

## ABSTRACT

STORY, ERICA NICOLE. The Effects of Green Tea and Epigallocatechin-3-gallate on the Growth of Probiotic Bacteria and LPS-Induced Inflammation in Differentiated Human Colon Cancer (HT-29) Cells. (Under the direction of Dr. Gabriel Keith Harris.)

Cancer is the second leading cause of death in the United States. Colon cancer was the third leading cause of U.S. cancer incidence and mortality in 2010. Chronic inflammation and diet have been linked with colon cancer incidence. Green tea (GT), epigallocatechin-3-gallate (EGCG) and probiotic bacteria have been reported to demonstrate anti-inflammatory activities, indicating that their increased consumption may modulate inflammatory responses. We studied the effects of GT and EGCG on probiotic bacteria growth and on inflammatory markers in a colon cancer cell line *in vitro*.

The objective of the first study was to determine the effect of GT and EGCG on the growth rate and total growth of the probiotic bacteria strains *Lactobacillus acidophilus* NCFM (*L. acidophilus*) and *Lactobacillus gasseri* ATCC 33323 (*L. gasseri*). Concentrations of GT and EGCG enhanced the maximum specific growth rate (MSGR) and increased the apparent cell density of both *L. acidophilus* and *L. gasseri* compared to the control. However, GT and EGCG did not significantly increase cell plate counts for *L. acidophilus* and *L. gasseri* relative to the controls. This study did not support the hypothesis that GT or EGCG function as prebiotics for *L. acidophilus* or *L. gasseri*.

The objective of the second study was to examine the effects of GT and EGCG on the production of cyclooxygenase-2 (COX-2) and prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) in lipopolysaccharide (LPS)-induced, differentiated human colon cancer (HT-29) cells. Cell viability results indicated that the GT and EGCG treatments were not toxic at the levels used. Alkaline phosphatase assay results confirmed that HT-29 cells were differentiated after 24 hours of

co-treatment with sodium butyrate. Increasing concentrations of GT and EGCG showed a trend toward increased PGE<sub>2</sub> and H<sub>2</sub>O<sub>2</sub> formation and the highest concentration of GT and EGCG (0.045 mg/ml) were significantly different from the control. H<sub>2</sub>O<sub>2</sub> formation was proposed as a possible mechanism for PGE<sub>2</sub> production. Replacing GT and EGCG treatments with corresponding amounts of H<sub>2</sub>O<sub>2</sub> showed a similar trend in the GT/EGCG PGE<sub>2</sub> ELISA results. Catalase treated cells did not show decreased PGE<sub>2</sub> and COX-2 formation, indicating that the observed increases in PGE<sub>2</sub> were not due to a H<sub>2</sub>O<sub>2</sub>-mediated inflammatory response. Additionally, differences in COX-2 expression and activity were not observed, indicating that COX-2 had not caused the observed increase in PGE<sub>2</sub> production. This study highlighted the need to further examine the complex effects of dietary constituents on reactive oxygen species and inflammatory responses.

The objective of the third study was to determine the effects of GT or EGCG on interleukin-8 (IL-8) formation and nuclear factor-κB (NF-κB) expression in LPS-induced, differentiated HT-29 cells. Increasing concentrations of GT and EGCG showed trends toward decreased IL-8 formation, however, only the highest EGCG treatment (0.045 mg/ml) was significant. NF-κB Western blot results were inconclusive. This experiment showed that EGCG, but not GT, demonstrated anti-inflammatory effects on IL-8 formation in this cell line, suggesting potential benefits relative to human colon cancer.

Overall, these results indicated that GT and EGCG may have different effects on health depending on the endpoint measured. GT and EGCG did not increase the growth of probiotic bacteria. GT and EGCG had opposing effects on inflammatory markers in HT-29 cells. Further research is needed to better understand the effects of whole foods versus their individual components.

The Effects of Green Tea and Epigallocatechin-3-gallate on the Growth of Probiotic Bacteria  
and LPS-Induced Inflammation in Differentiated Human Colon Cancer (HT-29) Cells

by  
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## **DEDICATION**

*To Mom and Dad, for always believing in me,*

*and*

*To Bryan, for your continuous encouragement, patience and love.*

## BIOGRAPHY

Erica Story was born in Cleveland, OH. She graduated from Strongsville High School and attended The College of Wooster in Wooster, OH where she graduated with a B.A in Biochemistry and Molecular Biology. She worked at Promerus, LLC for two years before realizing that her dream job was to work in the food industry. As a result, Erica decided to attend the North Carolina State University to pursue her M.S. in Food Science. At NC State, Erica was an active member of the Food Science Club, participating in numerous dairy bars, the wine and cheese committee and serving as Secretary in 2009. Erica was a member of the Product Development team that won 1<sup>st</sup> place in the IFTSA Product Development Competition for their instant smoothie product: *Shiverrs*. Erica took one semester off to participate in a 6 month Co-op at the Campbell Soup Company working on soup product development. This experience reconfirmed her desire to work in the food industry. Following graduation, Erica plans to pursue a career in food product development.

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## LIST OF ABBREVIATIONS

AA (arachidonic acid)  
ALP (alkaline phosphatase)  
AP-1 (activator protein-1)  
BCA (bicinchoninic acid)  
BSA (bovine serum albumin)  
COX (cyclooxygenase)  
cPGES (cytosolic PGE<sub>2</sub> synthase)  
CVD (cardiovascular disease)  
DI (deionized)  
DMEM (Dulbecco's modified eagle's medium)  
EGCG (epigallocatechin-3-gallate)  
ELISA (enzyme linked immuno-sorbent assay)  
FADD (fas-associated death domain)  
FBS (fetal bovine serum)  
GT (green tea)  
H<sub>2</sub>O<sub>2</sub> (hydrogen peroxide)  
HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)  
HPETE (hydroperoxy-6,8,11,14-eicosatetraenoic acid)  
IKK (I $\kappa$ B kinase)  
IL-8 (interleukin-8)  
LOX (lipoxygenase)  
LPS (lipopolysaccharide)  
MAPKKK (mitogen activated protein kinase kinase kinase)  
MD-2 (myeloid differentiation-2)  
mPGES (membrane bound PGE<sub>2</sub> synthase)  
MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenoltetrazolium)  
NaBt (sodium butyrate)  
NFDM (non-fat dry milk)  
NF- $\kappa$ B (nuclear factor kappa B)  
NO (nitric oxide)  
PG (prostaglandin)  
PGE<sub>2</sub> (prostaglandin E<sub>2</sub>)  
PBS (phosphate buffered saline)  
PVDF (polyvinylidene fluoride)  
ROS (reactive oxygen species)  
SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis)  
TBS-T (tris buffered saline with Tween)  
TLR4 (toll-like receptor-4)  
TNF- $\alpha$  (tumor necrosis factor alpha)  
TRADD (TNF-receptor death domain)

TRAF (TNF-receptor associated factor)  
TRAP (total peroxy radical trapping assay)  
TX (thromboxane)

## **CHAPTER 1: Literature Review**

## 1.1 INTRODUCTION

This master's thesis research consists of two separate projects: one on the effect of green tea (GT) and epigallocatechin-3-gallate (EGCG) on the growth rate and total growth of probiotic bacteria (Project 1) and one on the effect of GT and EGCG on lipopolysaccharide (LPS)-induced inflammation in differentiated human colon cancer (HT-29) cells (Project 2). Therefore, the following literature review contains information that is relevant to each project, with some information relevant to both projects.

Global causes of morbidity and mortality have shifted away from infectious diseases to chronic diseases like cardiovascular disease (CVD), cancer, type-2 diabetes and obesity. Chronic diseases are responsible for half the global morbidity and two-thirds of the global mortality. Over 35 million people die every year from chronic diseases (Bengmark 2004). There is strong evidence from epidemiological, prospective cohort and intervention studies that chronic disease incidence is linked to lack of exercise and inappropriate diet, such as consuming too many calorie-dense foods containing saturated fat and sugar and not consuming enough foods high in antioxidants like fruit and vegetables (Bengmark 2004; Roberts and Barnard 2005).

Even though chronic disease is a prevalent cause of death worldwide, it may be prevented through changes to everyday lifestyle like increasing exercise and consuming more antioxidant rich foods. Plant based polyphenols possess antioxidant function and their consumption may provide a way to help prevent chronic disease (Santangelo and others 2007). GT is one such plant that contains a variety of polyphenols and has been shown to possess antioxidant activity (Khan and Mukhtar 2007). There is strong evidence from *in*

*vitro* and animal studies that GT polyphenols have potentially beneficial effects on the pathogenesis of many chronic diseases (Khan and Mukhtar 2007).

