffeine and Theobromine

Procyanidins are exogenous antioxidants that have been known to have hormetic effects. This "double-edged" effect describes a compound that induces biologically opposite effects at different doses, most commonly a beneficial effect at low, physiological doses and pro-inflammatory effects at high doses (Calabrese and others 2007). It is marked by the "U-shaped" dose-response curve seen below in Figure 4.8. Hormesis has been well documented both *in vitro* and *in vivo* (Bouayed and Bohn 2010; Watjen and others 2005; Omenn and others 1996; Podmore and others 1998).

Physiological concentrations of procyanidin dimers in chocolate products are around 36 μM or 0.021 mg/g as found by Cooper and others (2007). Hormesis is seen when compounds go above physiological concentrations, and the increase in levels of pro-inflammatory biomarkers seen in cocoa extracts at 50 μM procyanidin-B2 is seen repeatedly in this study and is therefore indicative of a hormetic effect. However at 500 μM procyanidin-B2 in cocoa extracts, there is again significant inhibition of inflammatory biomarkers (including TG2), which may point to a synergistic effect with caffeine and theobromine occurring at this concentration.

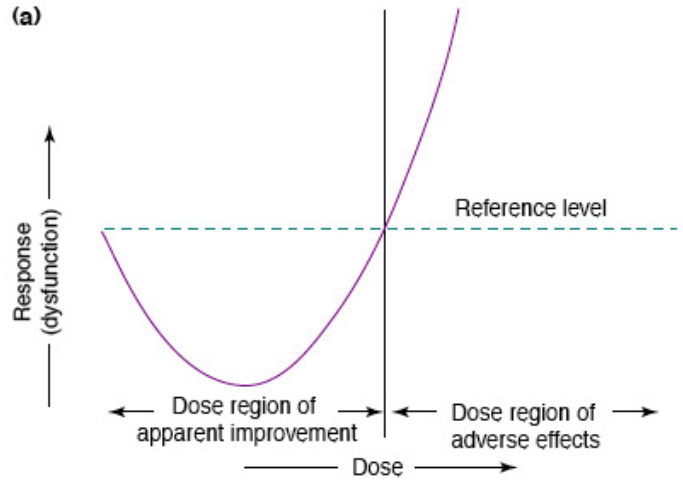

Figure 4.8: U-shaped dose-response curve observed in hormesis (Calabrese and Baldwin 2001)

Caffeine and theobromine have low molecular weights compared to procyanidin-B2, making their physiological doses much higher. A high-phenolic chocolate product may contain about 1 mg/g caffeine (5 mM) and 5 mg/g theobromine (27 mM), therefore the cocoa extracts with higher procyanidin-B2 content are closer to the concentrations these compounds are normally consumed (Sarria and others 2015).

Figure 4.9 shows the result of incubating IFN- and gliadin-treated cells with caffeine or theobromine alone at two different concentrations. The lower concentration (50 μM) is close to what would be expected with the cocoa extracts standardized to 8.5-20 μM procyanidin-B2, and the higher concentration (500 μM) is close to what would be seen with cocoa extracts containing 500 μM procyanidin-B2.

Caco-2 cells incubated with IFN showed increased TG2 inhibition with increased concentrations of caffeine and theobromine. As seen in the WB for TG2 with cells incubated with gliadin, higher concentrations of procyanidin-B2 did not show an increased effect on TG2 inhibition, which is again demonstrated here with caffeine and theobromine. It can be concluded

that the hormetic effect is therefore more apparent when gliadin induces inflammation in Caco-2 cells rather than IFN.

At 50 μM caffeine the relative TG2 level in Caco-2 cells was 37% compared to IFN alone and 35% compared to gliadin alone. This means that in the presence of 50 μM caffeine, TG2 levels decreased 63% and 65%, respectively. At 50 μM theobromine the relative TG2 level was 44% compared to IFN alone and 16% compared to gliadin alone, resulting a in 56% and 84% decrease in TG2 levels respectively. These were pure compounds and their effects were slightly decreased in the cocoa extracts, though the results from WB for TG2 shows that inhibition was still achieved and is attributed to these compounds.

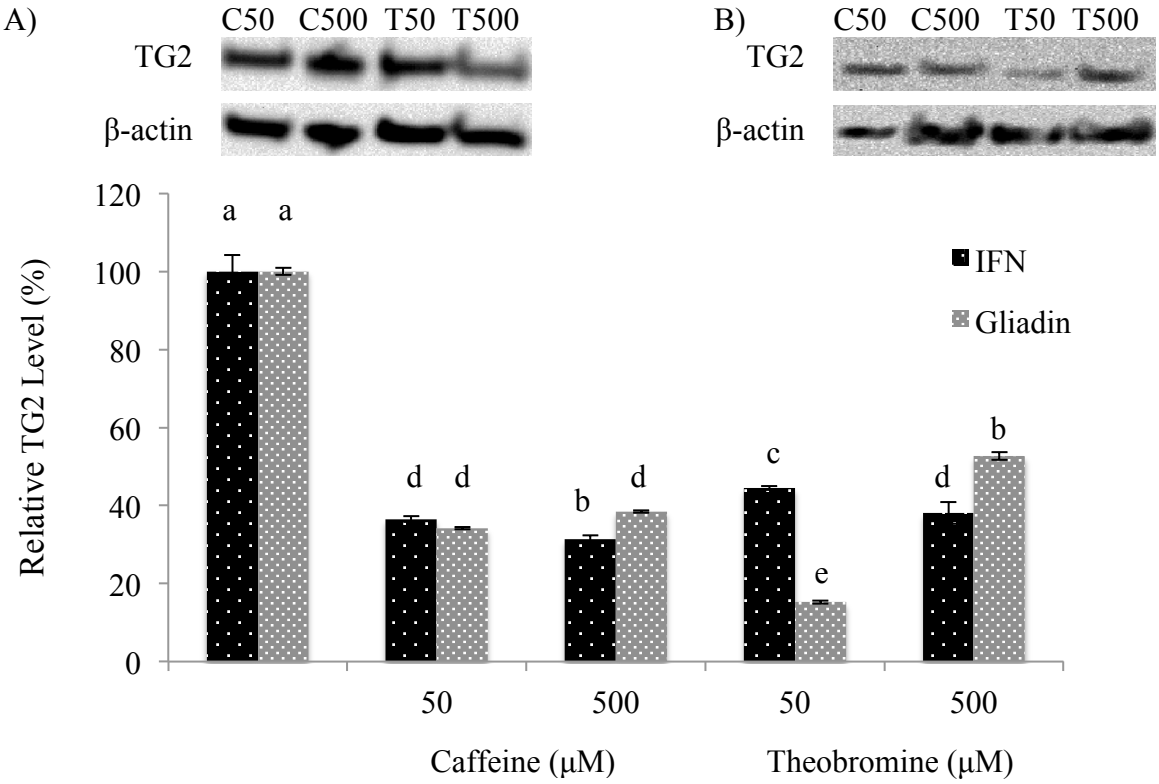

Figure 4.9: TG2 inhibition by Caffeine and Theobromine as resulted by Western blotting in A) IFN- and B) gliadin-treated cells. TG2 levels are expressed relative to gliadin or IFN (100%). The figures represent mean densitometric value ± standard deviation of TG2 bands, normalized for corresponding actin density. Letters abcde are significantly different ($p<0.05$) C = Caffeine T = Theobromine