GT has been consumed for its attributed health effects for over 50 centuries, but, only in the last three decades have scientific experiments been conducted to confirm the health benefits of GT consumption (Khan and Mukhtar 2007). Tea in the form of green or black tea is the second most consumed beverage worldwide, second only to water (Chen and others 2008). Therefore, if positive health benefits, including a reduction in chronic disease incidence or mortality, could be attributed to GT consumption, the benefits would be widespread.

In addition to studying the health benefits elicited by consuming GT as a whole food, it is also important to study the individual compounds that comprise GT to determine whether the observed health effects are a result of consuming GT as a whole food or from the individual components. Of particular interest is the largest phenolic component of GT, EGCG, which has been shown to be mainly responsible for the observed health benefits of GT (Khan and Mukhtar 2007).

### ***1.1.1 Introduction to Project 1***

Like GT and EGCG, many health benefits have been attributed to the consumption probiotic bacteria. Probiotic bacteria are known to stimulate and regulate numerous immune responses that include the prevention or attenuation of diarrhea, acute gastroenteritis and Crohn's disease. However, the preventative effect of probiotic bacteria against

immunological disorders like cancer, asthma and arthritis has not yet been proven (Gill and Prasad 2008).

Prebiotics are dietary components that help stimulate the growth of probiotic bacteria in the gut (Grizard and Barthomeuf 1999). Previous studies have shown Chinese GT to possess small but not significant prebiotic activity as it slightly stimulated the growth of the probiotic bacteria strain *Lactobacillus rhamnosus* (Molan and others 2009). It is important to test other types of GT, like Japanese GT, to determine whether they have an effect on different types of probiotic *Lactobacillus* spp.

GT and probiotic bacteria have both been associated with positive health benefits separately, but, little evidence exists on the combined health effects of GT and probiotic bacteria. Therefore, it is important to study whether there is an additive effect of the health benefits of GT and probiotic bacteria when consumed together.

The objective of the first project was to determine whether GT and EGCG have an effect on the growth rate or total growth of the probiotic bacteria *Lactobacillus acidophilus* and *Lactobacillus gasseri*. Our hypothesis was that GT and EGCG will cause an increase in growth rate and total growth of the probiotic bacteria *L. acidophilus* and *L. gasseri*. We predicted that the increase in growth will result from hormesis (increased vigor due to a mild stress) or that GT and EGCG will act as a prebiotic.

### ***1.1.2 Introduction to Project 2***

Of the chronic diseases, cancer is one of the leading causes of death in the United States, second only to CVD. Colon cancer was ranked third in new types of cancer

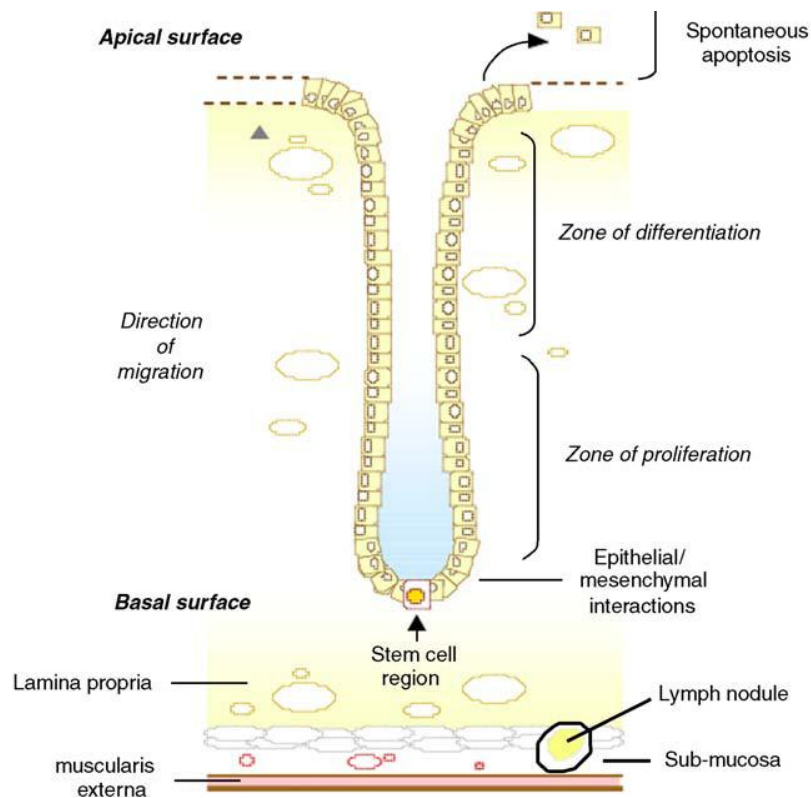
diagnosed and cancer related deaths in 2010 in the U.S. (American Cancer Society 2010). The incidence of cancer has been linked with inflammation and diet (Williams and others 1999). Therefore, it is important to look at compounds known to possess antioxidant and anti-inflammatory properties, like GT and EGCG, as possible ways to help prevent colon cancer.

The objective of the second project was to determine the effect of GT and EGCG on LPS-induced inflammation in differentiated HT-29 cells. Different markers of inflammation associated with colon cancer were measured after treatment with varying concentrations of GT and EGCG. We hypothesized that GT and EGCG will decrease the concentration or expression of the inflammatory markers interleukin-8 (IL-8), nuclear factor- $\kappa$ B (NF- $\kappa$ B), cyclooxygenase-2 (COX-2) and prostaglandin E<sub>2</sub> (PGE<sub>2</sub>). If GT or EGCG has an effect on the above markers, this will increase the understanding of how these compounds help prevent colon cancer incidence and other inflammatory diseases associated with the markers. Additionally, if GT or EGCG has a greater effect on the inflammatory markers than the other, it will help determine whether it is more beneficial to consume EGCG as part of a GT beverage or as a pure compound in order to elicit the greatest health benefits.

## **1.2 Human Intestinal Anatomy**

The human colon is approximately 3.5 feet long with a diameter of 2.5 inches. It is comprised of the cecum, ascending colon, transverse colon, descending colon and sigmoid colon (Byrd-Bredbenner and others 2009). The colon has three main functions: the absorption of water and electrolytes, the housing of bacterial flora and the formation of feces.

The inner-wall of the colon is lined with epithelial cells and a layer of mucosa (Byrd-Bredbenner and others 2009). The inner-surface of the colon also contains numerous blunt ending crypts, known as crypts of Leiberkhun, which are about 50 cells deep (Figure 1.1). These crypts are lined with a single layer of epithelial cells and help to increase the overall surface area of the large intestine and its ability to re-absorb nutrients (Chell and others 2006).



**Figure 1.1** Colonic crypt cell life cycle (Chell and others 2006)

### 1.2.1 Intestinal Epithelial Cell Life Cycle

Homeostasis of the colonic epithelium and colon crypts is achieved when cell proliferation, differentiation and cell death are appropriately regulated. Cell proliferation begins as the basal stem cells replicate at the base of the crypt (the basal surface). As



replication continues, these cells migrate up the crypt wall towards the apical surface. Regenerative cells differentiate into absorptive enterocytes, goblet cells (that secrete mucous) and enteroendocrine cells. When cells reach the apical surface of the crypt, they are fully differentiated. Therefore, the differentiation and migration process are highly coupled. Once cells reach the apical surface they are exfoliated into the colon lumen. As a result, there is a high turnover rate for the epithelium cells lining the human colon, with cells regenerating every 6-7 days, making these cells more susceptible to mutations (Chell and others 2006).

### ***1.2.2 Differentiation***

Differentiation is a step in the life cycle of a colonic epithelial cell. Varying levels of differentiation affect the sensitivity of cells to a multitude of stimuli (Kovarikova and others 2004). During differentiation many changes occur in the cell, such as changes in sensitivity to cytotoxic agents, inducers of apoptosis and inhibitors of proliferation (Kovarikova and others 2004). Measurement of alkaline phosphatase (ALP) production is a common method for determining cell differentiation (Heerdt and others 1994). In the present study, human colon cancer cells were differentiated to more closely approximate the actual colon lumen environment by causing the cells act like normal colon epithelial cells.

### ***1.2.3 Sodium Butyrate***

In this study sodium butyrate was used to differentiate HT-29 cells since butyrate is known to induce cell differentiation and apoptosis (Heerdt and others 1994). Butyrate is a short chain four carbon fatty acid naturally found in the colon. It is a by-product of

carbohydrate fermentation and is found in millimolar concentrations in the colon lumen (Cummings 1981). Butyrate serves as the main energy source for the colonic epithelium, with more than 70% of the oxygen consumed by colonocytes coming from the oxidation of n-butyrate (Roediger 1980).

#### ***1.2.4 Natural Intestinal Bacteria Flora***

The human colon houses approximately 100 trillion microbial cells, which is more than ten times the number of (human) cells in the body. Of these bacteria, there are around 400 different species, which can be either beneficial or pathogenic (Byrd-Bredbenner and others 2009). Potentially beneficial bacterial are called probiotic bacteria.

##### ***1.2.4.1 Probiotic Bacteria***

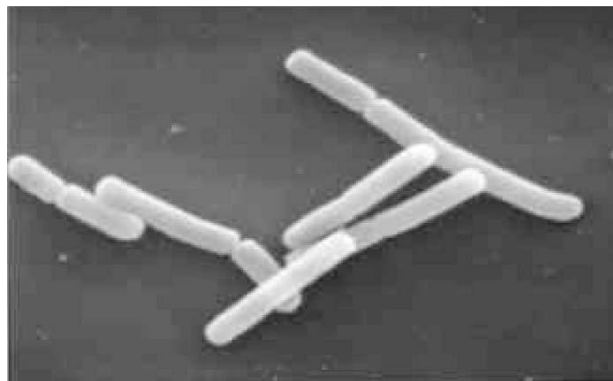
Probiotic bacteria were first defined by Fuller as a “live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance” (Fuller 1989). Probiotics are typically found in fermented foods like yogurt and miso (Byrd-Bredbenner and others 2009). Probiotics are a type of lactic acid bacteria, typically of the *Lactobacillus* and *Bifidobacterium* genera (Byrd-Bredbenner and others 2009). Lactic acid bacteria are typically gram positive, acid tolerant, non-sporulating, anaerobic bacteria (Klaenhammer and others 2005).

Probiotics are beneficial because they confer health benefits when consumed in large enough quantities (FAO/WHO 2001). For example, probiotics help prevent and treat diarrhea, prevent food allergies and treat irritable bowel syndrome (Byrd-Bredbenner and

others 2009). Probiotics are also helpful in the treating of lactose intolerance, *Candida* vaginitis, cryptosporidiosis, *Helicobacter pylori* gastroenteritis, urinary tract infections and hepatic encephalopathy (Chow 2002). Additionally, probiotics have been shown to help reduce the risk of colon cancer through protecting against colonic DNA mutations (Tuohy and others 2005).

#### 1.2.4.1.1 *Lactobacillus acidophilus* NCFM

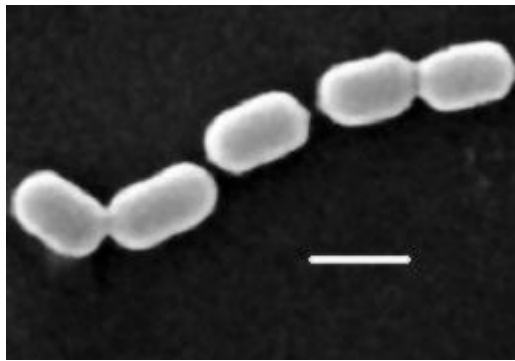
*Lactobacillus acidophilus* NCFM is a gram positive, rod shaped, homofermentative, commercially available strain of probiotic bacteria that is widely available for use in milk, yogurt and toddler formula (Figure 1.2). NCFM is a member of the type A1 *L. acidophilus* species. *L. acidophilus* NCFM is ideal for use in cell culture studies as it is known to adhere to both Caco-2 and HT-29 cell lines (Sanders and Klaenhammer 2001). Additionally, the complete genome sequence of the NCFM strain has been determined, making it extremely relevant in future studies of genomics and probiotic functionality (Sanders and Klaenhammer 2001; Altermann and others 2005).



**Figure 1.2** *Lactobacillus acidophilus* NCFM cells (Sanders and Klaenhammer 2001)

#### 1.2.4.1.2 *Lactobacillus gasseri* ATCC 33323

*Lactobacillus gasseri* ATCC 33323 is another gram positive, rod shaped species of lactic acid bacteria that is a probiotic. It is a member of the *L. acidophilus* complex (Figure 1.3) (Kullen and others 2000). Additionally, *L. gasseri* is the major homofermentative *Lactobacillus* species found in the human gastrointestinal tract (Kullen and others 2000). Like *L. acidophilus*, *L. gasseri* is also able to adhere to human intestinal cells (Conway and others 1987; Greene and Klaenhammer 1994).



**Figure 1.3** *Lactobacillus gasseri* ATCC 33323 (Dykstra and others)

#### 1.2.4.2 Effect of Green Tea and EGCG on Intestinal Probiotic Bacteria

There are few *in vitro*, animal and human studies that have investigated the effect of green tea (GT) or epigallocatechin-3-gallate (EGCG) on intestinal probiotic bacteria growth. The survival of probiotic bacteria *Lactobacillus rhamnosus*, *Bifidobacterium lactis* and *Lactobacillus paracasei* was examined in a fruit juice system with and without the addition of vitamins and antioxidants (Shah and others 2010). This study found that the probiotic bacteria in fruit juice samples containing GT extract (0.001% w/v) had better survival rates compared to the control (Shah and others 2010). Another *in vitro* study examined the effect of GT with probiotic strains on the growth of pathogenic bacteria like *Staphylococcus aureus*

and *Streptococcus pyogenes*. This study found that GT in combination with *L. acidophilus* LAFTI® L10 significantly reduced the viable count of both *Staphylococcus aureus* and *Streptococcus pyogenes* after 4 hours; after 24 hours neither pathogen was viable (Su and others 2008).

An animal study on Holstein calves found that GT extracts maintained high faecal counts of probiotic bacteria from the *Bifidobacterium* spp. and *Lactobacillus* spp. (Ishihara and others 2001). This helped decrease the number of calf deaths from digestive and respiratory organ diseases. In contrast, an animal study looking at the effect of GT on caecal microflora in broiler chickens found that supplementation with GT polyphenols (4.1 g/kg broiler weight) caused a significant reduction in total bacteria count including those of *Bifidobacterium* spp. and *Lactobacillus* spp. (Cao and others 2005).

An *in vivo* study on the effect of GT extract (0.4 g/day) consumption on human fecal microflora composition found that *Bifidobacterium* spp. levels increased significantly after 4 weeks, however, returned to normal 2 weeks after ceasing the GT treatment (Okubo and others 1992). There has also been a study conducted on the effect of black tea (BT) on fecal microflora composition and fecal bile acid profile in humans. This study found that each individual subject had a unique bacterial profile and that this profile did not change significantly due to the consumption of BT for 2 weeks (Mai and others 2004). In conclusion, these studies indicate a need for more research on the effect of GT or EGCG on probiotic bacteria growth; this would determine whether the consumption of both at the same time has greater beneficial health effects than the consumption of each component individually.

### ***1.2.5 Intestinal Cell Culture Models***

In order to explore the effects of compounds on the human GI tract *in vitro*, there must be cell culture models that accurately represent the environment of the human intestine. Two of the most commonly used GI cells lines are Caco-2 cells and HT-29 cells. Depending on the research being done one cell line is probably better suited to different research scenarios.

#### ***1.2.5.1 Caco-2 Cell Line***

Caco-2 cells are an adherent, heterogeneous, epithelial cell line isolated from human colorectal adenocarcinoma cells (Fogh and others 1977) . Caco-2 cells are derived from human colon cells, however, when cultured and differentiated they simulate the phenotype of small intestine enterocytes and can be used for *in vitro* cell culture studies (Pinto and others 1983; Hidalgo and others 1989).

#### ***1.2.5.2 HT-29 Cell Line***

Although Caco-2 cells are more widely used in cell culture studies modeling the human gastrointestinal tract, HT-29 cells were used in this study to more closely model the human colon and the effect of treatments on human colon cancer markers. Like Caco-2 cells, HT-29 cells are also adherent, heterogeneous, epithelial cells isolated from a human colorectal adenocarcinoma (Fogh and others 1977). HT-29 cells differ from Caco-2 cells because they secrete mucous and more closely simulate the human colon by exhibiting an

enterocyte-like phenotype when differentiated (Siavoshian and others 1997; Kovarikova and others 2004).