Alone, 500 μM caffeine resulted in a relative TG2 level of 32% compared to IFN alone and 38% compared to gliadin alone, which means that TG2 was reduced by 68% in IFN-treated cells and 62% in gliadin-treated cells. As seen in Figure 4.9, 500 μM theobromine alone exhibited 38% relative TG2 level compared to IFN alone and 53% compared to gliadin alone. The decreases in TG2 levels are therefore 62% and 47% in IFN- and gliadin-treated cells, respectively. A synergistic effect is seen when compounds increase each other's effectiveness in a matrix. This can be observed in cocoa extracts containing 500 μM procyanidin-B2 where caffeine and theobromine are closer to their physiological concentrations. When the cocoa contained high concentrations of theobromine and caffeine along with procyanidin-B2, the reduction in relative TG2 level was greatest (Figure 4.10). This synergism of caffeine and theobromine explains why cocoa extracts containing 500 μM procyanidin-B2 displayed greater anti-inflammatory effects than lower concentrations, and the effect is seen several times throughout the study.

4.3.2 Comparison of Cocoa Extracts to Cysteamine

There are several known inhibitors of TG2, cystamine (and its derivative, cysteamine) being one of the most effective. Cystamine (β,β'-diaminodietyl disulfide) inhibits TG by blocking this enzyme at its active site, which is a cysteine thiol residue. Gliadin peptides contain repetitive glutamine sequences, making them a preferred substrate for TG2, whose main function is to catalyze the cross-linking of glutamine residues by transferring an acyl group. This action creates immunotoxic gliadin peptides that bind to a lysine residue on glutamine-acceptor proteins such as HLA-DQ2, which as previously mentioned elicits the inflammatory response in T-cells in CD (Ferretti and others 2012).

Cystamine is an irreversible TG inhibitor whose mechanism of action is a thiol-disulfide exchange that inactivates TG at its active site (a cysteinyl residue) (Jeitner and others 2005). While cystamine is a more potent inducer of TG2, intracellular conditions reduce cystamine to cysteamine (β-mercaptoethylamine) therefore it is more appropriate to study this form to ensure the exact concentration of the inhibitor in the cytoplasm. The inhibitory action of cysteamine on TG2 has been demonstrated both *in vitro* and *in vivo* (Jeon and others 2005).

Cysteamine was tested in concentrations of 250 μM and 500 μM for both gliadin and IFN treated cells and showed a decrease in levels of TG2 at both concentrations (Figure 4.10). Compared to gliadin (20 μg/mL) alone, incubation with 250 μM and 500 μM cysteamine resulted in relative TG2 levels of 49.1% and 35.9%, respectively. This means there was a 50.9% decrease in TG2 levels at 250 μM and a 64.1% decrease at 500 μM cysteamine.

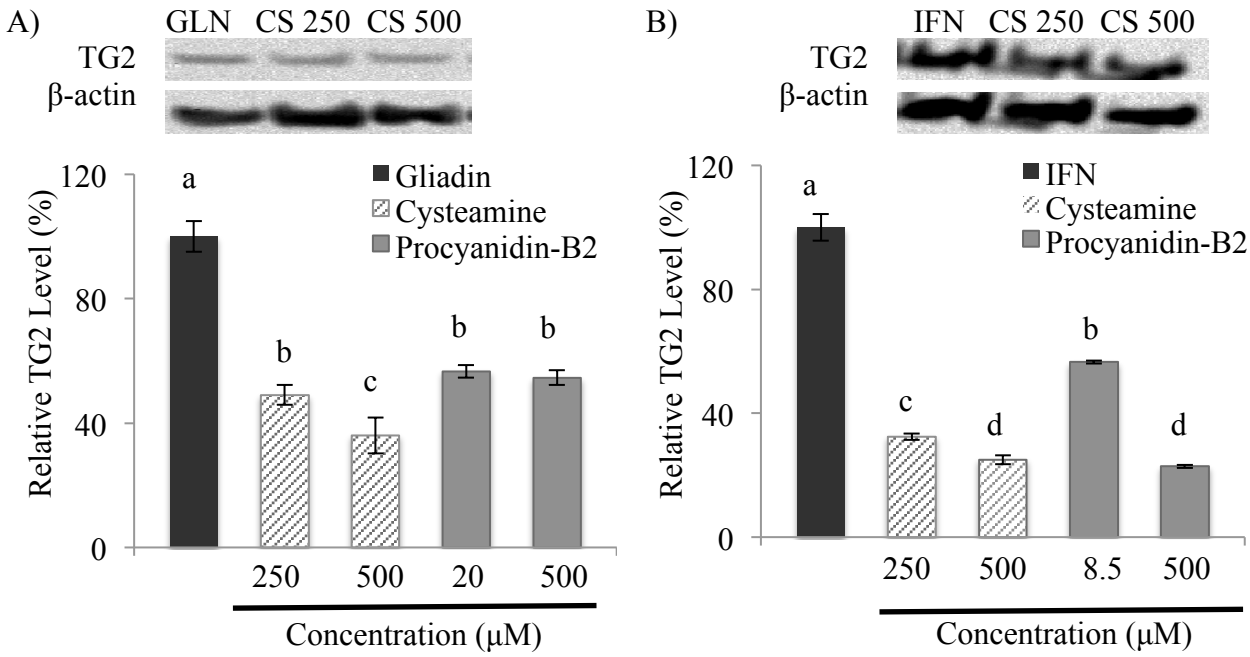

Figure 4.10: Comparison of TG2 inhibition between cysteamine and procyanidin-B2 (cocoa extracts) as resulted by Western blotting in A) gliadin-treated cells and B) IFN-treated cells. TG2 levels are expressed relative to gliadin or IFN (100%). The figures represent mean densitometric value ± standard deviation of TG2 bands, normalized for corresponding actin density. Letters abcd are significantly different ($p<0.05$) CS=Cysteamine

Cysteamine had even greater effects in cells induced by IFN; 250 μM expressed a relative TG2 level of 32.4% and 500 μM had a relative TG2 level by 25%, meaning the decreases in TG2 level by 250 and 500 μM cysteamine were 67.6% and 75% respectively. The results of cysteamine were compared to the cocoa extracts by using the concentrations in both gliadin- and IFN-treated cells that resulted in the greatest TG2 inhibition. For gliadin-treated cells these concentrations were 20 μM and 500 μM procyanidin-B2, which had relative TG2 levels of 56.6% and 54.6% respectively. Therefore compared to gliadin alone, TG2 levels decreased 43.4% and 45.4% which was comparable to the effects of 250 μM cysteamine. In Caco-2 cells incubated with IFN (10 ng/mL) and cocoa extracts, TG2 inhibition was greatest at 8.5 μM and 500 μM procyanidin-B2. Again, Figure 4.10 shows that the relative TG2 level at these concentrations compared to IFN alone was 55.7% and 22.8% respectively. Therefore TG2 levels decreased 44.3% and 77.2%, respectively with cocoa extracts compared to IFN alone. Statistical analysis showed that the effects of 500 μM procyanidin-B2 in the cocoa extracts were comparable to 500 μM cysteamine on TG2 inhibition.