### **1.3 Tea Consumption**

Tea is one of the most consumed beverages worldwide, second only to water (Chen and others 2008). The per capita consumption estimate of brewed tea is 120 ml/day (Katiyar and Mukhtar 1996). Of the types of tea (black, oolong and green), black tea (BT) is the most consumed at 78% of total tea consumption and is most popular in Western countries. Oolong tea is the least consumed type of tea at 2% of total tea consumption and is most popular in Southeastern China. Finally, GT makes up 20% of the total tea consumption and is widely consumed in Asian countries such as Japan, China, Korea and India (Katiyar and Mukhtar 1996).

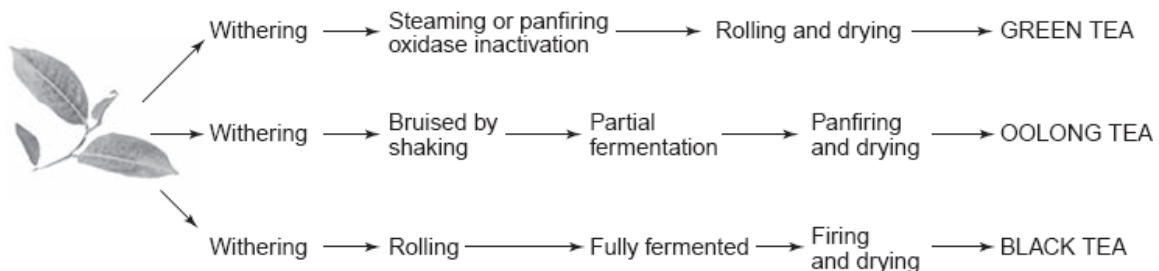
### **1.4 Tea Production**

All types of tea are made from an infusion of *Camellia sinensis* plant leaves (Figure 1.4), which has been grown in Southeast Asia for thousands of years and is now grown in over 30 countries worldwide (Butt and Sultan 2009). About 2.5 million metric tons of tea are manufactured each year (Katiyar and Mukhtar 1996). The leaves of the *Camellia sinensis* plant undergo different fermentation and enzymatic oxidation processes to produce BT, GT and oolong tea, which have varying chemical compositions (Figure 1.5). BT is produced by a full fermentation process, which allows for the polyphenol oxidase enzyme found in the plant to fully oxidize the catechins in the tea leaves to theaflavins and thearubigens. GT is

not prepared by a fermentation process, but is instead made by steaming and drying the tea leaves, which inactivates polyphenol oxidase, thereby preserving the catechins present in the *Camellia sinensis* leaves. Oolong tea is partially fermented, therefore, having a chemical makeup that is in between that of black and GT with some catechins and oligocatechins present (Chen and others 2008).



**Figure 1.4** *Camellia sinensis* plant (Butt and Sultan 2009)



**Figure 1.5** Processing of black, oolong and green teas (Khan and others 2008)

### 1.5 Chemical Composition of Green Tea

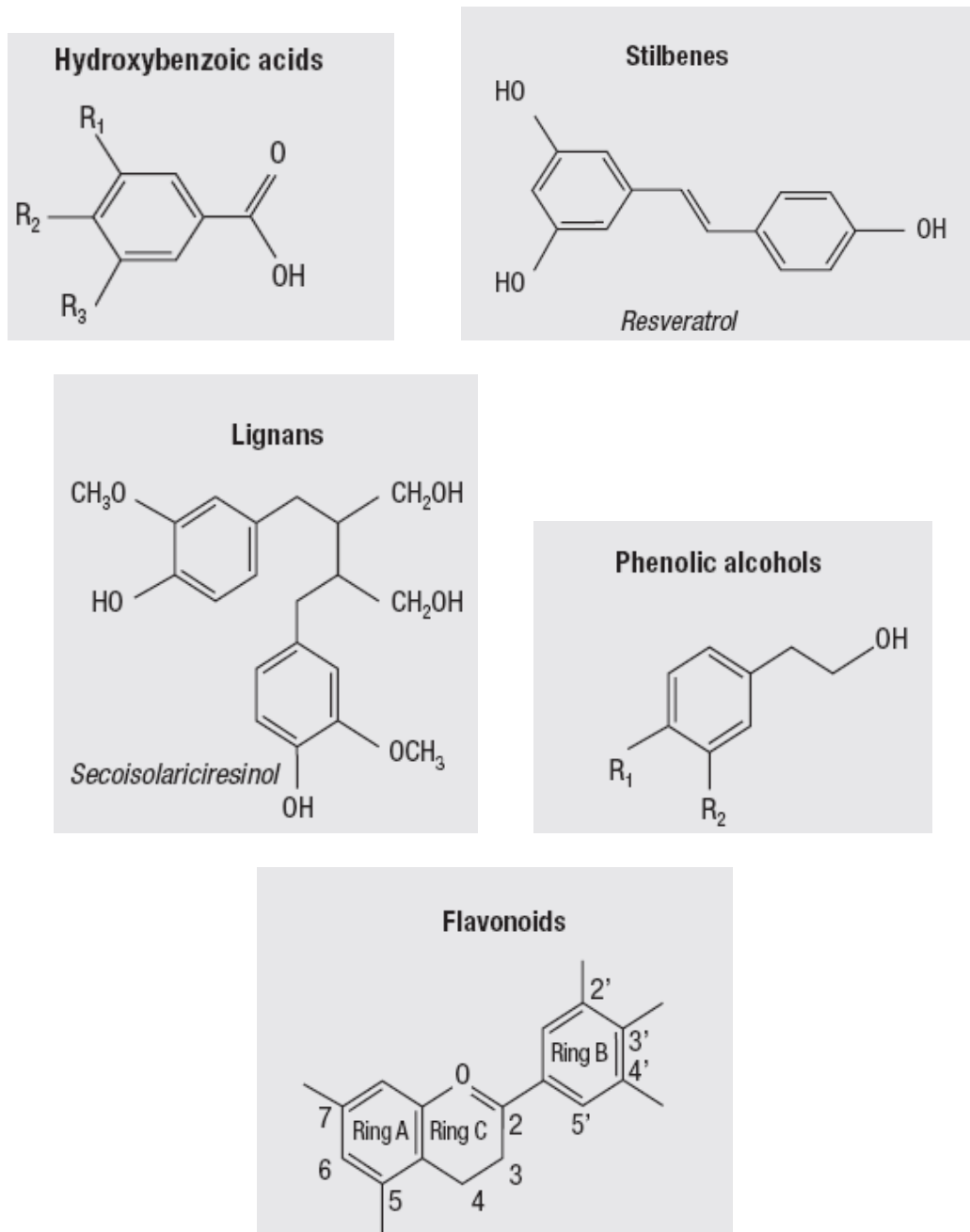
The exact composition of dried GT leaves varies depending on the location of growth, time of harvest and variations in processing. A typical GT beverage prepared by brewing 1 g of dried leaves in 100 ml of water for 3 minutes contains 250-350 mg tea solids (Balentine and others 1997). Of these solids, the typical dried GT leaf is mainly comprised of



polyphenols (10-25% dry weight), which include flavonols, flavonoids and flavonidials. GT leaves also contain alkaloids (4% dry weight) such as caffeine, theobromine and theophylline; antioxidant compounds such as carotenoids, tocopherols and ascorbic acid; minerals such as potassium, manganese, calcium, fluoride and aluminum; proteins, carbohydrates, phenolic acids, and fiber (Balentine and others 1997; Khan and others 2008; Butt and Sultan 2009).

### ***1.5.1 Polyphenols***

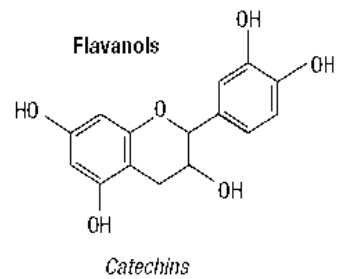
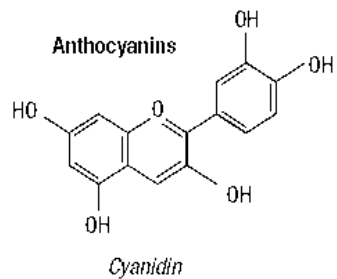
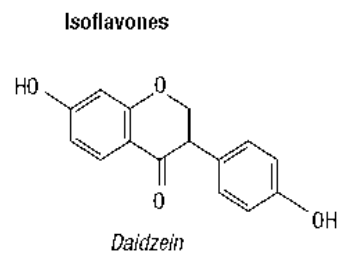
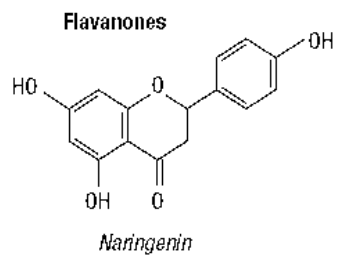
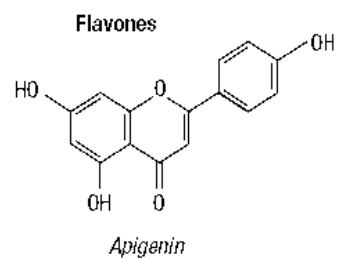
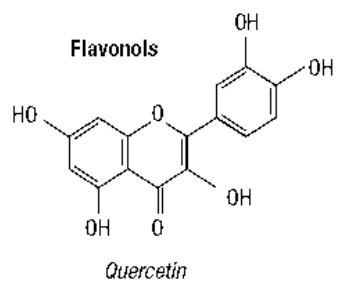
GT contains a variety of polyphenols, which are a group of phytochemicals commonly found in plants and human diets (Cheynier 2005). The average intake of polyphenols from food is about 1g/day (Biesalski 2007). In plants, polyphenols serve as secondary metabolites by providing anti-microbial and anti-fungal functions, as well as protection from insect and UV-radiation damage (Stevenson and Hurst 2007). The structure of polyphenols is characterized by a benzene ring with two or more hydroxyl groups attached (Stevenson and Hurst 2007). The main groups of polyphenols are flavonoids, phenolic acids, phenolic alcohols, stilbenes and lignans (Figure 1.6). The flavonoid group is of importance to this review as it contains the main polyphenolic component of GT, epigallocatechin-3-gallate (EGCG).



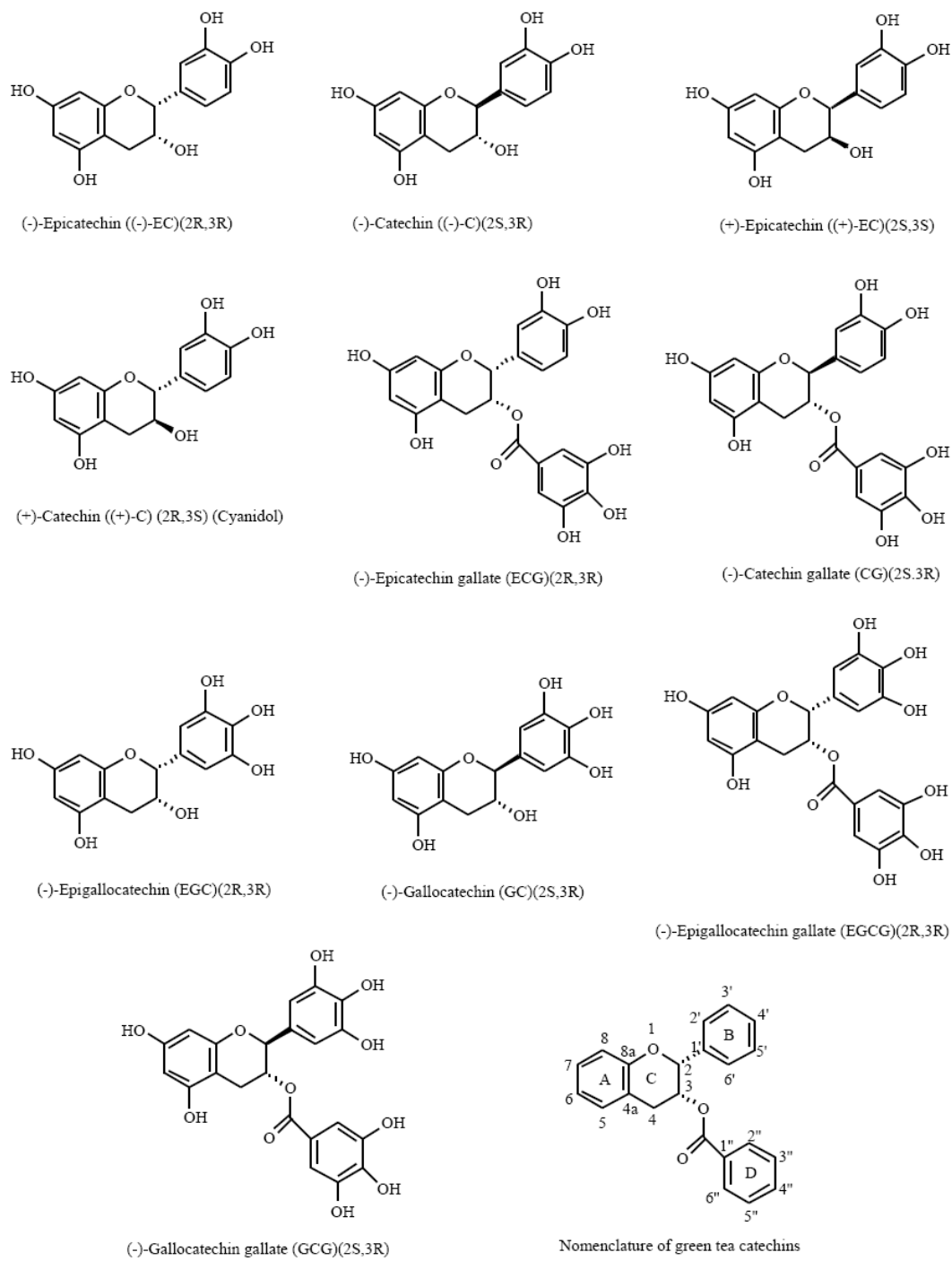
**Figure 1.6** Structures of polyphenols (D'Archivio and others 2007)

### ***1.5.2 Flavonoids***

Over 6,000 types of flavonoids have been identified in plants, with the list continuously growing (Geleijnse and Hollman 2008). Flavonoids have a diphenylpropane skeleton structure ( $C_6C_3C_6$ ) (RiceEvans and others 1996). The aromatic 'A' ring is attached to heterocyclic benzopyran 'C' ring and the 'B' ring is a phenyl constituent (Figure 1.6) (Aron and Kennedy 2008). The flavonoids can be further divided into 6 subclasses depending on the oxidation state of the central pyran 'C' ring, which include: flavonols, flavones, flavanones, isoflavones, anthocyanidins and flavanols (Figure 1.7) (D'Archivio and others 2007). Flavanols include catechins like epicatechin (EC), galliccatechin (GC), epigallocatechin (EGC) and epigallocatechingallate (EGCG) molecules, which can be found in foods like red wine, dark chocolate, BT and GT (Figure 1.8) (Manach and others 2005).



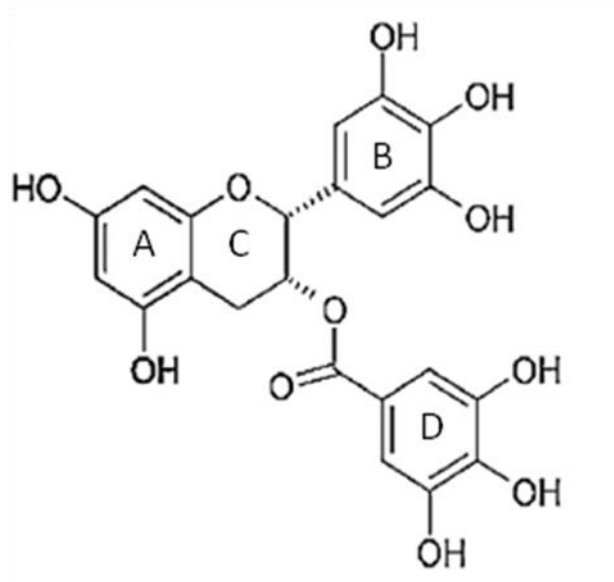
**Figure 1.7** Structures of flavonoids (D'Archivio and others 2007)



**Figure 1.8** Structures of catechins (Feng 2006)

### 1.5.3 Catechins

Catechins are the main polyphenolic components of GT, comprising approximately 30% of the dry leaf weight (Graham 1992). The structure of a catechin consists of a benzopyran skeleton substituted with a phenyl ring at the 2-position and a hydroxyl (or ester) at the 3-position (Chen and others 2008). Of the catechins present in GT, EGCG is the most abundant comprising 50-80% of the total catechins (Figure 1.9) (Feng 2006).