In summary, both gliadin- and IFN-induced cells that contained 500 μM procyanidin-B2 exhibited the greatest decrease in TG2 levels for cells treated with cocoa extracts (45.4% and 77.2%, respectively). The results from cocoa extracts at 500 μM procyanidin-B2 and was comparable to cysteamine, which is a known inhibitor of TG2 and decreased TG2 levels up to 64.1% compared to gliadin alone and 75% compared to IFN alone. The cocoa extracts may exhibit a synergistic effect with caffeine and theobromine at this concentration, whereas lower concentrations may be the action of procyanidin-B2 alone.

In CD, TG2 inhibition could reduce the inflammatory response to gliadin thus preventing damage to intestinal mucosa and protecting against the major symptoms. With the central role

that TG2 plays in disease pathogenesis, these results show the potential of highly concentrated cocoa extracts as an alternative therapy in CD.

4.4 Effect of Cocoa Extracts on CD Inflammatory Biomarkers

To further analyze the ability of cocoa extracts to attenuate gluten related toxicity in CD, other important markers of inflammation were also assessed. Both cyclooxygenase-2 and IL-15 are expressed early in CD pathogenesis and therefore have crucial roles in the innate immune response (Capozzi and others 2013; Barone and others 2011). The pro-inflammatory actions and oxidative stress caused by IFN and TG2 in enterocytes also contribute to elevated levels of these biomarkers (Ferretti and others 2012). The role these cytokines have in CD pathogenesis is summarized in Figure 4.11. To further analyze the potential of cocoa as an alternative therapy in CD, COX-2 levels in IFN and gliadin-treated Caco-2 cells were assessed by WB and IL-15 was analyzed by ELISA.

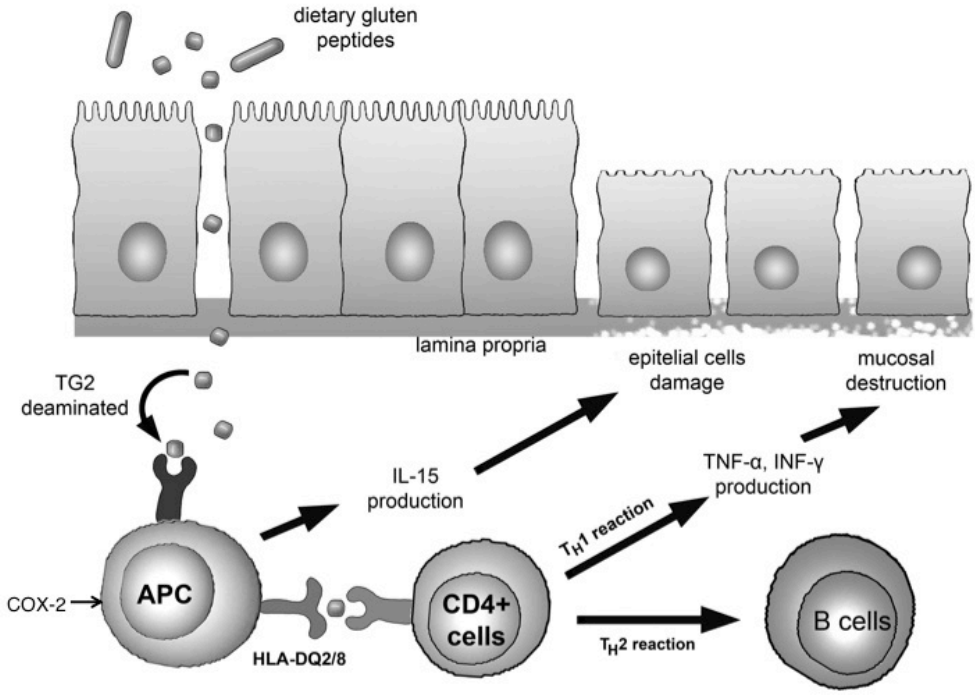

Figure 4.11: Schematic representation of CD pathogenesis (Torres and others 2015)

4.4.1 Cyclooxygenase-2 (COX-2)

The rapid enzymatic activity of COX-2 promotes inflammation through prostaglandins and is activated by stimuli such as gliadin. Maiuri and others (2003) demonstrated the ability of gliadin peptides to increase levels of COX-2 in the duodenum of CD patients, showing the role of this enzyme in innate immunity. The effect of IFN on COX-2 levels in Caco-2 cells captures the adaptive immune response (Figure 4.12).

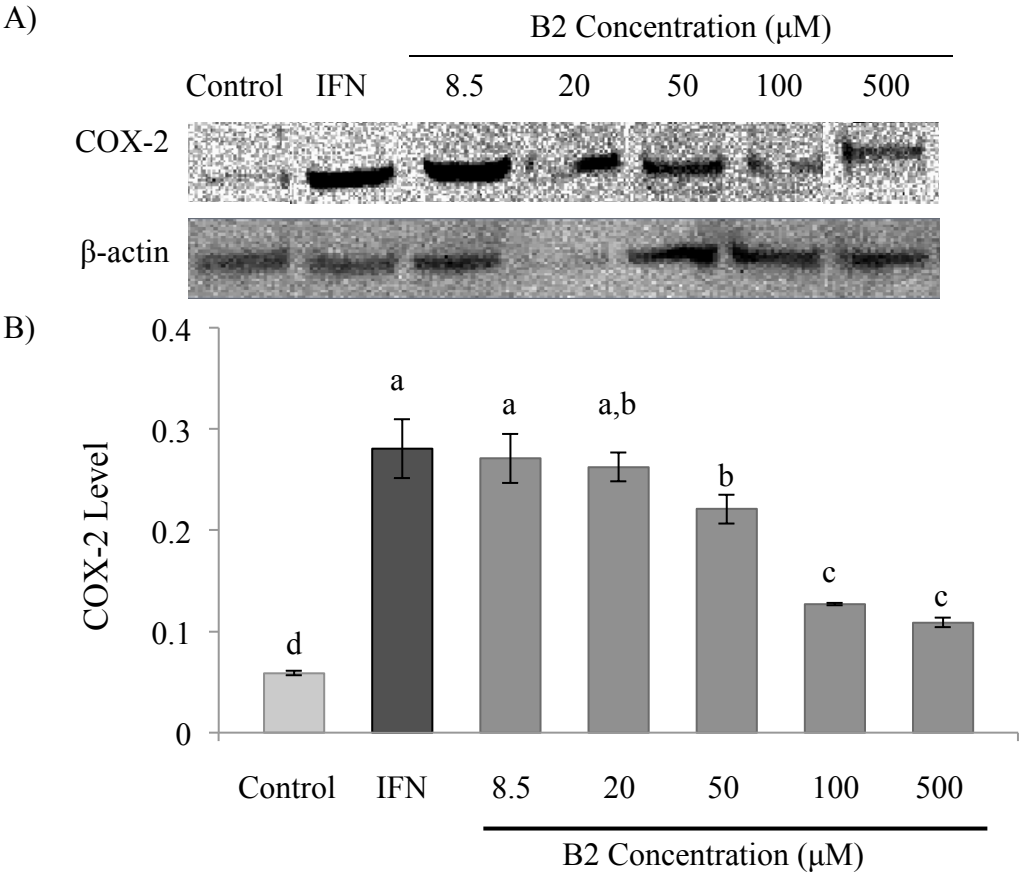

Figure 4.12: COX-2 levels in Caco-2 cells exposed to IFN (10 ng/mL) or medium alone (Control) as resulted by Western blotting. A) Image from WB experiment from one of three exposures. B) COX-2 levels were measured by mean densitometric value ± standard deviation of COX-2 bands, normalized for corresponding actin density (ratio of COX-2:actin). Letters abcd are significantly different ($p<0.05$)

IFN-treated Caco-2 cells saw a significant increase in levels of COX-2. Levels decreased significantly in cells with cocoa extracts containing between 50 μM-500 μM procyanidin-B2.

Below 50 μM was not effective at significantly decreasing levels of COX-2. Cocoa extracts containing the higher concentrations (100 and 500 μM procyanidin-B2) were again more effective at decreasing levels of an inflammatory biomarker to levels closer to the control (media alone). Gliadin (peptide sequence p31-43) also modulates levels of COX-2 in Caco-2 cells (Figure 4.13).

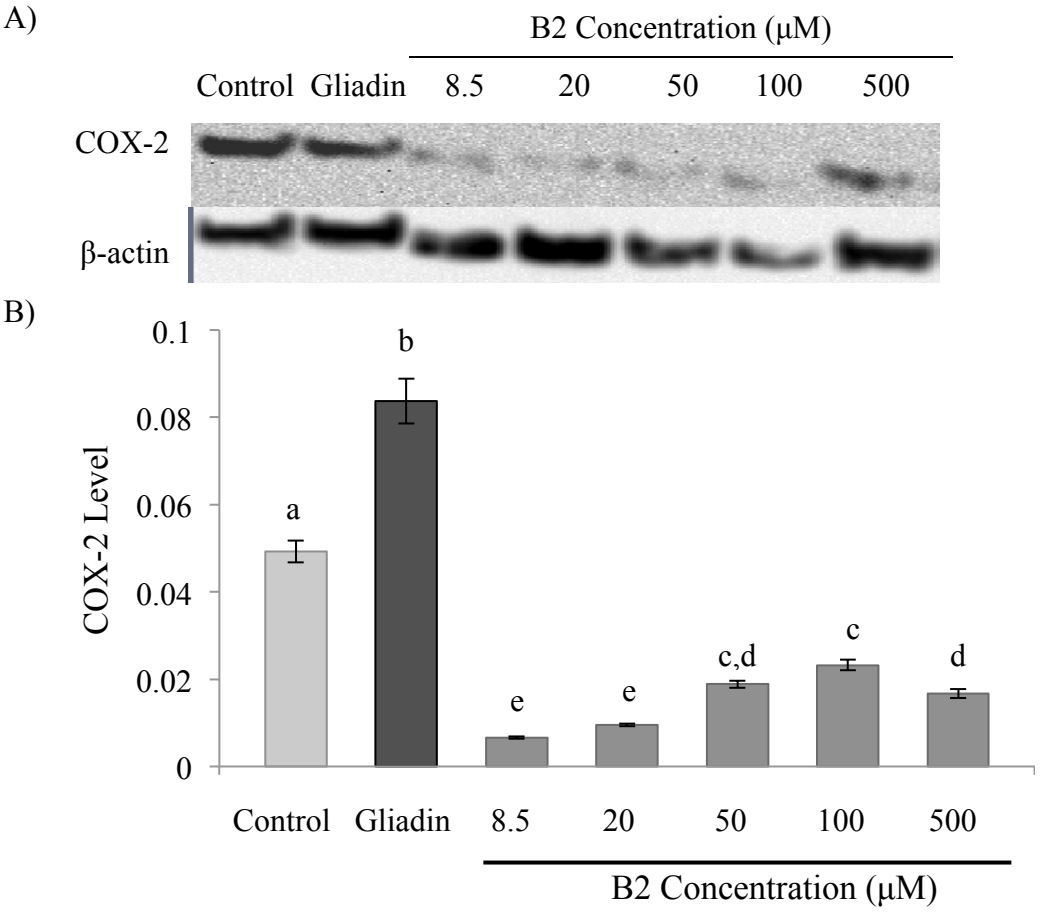

Figure 4.13: COX-2 levels in Caco-2 cells exposed to gliadin (20 μg/mL) or medium alone (Control) as resulted by Western blotting. **A)** Image from WB experiment from one of three exposures. **B)** COX-2 levels were measured by mean densitometric value ± standard deviation of COX-2 bands, normalized for corresponding actin density (ratio of COX-2:actin). Letters abcde are significantly different ($p<0.05$)

Compared to the IFN-induced cells, overall levels of COX-2 were much lower in cells incubated with α-gliadin. Because of its function in the innate (immediate) immune response, the

levels of COX-2 are typically tested after 24 h of treatment rather than the 72 h of incubation that was done for this study. Even so, COX-2 levels were still detectable and was significantly higher in the cells incubated with α-gliadin alone compared to the control and treatments. Treatment with cocoa extracts decreased levels of COX-2, and the greatest decreases were seen at the lower concentrations of 8.5 and 20 μM procyanidin-B2.

4.4.2 Interleukin-15 (IL-15)

IL-15 is a cytokine that, like TG2, plays a central role in CD pathogenesis. While TG2 captures the adaptive immune response in CD, IL-15 dominates innate immunity (Vincentini and others 2015). Rather than being secreted by cells, this cytokine is present at the cell surface and functions by stimulating intraepithelial lymphocytes (IELs), which are mediators of cytotoxicity in epithelial cells. IL-15 also induces T-cell proliferation and levels of this cytokine correlate to the degree of mucosal damage in CD (Di Sabatino and others 2006). When IL-15 is blocked cytotoxicity in Caco-2 cells is reduced, making inhibition of this cytokine an important factor to preventing CD inflammation.

IL-15 was significantly higher in cells treated with IFN alone (Figure 4.14). Incubating with cocoa extracts decreased levels of IL-15 similar to that seen in the control (medium alone), though the effect was not dependent on procyanidin-B2 concentration. The α-gliadin (p31-43) sequence directly induces IL-15 production and significantly increased levels of IL-15 in Caco-2 cells compared to the control (Figure 4.15). Unlike the IFN treated cells, cocoa extracts had different effects with varying procyanidin-B2 concentration. Concentrations of at least 50 μM procyanidin-B2 were required to achieve inhibition of IL-15.