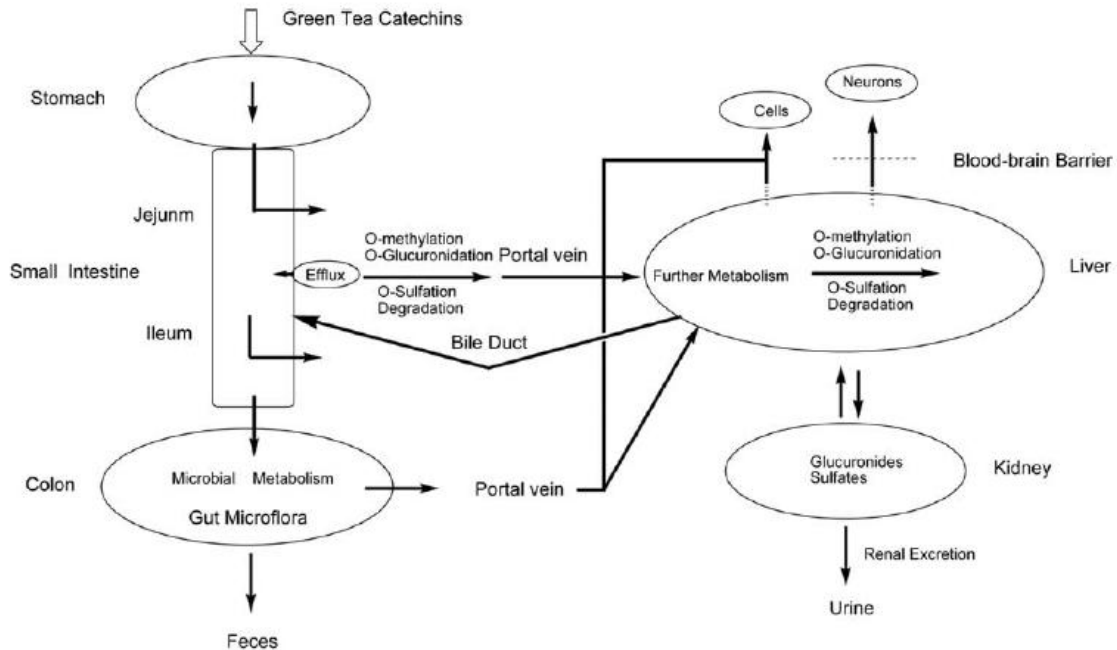
**Figure 1.9** Structure of EGCG (Feng 2006)

### 1.6 Metabolism and Absorption of Green Tea Catechins

It is important to identify the manner by which GT catechins, like epicatechin (EC), epicatechin gallate (ECG), epigallocatechin (EGC) and EGCG, are metabolized and absorbed in order to understand how these metabolites function *in vivo* to elicit beneficial health effects. In humans and animals the metabolism of GT catechins depends on intestinal metabolism, microbial metabolism, hepatic metabolism, transporters and chemical stability

(Feng 2006). Once ingested, GT catechins can be found in the forms of methylated, sulfated and glucuronidated metabolites, microbial metabolites or degradants (Feng 2006).

A proposed mechanism for the metabolism of GT catechins can be found in Figure 1.10. The gastrointestinal (GI) tract plays an important role in the metabolism and bioavailability of GT catechins. After GT catechins are ingested, the metabolites that are formed in the small intestine have two possible metabolic pathways. In one route, the metabolites are transported to the intestinal lumen, which leads to the large intestine where the gut microflora further metabolize them into small phenolic acids and valerolactones (Feng 2006). These metabolites can then be reabsorbed by the portal vein, further metabolized by the liver or excreted in fecal matter. In another route, the metabolites formed in the small intestine are methylated, sulfated, glucuronidated or degraded and then further metabolized by the liver. Once metabolites reach the liver, they can either be excreted through urine or absorbed by cells and neurons (Feng 2006).



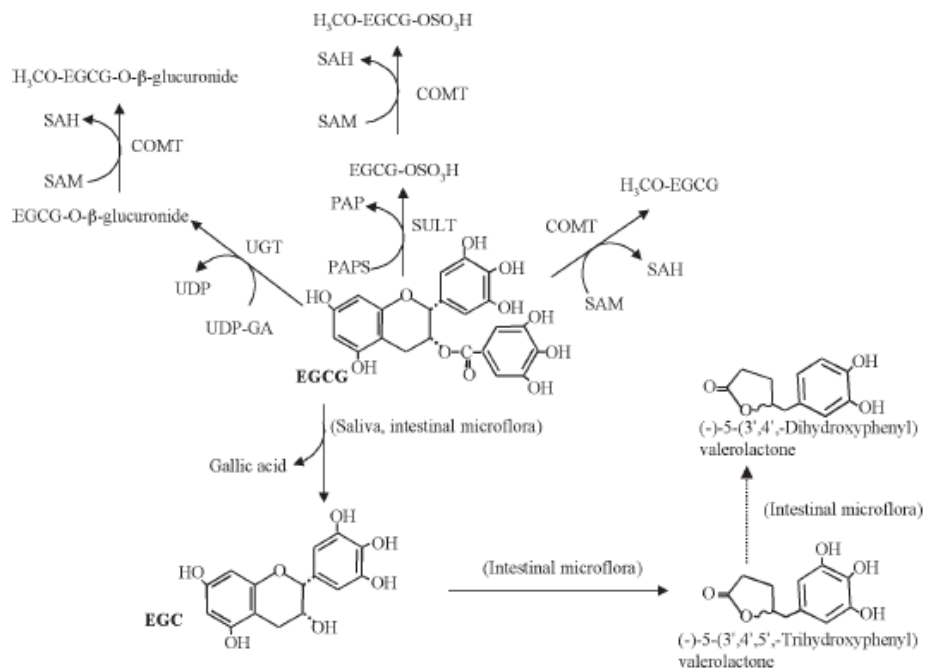
**Figure 1.10** Proposed metabolic pathway of GT catechins (Feng 2006)

There are many enzymes that are important to the metabolism of GT catechins. These include catechol-*O*-methyltransferase, UDP-glucuronosyltransferase, and phenolsulfotransferase (Higdon and Frei 2003). In a study using rats, the highest activity of catechol-*O*-methyltransferase was found in the liver and kidney, the highest amount of UDP-glucuronosyltransferases was found in the mucosa of the small and large intestine and the highest amount of phenolsulfotransferase was found in the liver (Piskula and Terao 1998).

The metabolism of GT catechins is very complex and results in a variety of metabolites (Figure 1.11). Catechins are metabolized extensively and quickly with peak blood plasma levels of GT catechins occurring four hours after administration (Chow and others 2001). A study by Yang and others showed that the metabolism of EGCG begins as soon as it enters the oral cavity and comes into contact with saliva (Yang and others 1999).



Catechin esterase activity was found to be present in the saliva resulting in the degalloylation of EGCG in the mouth and esophagus (Yang and others 1999). Also, EGCG has been shown to be methylated to 4''-O-methyl-EGCG and then to 4'',4'-di-O-methyl-EGCG by liver cytosolic catechol-*O*-methyltransferase (Feng 2006). Metabolites of EC and EGC, (-)-5-(3', 4', 5'-trihydroxyphenyl)- $\gamma$ -valerolactone and (-)-5-(3',4'-dihydroxyphenyl)- $\gamma$ -valerolactone, were recently found to be formed in the colon, absorbed, then excreted in human urine (Li and others 2000). It is important to note that the amount and type of microflora bacteria in the intestine affect how GT catechins are metabolized, creating inter-individual variation (Feng 2006).



**Figure 1.11** Potential metabolism pathways of EGCG (Yang and others 2000; Higdon and Frei 2003)

## **1.7 Tissue Distribution of Green Tea Catechins**

Tissue distribution of GT catechins appears to be mostly dependent on whether that tissue comes into direct contact with the catechins during the metabolism process. Rats given 0.6% GT polyphenols for 28 days showed high concentrations of EGC and EC in the esophagus, large intestine, kidney, bladder, lung and prostate. In contrast, low concentrations of EGC and EC were found in the liver, spleen, heart and thyroid (Kim and others 2000). For example, mice and rats given the same 0.6% GT polyphenol mixture for 28 days reported different tissue distribution results. For the mice, higher concentrations of EGCG were found compared to EGC in the lungs, while a comparable amount of EGCG and EGC were found in the liver. This suggests that EGCG has a greater bioavailability in mice than rats (Kim and others 2000).

There is little available data on the tissue distribution of catechins after tea consumption in humans. However, one recent study found EGCG, 4'-O-methyl EGCG and (-)-epicatechin-3-gallate in prostatectomy tissues of men with prostate cancer after they had consumed 6 cups of GT for 3-6 weeks. GT catechins were not found in the prostatectomy tissue of men that had consumed water for the same period of time (Wang and others 2010a).