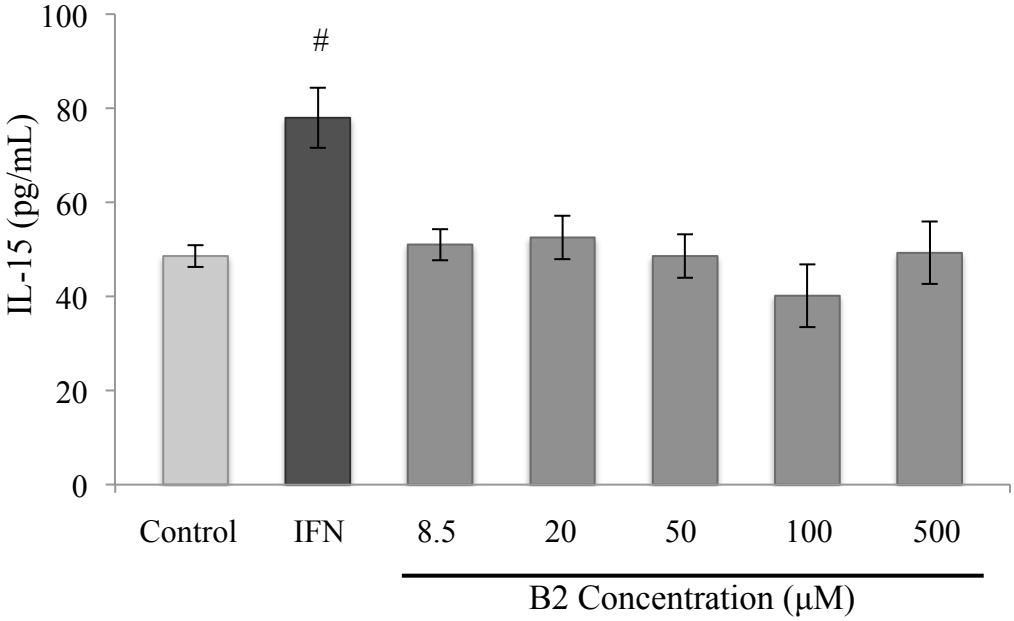

Figure 4.14: Levels of IL-15 in Caco-2 cells incubated with IFN (10 ng/mL) or medium alone (Control) as resulted by ELISA. # denotes significantly different ($p<0.05$)

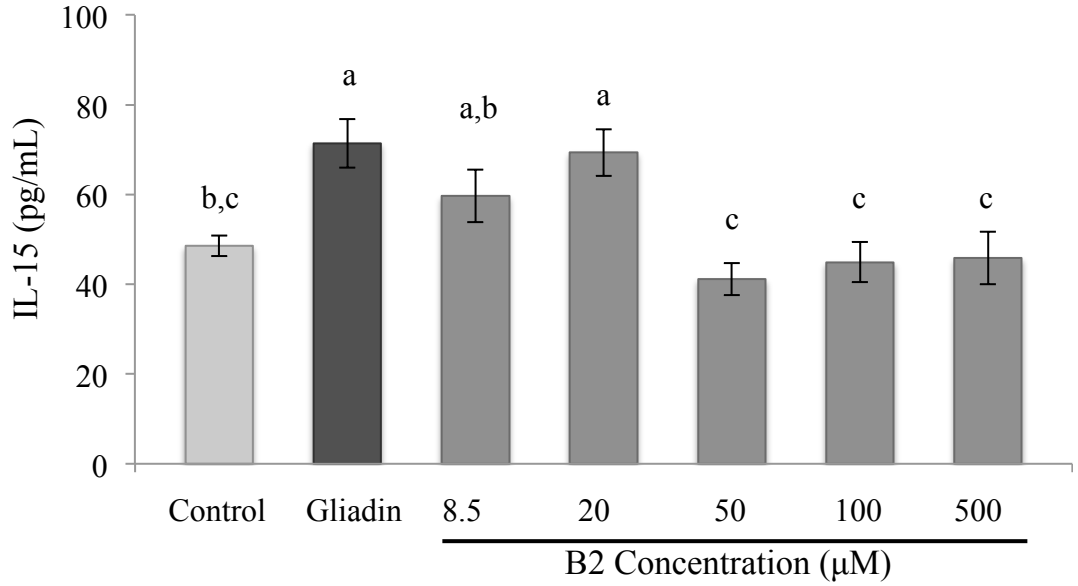

Figure 4.15: Levels of IL-15 in Caco-2 cells incubated with gliadin (20 μg/mL) or medium alone (Control) as resulted by ELISA. Letters abc are significantly different ($p<0.05$)

Blocking expression of IL-15 could be protective against the toxicity of gliadin by preventing tissue damage. These findings suggest that highly concentrated cocoa extracts are

effective in decreasing IL-15 levels. Due to its pivotal role in CD pathogenesis, inhibiting IL-15 as well as TG2 and COX-2 could be an effective therapy and using a safe treatment like a cocoa product has great potential.

4.5 Effect of Cocoa Extracts on Serum Cytokines

Both gliadin and IFN are responsible for the secretion of inflammatory cytokines in CD. Cytokine profiles can vary depending on the level of enteropathy and compliance to a GFD, although IL-6 and IL-8 are two of the most common serum cytokines elevated in CD (Kapoor and others 2013). Elevated IL-1β is associated with individuals with refractory CD, or those who do not respond to a GFD. Positive correlations have been found between the levels of TG2 and serum cytokines (Manavalan and others 2010). The elevation of TG2 in response to IFN and gliadin has been demonstrated through Western blotting, and increased levels of IL-6, IL-8 and IL-1β were expected. Enzyme-linked immunosorbent assay (ELISA) analysis of serum is commonly done in CD diagnosis and is also used to monitor dietary compliance; in this study we investigated whether treatment with cocoa extracts was effective in decreasing levels of common CD cytokines.

4.5.1 Interleukin-6 (IL-6)

The inflammatory cytokine IL-6 plays a central role in immune responses, and evidence of increased serum IL-6 in untreated CD has been well documented (Romaldini and others 2002, O'Keeffe and others 1999). It is a macrophage-derived cytokine and increases production of other inflammatory cytokines by up-regulating T-cell functions. Serological testing for IL-6 is a reliable, non-invasive measure to assess adherence to a GFD in CD, as when gluten is introduced into the diet there is a significant increase in the levels of IL-6. A significant correlation exists between IL-6 and tissue Transglutaminase (tTG) in CD patients (Kapoor and others 2013). Due

to this correlation the pattern seen in Western blotting for TG2 is expected to be similar to levels of IL-6 in Caco-2 cell supernatants in response to treatment with cocoa extracts.

IFN significantly increased the levels of IL-6 (Figure 4.16). IFN-γ is known to greatly increase production of IL-6, therefore these high levels were expected (Biondillo and others 1994). In Caco-2 cells, cocoa extracts with concentrations containing at least 8.5 μM procyanidin-B2 significantly reduced levels of IL-6. The greatest decrease in IL-6 was seen in cells with cocoa extracts containing 500 μM procyanidin-B2, which was similar to what was seen in Western blotting for TG2.

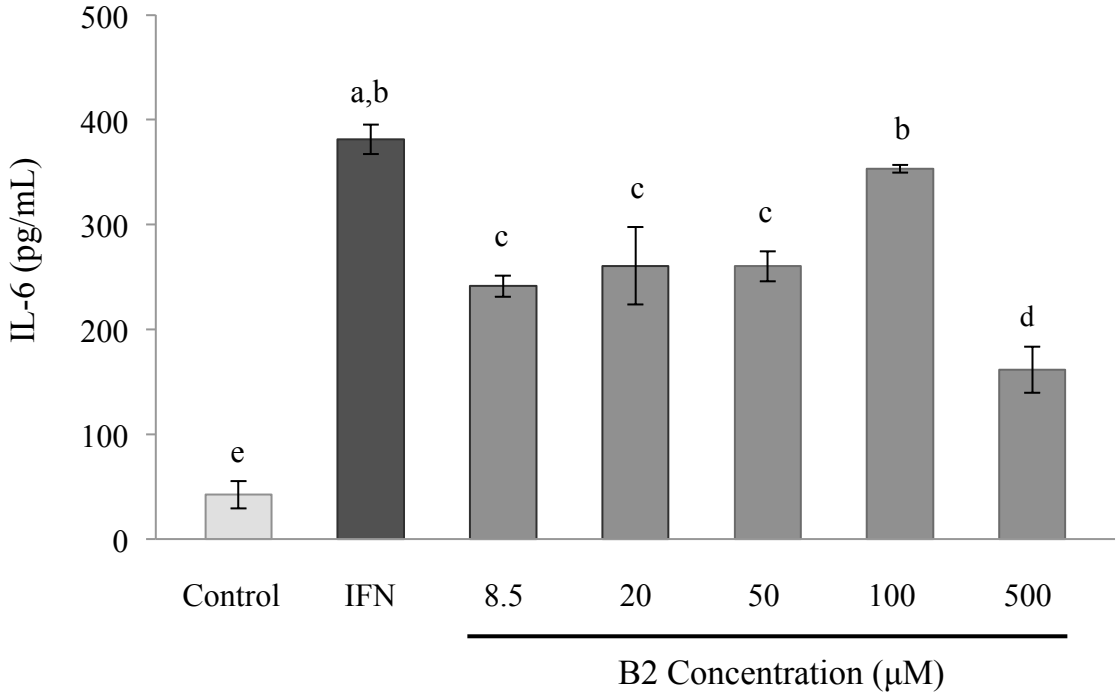

Figure 4.16: Levels of IL-6 in Caco-2 cells incubated with IFN (10 ng/mL) or medium alone (Control) as resulted by ELISA. Letters abcde are significantly different ($p<0.05$)

Gliadin significantly increased levels of IL-6 in Caco-2 cells (Figure 4.17). Treatment with cocoa extracts does not appear to result in lower levels of IL-6, since these differences are not significant. Though the amount of IL-6 is much lower than those seen in the IFN-induced cells, these levels are closer to what is seen in literature. Newly diagnosed CD patients not on a

GFD have between 12-28 pg/mL IL-6, though levels are higher (80-120 pg/mL) in patients who follow a GFD but still experience persistent symptoms, also called refractory CD (Kapoor and others 2013).

The results from IL-6 ELISA follow patterns similar to results from TG2 Western blotting for IFN-treated cells. This is not unexpected, as there is sufficient evidence of the correlation between TG2 and serum cytokines such as IL-6. Cells treated with gliadin did not show significant decreases of IL-6 when incubated with any concentration of procyanidin-B2. The findings suggest that adaptive immunity (response captured by IFN) may therefore be a more effective target for treatment using cocoa extracts.

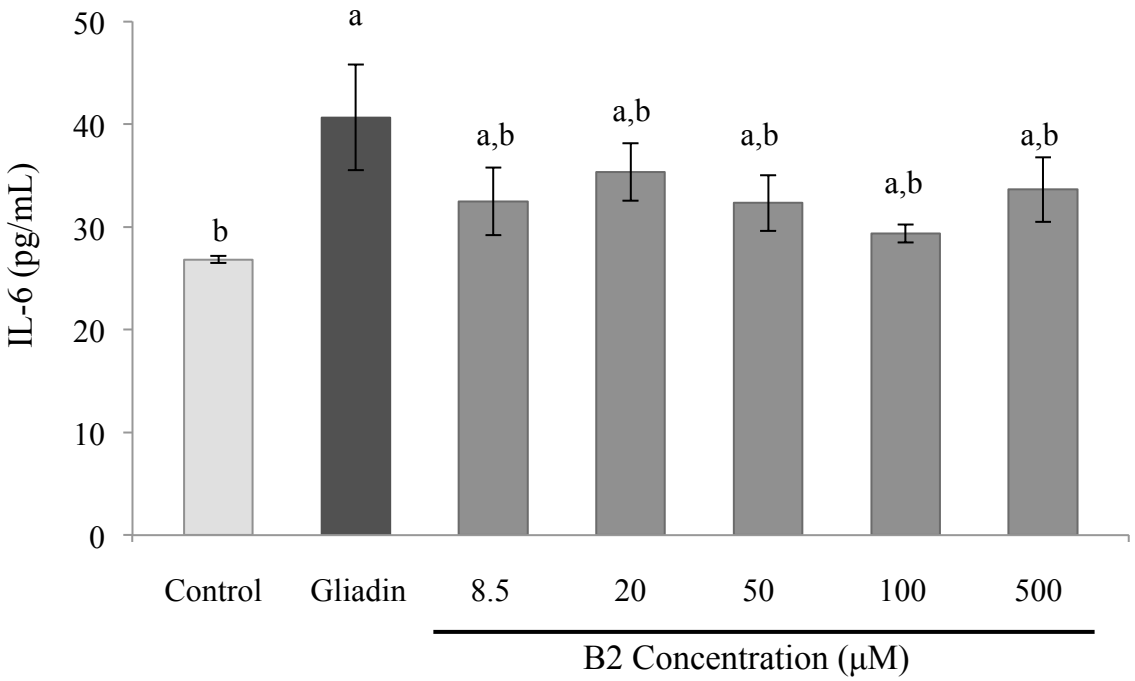

Figure 4.17: Levels of IL-6 in Caco-2 cells incubated with gliadin (20 μg/mL) or medium alone (Control) as resulted by ELISA. Letters ab are significantly different ($p<0.05$)

IFN-induced production of IL-6 is seen in many types of cells, therefore the results from the gliadin-treated cells may be more specific to CD. IL-6 is an important biomarker in CD due

its common use in serological testing and diagnosis, and since IFN is a part of the adaptive immune response in CD the data from both gliadin and IFN treated cells are relevant.

4.5.2 Interleukin-8 (IL-8)

The inflammatory cytokine IL-8 is overexpressed in response to gluten in CD patients and is also expressed in stressed Caco-2 cells (Hall and others 2007). Similar to IL-6, when serum IL-8 levels are elevated and influence the production of other inflammatory cytokines through modulation of T-cell functions.

Treating Caco-2 cells with IFN significantly increased levels of IL-8 (Figure 4.18). Cocoa extracts containing procyanidin-B2 decreased levels of IL-8 starting at concentrations of 8.5 μM. Concentrations ranging from 50-500 μM were not significantly different from the control levels of IL-8 (pg/mL).