## **1.8 Bioavailability of Green Tea Catechins**

Bioavailability is defined as the proportion of a nutrient that is digested, absorbed and metabolized through normal pathways (D'Archivio and others 2007). It is important to understand the bioavailability of the metabolites of GT, like EGCG, in order to determine whether these metabolites are present at high enough physiological concentrations to elicit a

health effect. The estimated daily intake of catechins is 18-50 mg/day with the main sources being tea, chocolate, apples, pears, grapes and red wine (Arts and others 2000). Most catechins (75-99%) are not excreted in the urine when consumed as part of the diet (Scalbert and Williamson 2000). This indicates that they are being absorbed and excreted in the bile or that they are being metabolized by intestinal microflora or intestinal tissues (Scalbert and Williamson 2000). For example, in humans, EGC consumed as a GT infusion (82 mg ingested) resulted in a maximum plasma concentration of 0.67  $\mu\text{M}$ , of which 3.6% was excreted in the urine (Lee and others 1995). Also, EC consumed as a GT infusion (32 mg ingested) resulted in a maximum plasma concentration of 0.27  $\mu\text{M}$ , of which 6.2% was excreted in the urine (Lee and others 1995). Additionally, approximately 2 hours after consuming 4.5 g of decaffeinated GT solids, the average plasma concentration of EGCG was observed to be 326 ng/ml (711 nM) for 18 human volunteers (Yang and others 1998). In addition, a study that administered 800 mg of EGCG found that the plasma concentration of EGCG reached a maximum level of 438.5 ng/ml (956 nM) 4 hours after consumption (Chow and others 2001).

Rats given 0.6% GT polyphenol solution for 28 days reached a peak catechin level 2 weeks into the study, which then continually decreased to the day 1 catechin levels by day 28 (Kim and others 2000). It was also found that even though the amount of EGCG was greater than EGC in the GT polyphenol solution in a ratio of 5:1, the concentration of EGCG in the plasma of the rats was still significantly less than that of EGC and EC (Kim and others 2000). In contrast, when the same GT polyphenol solution was administered to mice, the

concentration of EGCG was higher than that of EGC and EC, thus, providing evidence for varying bioavailability of GT catechins among different species (Kim and others 2000).

It is important to note that many bioavailability studies of GT catechins report large inter-individual differences in catechin plasma concentrations (Higdon and Frei 2003).

Possible reasons for these differences include a polymorphism in catechol-*O*-methyltransferase that may result in a 3-4 fold activity increase of this enzyme, as well as dietary, environmental and behavioral factors (Higdon and Frei 2003). Therefore, it is important for the administered catechin as well as its metabolites to be measured in order to accurately determine the bioavailability of a molecule.

## **1.9 Antioxidant Effects of Green Tea and EGCG**

GT and EGCG possess potent antioxidant and anti-inflammatory effects (RiceEvans and others 1996; Rahman and others 2006). For this reason GT and EGCG have been implicated in the prevention of diseases associated with oxidative stress and inflammation such as CVD and cancer (Butt and Sultan 2009).

Reactive oxygen species (ROS) are produced naturally during the oxidation process. ROS include oxygen radicals like superoxides anions, hydroxyls, alkoxyls, peroxy, nitric oxide, as well as non-radicals like hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), hypochlorous acid, ozone, singlet oxygen and peroxynitrite (Aron and Kennedy 2008). The ROS are usually neutralized by antioxidants and antioxidant enzymes before they cause damage to proteins, lipids and DNA (Chen and others 2008). However, if not counteracted, these molecules can

lead to oxidative tissue damage, which has been associated with diseases like cancer, heart disease, diabetes, Alzheimer's disease and aging (Cutler 2005; Cutler and others 2005).

Polyphenols possess antioxidant activity through several different mechanisms, which include reactive oxygen species (ROS) scavenging, metal chelation and enzyme inhibition (Cos and others 2004). Antioxidant activity is determined by a molecule's hydrogen or electron-donating ability, ability to stabilize or delocalize an unpaired electron, reactivity with other antioxidants and potential to chelate transition metals (RiceEvans and others 1997). Another related factor in determining a molecule's antioxidant activity is its reduction potential. A lower reduction potential indicates greater antioxidant activity as it requires less energy for the molecule to donate a hydrogen or electron (Higdon and Frei 2003).

The structure of polyphenols is ideal for free radical scavenging. The free radical scavenging ability of polyphenols largely depends on the number and accessibility of the phenol moieties (Cheynier 2005). For EGCG and EGC the trihydroxyphenyl B ring has been shown to be the principle site of antioxidant reaction, not the 3-galloyl group as previously hypothesized (Valcic and others 1999; Valcic and others 2000). Another study indicated that the hydroxyl groups on the A ring may also act as antioxidant sites for EGCG and EGC (Zhu and others 2000).

Polyphenols are especially good at chelating iron and copper molecules which prevents the formation of transition metal catalyzed radicals (RiceEvans and others 1997). There are two main points on the flavonoid structure that affect its metal chelation ability. These are the o-dihydroxyl groups in the 3',4'-dihydroxy positions in the B ring and the ketol structures 4-keto,3-hydroxy or 4-keto and 5-hydroxy in the C ring (RiceEvans and others

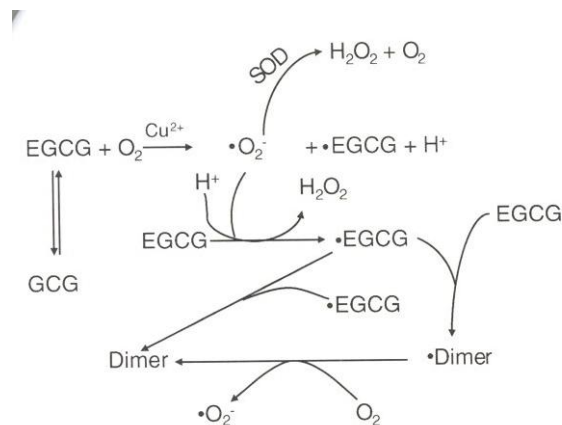
1997). Whether these positions are glycosylated also affects the antioxidant activity of the flavonoid.

The catechins present in GT have greater antioxidant activities than Vitamin C or Vitamin E as measured by the trolox equivalent antioxidant activity (TEAC). Of the GT catechins, ECG (4.9 mM) has the greatest antioxidant activity, followed by EGCG (4.8 mM), EGC (3.8 mM) and EC (2.4 mM), while Vitamin C and Vitamin E each have an antioxidant activity of 1.0 mM (RiceEvans and others 1997). In fact, 78% of the antioxidant activity in GT can be attributed to the catechin and catechin gallate ester molecules (RiceEvans and others 1997).

*In vitro* experiments show that GT polyphenols (GTPs) have antioxidant effects. For example, GTPs (10 µg/ml) preincubated with human microvascular endothelial cells (HUMVECs) for one hour completely prevented the oxidative damage caused by H<sub>2</sub>O<sub>2</sub> and xanthine oxidase in the absence of GTPs (Rah and others 2005).

In contrast, some *in vitro* studies have shown that GT and EGCG possess pro-oxidant instead of antioxidant activities. These pro-oxidant activities are mainly due to the instability of EGCG in cell culture media. EGCG has a half life of 30 minutes in McCoy's 5A culture media at 37°C and 130 minutes in the presence of HT-29 cells (Hong and others 2002). When EGCG is exposed to cell culture media and oxygen under neutral to slightly alkaline pH conditions, it undergoes an auto-oxidation process where EGCG donates an electron to molecular oxygen forming the superoxide radical (Sakagami and others 2001; Hou and others 2004). The superoxide radical can cause the formation of theasinensin (mw=914), an EGCG dimer (mw=884) or H<sub>2</sub>O<sub>2</sub> by a dismutation reaction (Figure 1.12) (Hong and others

2002; Hou and others 2004). The production of  $\text{H}_2\text{O}_2$  by EGCG is a significant contributing factor to the pro-oxidant activity of EGCG. For example, the addition of EGCG ( $40 \mu\text{M}$ ) to the cell culture media caused the production of  $\text{H}_2\text{O}_2$  ( $25 \mu\text{M}$ ) after 2 hours with less  $\text{H}_2\text{O}_2$  forming in the presence of HT-29 cells (Hong and others 2002). Additionally, EGCG ( $1 \text{ mM}$ ) produced  $\text{H}_2\text{O}_2$  ( $>400 \mu\text{M}$ ) after 2 hours in Dulbecco's modified eagle's medium (DMEM) without cells (Long and others 2000). These studies highlight the importance of performing mechanistic studies *in vivo* to determine whether high consumption levels of GT and EGCG should be recommended to humans.