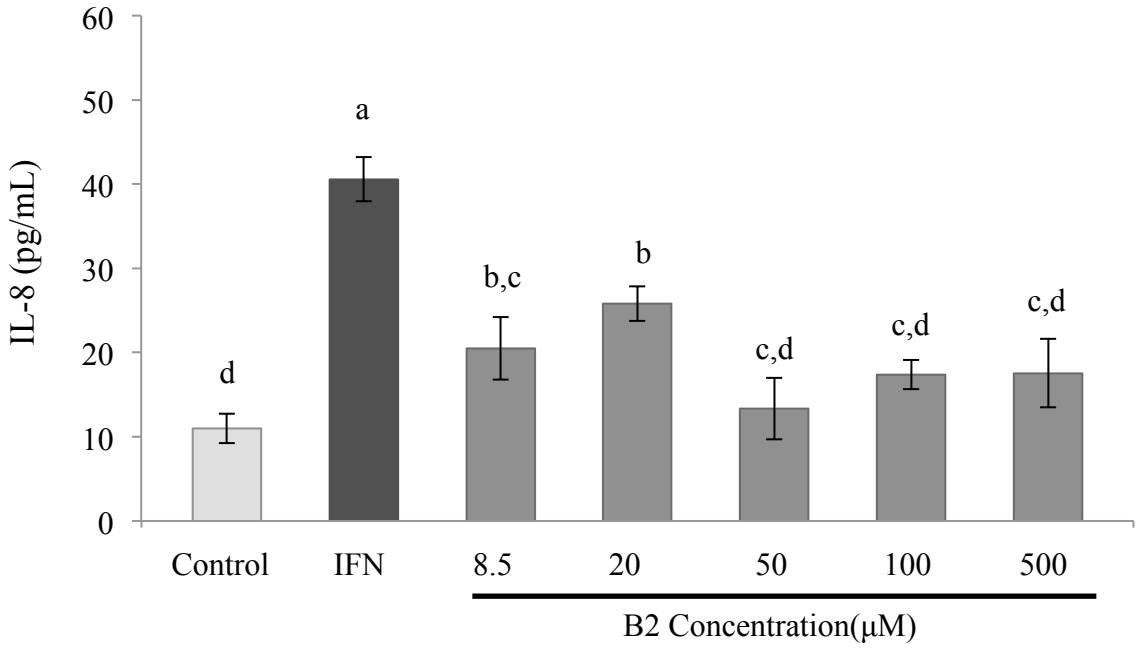

Figure 4.18: Levels of IL-8 in Caco-2 cells incubated with IFN (10 ng/mL) or medium alone (Control) as resulted by ELISA. Letters abcd are significantly different ($p<0.05$)

IL-8 was elevated in higher levels in Caco-2 cell supernatants treated with gliadin (p31-43) (Figure 4.19). Treatment with cocoa extracts decreased IL-8 levels starting at 8.5 μM procyanidin-B2 and the most effective treatment was 500 μM, which resulted in levels closest to the control. Similar to what was seen in previous experiments for the cells incubated with gliadin, treatment with cocoa extracts did not vary greatly among different concentrations.

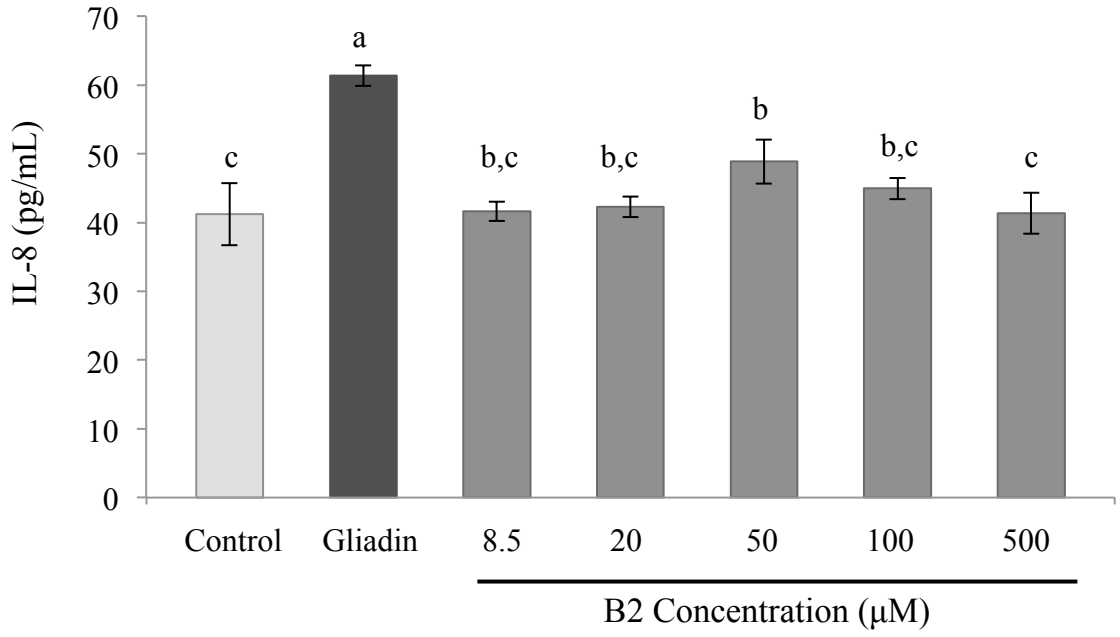

Figure 4.19: Levels of IL-8 in Caco-2 cells incubated with gliadin (20 μg/mL) or medium alone (Control) as resulted by ELISA. Letters abc are significantly different ($p<0.05$)

The results from IL-8 ELISA are in agreement to the other analyses of CD biomarkers. Levels of IL-8 were not dependent on concentration of procyanidin-B2 in IFN-treated cells, though all treatments did show significant decreases. Similar to analysis of TG2 levels in gliadin-treated Caco-2 cells, incubating with cocoa extracts still resulted in significantly decreased levels of the IL-8, indicating that there is some potential in modulating inflammation in innate immunity.

4.5.3 Interleukin-1β (IL-1β)

The cytokine IL-1β is an important mediator in intestinal inflammation and is increased in the serum of those with refractory CD (Andersen and others 2013; Manavalan and others 2010). Though the cytokines IL-6 and IL-8 are more commonly used for CD diagnostic criteria, IL-1β has been shown to increase tight junction permeability in Caco-2 cells and cause an increased expression of other CD biomarkers including COX-2 (Al-Sadi and Ma 2007; Neeb and others 2011).

A significant increase in the levels of IL-1β found between cells was found treated with IFN compared to medium alone (Figure 4.20). Cocoa extracts containing 8.5 and 50 μM B2 did express the highest levels for IFN-treated cells, though the concentrations are may not be considered elevated since these levels (up to 20 pg/mL IL-1β) have been seen control subjects without CD (Manavalan and others 2010). Although the levels of IL-1β were very low across all treatments, incubating with the higher concentrations 100 and 500 μM B2 appeared to show significant decreases in IL-1β compared to the other concentrations tested in cocoa extracts.

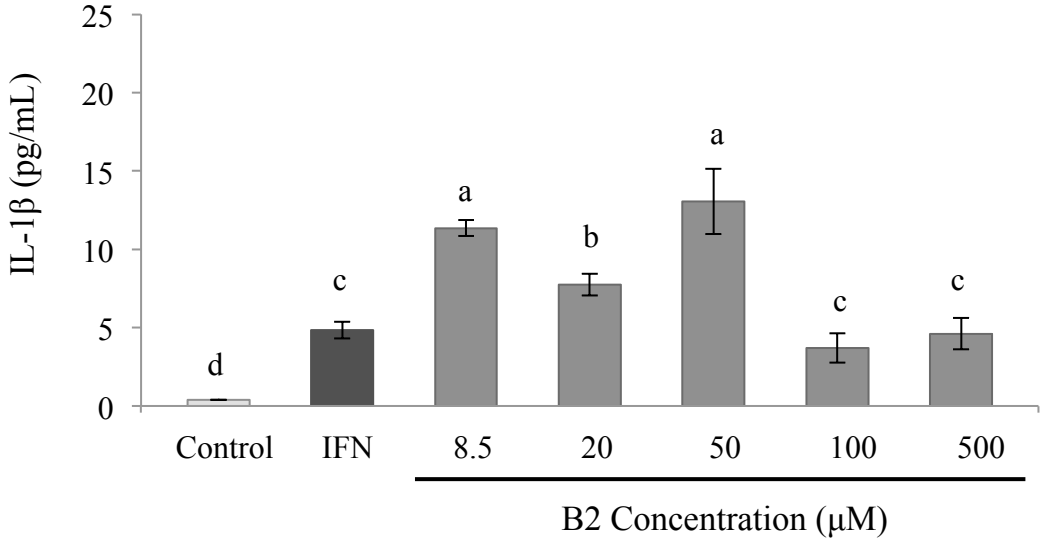

Figure 2.20: Levels of IL-1β in Caco-2 cells incubated with IFN (10 μg/mL) or medium alone (Control) as resulted by ELISA. # denotes concentrations found to be significantly different ($p<0.05$)

Treatment with gliadin (p31-43) significantly elevated levels of IL-1β in cell supernatants (Figure 4.21). Decreases in IL-1β were seen in cells treated with cocoa extracts containing at least 8.5 μM procyanidin-B2. As stated earlier, IL-1β induces COX-2. The response to incubation with gliadin plus cocoa extracts shown here is similar to the pattern seen in the WB for COX-2 levels, with 8.5 and 20 μM procyanidin-B2 resulting in greater inhibition than higher concentrations. COX-2 and therefore IL-1β has a larger role in innate immunity, which can explain why IL-1β showed a greater response to gliadin rather than IFN. Treatment with cocoa extracts was able to significantly affect the inflammatory response to gliadin by decreasing levels of both IL-1β and COX-2, two biomarkers along with IL-15 that capture innate immunity in CD.

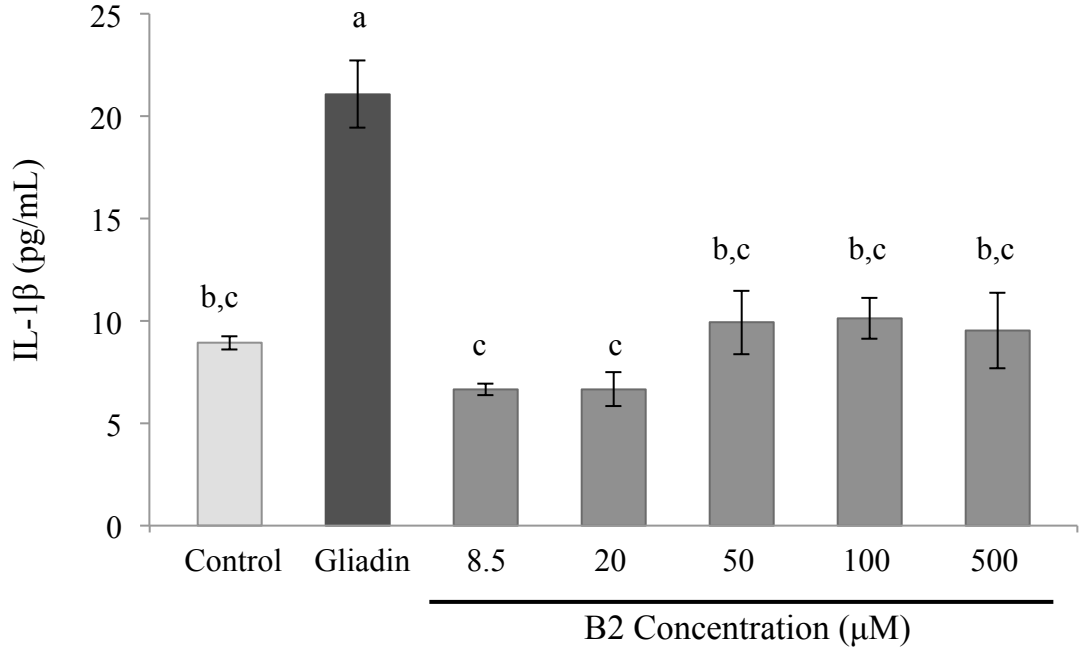

Figure 4.21: Levels of IL-1β in Caco-2 cells incubated with gliadin (20 μg/mL) or medium alone (Control) as resulted by ELISA. Letters abc are significantly different ($p<0.05$)

CHAPTER 5: SUMMARY AND CONCLUSIONS

This research demonstrates the potential of bioactive-rich foods to inhibit TG2-induced inflammation in enterocytes. A treatment using highly concentrated cocoa extracts could mitigate gluten toxicity and reduce inflammation in CD, presenting a possible alternative to a gluten-free diet, which places a large economic burden on individuals with CD stressing the need for additional therapies. This was a proof-of-concept study that requires further investigation into its effects on preventing inflammation and subsequent villous atrophy in CD.

The major finding of this study is that cocoa extracts containing high concentrations of procyanidin-B2, caffeine and theobromine were able to reduce levels of CD biomarkers in models of both innate and adaptive immunity. Incubating Caco-2 cells with IFN-γ or α-gliadin increased levels of TG2 as well as two markers prominent in innate immunity IL-15 and COX-2. Dose dependent effects were observed, though in each case cocoa extracts containing 500 μM procyanidin-B2 significantly decreased levels of these biomarkers. Both gliadin- and IFN-induced Caco-2 cells that contained 500 μM procyanidin-B2 exhibited the greatest decrease in TG2 levels for cells treated with cocoa extracts (45.4% and 77.2%, respectively). Reductions in the serum cytokines IL-6, IL-8 and IL-1β were also observed, which confirms the decreased levels of these biomarkers since a direct relationship exists between them.

The anti-inflammatory effects of cocoa extracts have been demonstrated in an *in vitro* model of CD. Studying these effects *in vivo* will further support the potential of these dietary inhibitors of TG2, an enzyme with a central role in pathogenesis of this disease.

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