**Figure 1.12** Auto-oxidation of EGCG (Hou and others 2004)

Similar to *in vitro* studies, *in vivo* studies also report conflicting results on the antioxidant activity of GT and EGCG. One study found that after consumption of 300 ml of GT, the human plasma antioxidant capacity, measured by total peroxy radical trapping assay (TRAP), significantly increased and peaked at 30-50 minutes (Serafini and others 1996). However, when GT was consumed with milk, there was not an increase in TRAP, which was due to the complexation of the polyphenols with milk proteins. When humans consumed a significant amount of GT (1 liter/day for 4 weeks) it was found that two serum markers of

lipid peroxidation, malonyldialdehyde and malonyldialdehyde+4-hydroxy-2(E)-nonenal, were significantly lower than controls (30 % and 39% respectively) (Coimbra and others 2006). Membrane bound hemoglobin, a marker of oxidative stress in erythrocyte membranes, also had a significantly reduced value (25%). In contrast, subjects that consumed GT (1000 ml/day) for 7 days did not show a significant difference in levels of urinary F<sub>2</sub>-isoprostane, a measure of *in vivo* lipid peroxidation (Hodgson and others 2002).

There are numerous ways that researchers can choose to measure antioxidant activity as can be seen in the studies listed above. Therefore, one must be careful in comparing studies that do not measure antioxidant activity in the same manner. The plasma and intracellular flavonoid concentrations after humans consume large amounts of GT catechins, are 100-1000 times less than other antioxidants like Vitamin C and Vitamin E, making it questionable as to whether these molecules can exert an antioxidant effect at these concentrations (Williams and others 2004). More *in vivo* research needs to be conducted to further determine whether GT has significant antioxidant activity in humans.

## **1.10 Anti-Inflammatory Effects of Green Tea and EGCG**

### ***1.10.1 Inflammation***

Inflammation is a non-specific response by the body to stimuli and injury. Its symptoms include swelling, redness, pain and fever (Larsen and Henson 1983). During an inflammatory event inflammation is typically a beneficial response, however, chronic inflammation can lead to a variety of diseases including cancer, obesity, type-2 diabetes, CVD, rheumatoid arthritis, and aging (Santangelo and others 2007). Epidemiological studies



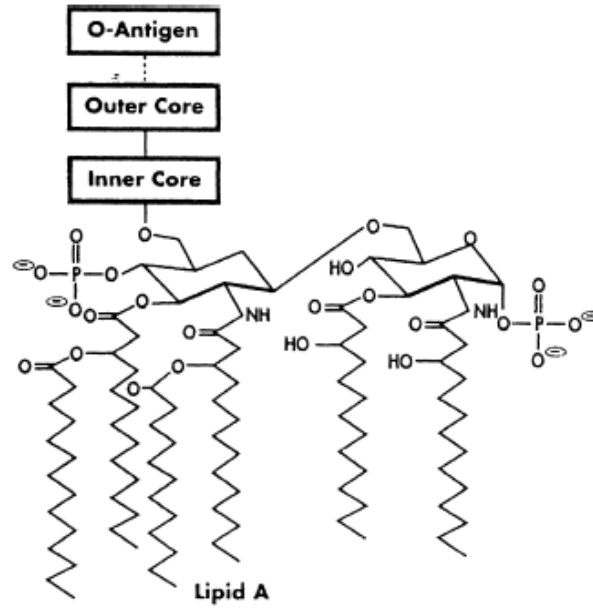
have shown that populations that consume diets high in specific polyphenols have a lower incidence of inflammatory related diseases (Biesalski 2007).

### ***1.10.2 Stimulators of Inflammation: LPS and TNF- $\alpha$***

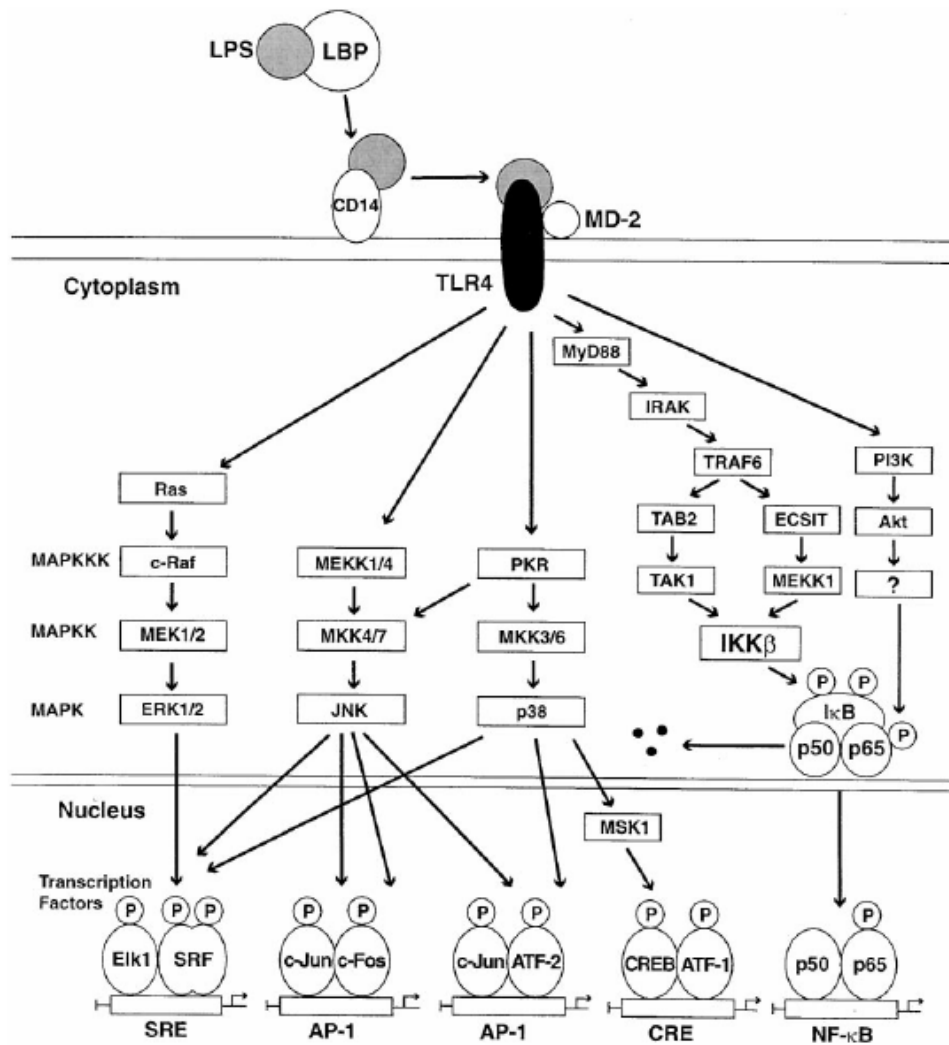
There are a variety of compounds that can cause an inflammatory response in human colon cells. For this study two compounds, lipopolysaccharide (LPS) and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), were chosen to stimulate inflammation in human colon cancer cells.

#### ***1.10.2.1 Mechanism of Inflammation by LPS***

LPS is an endotoxin found in gram negative bacteria. It is comprised of three parts: the O-antigen, the core oligosaccharide and the lipid A (endotoxin) moiety (Figure 1.13) (Raetz and Whitfield 2002). LPS causes inflammation through the binding of the Lipid A portion of LPS to the toll-like receptor-4 (TLR4) (Figure 1.14) (Poltorak and others 1998). The recognition of LPS by a cell begins with the transfer of LPS to CD-14 by the LPS binding protein (Ulevitch and Tobias 1995). The LPS/CD-14 complex is then recognized by the myeloid differentiation (MD)-2 receptor, an essential co-receptor, which causes the activation of TLR4 (Teghanemt and others 2008). The binding of LPS to TLR4 through the MD-2 receptor causes a signaling cascade that leads to the activation of numerous transcription factors including nuclear factor- $\kappa$ B (NF- $\kappa$ B), activator-protein-1 (AP-1), serum response element (SRE) and cyclic AMP response element (CRE) (Figure 1.14) (Guha and Mackman 2001).



**Figure 1.13** Structure of LPS (Guha and Mackman 2001)



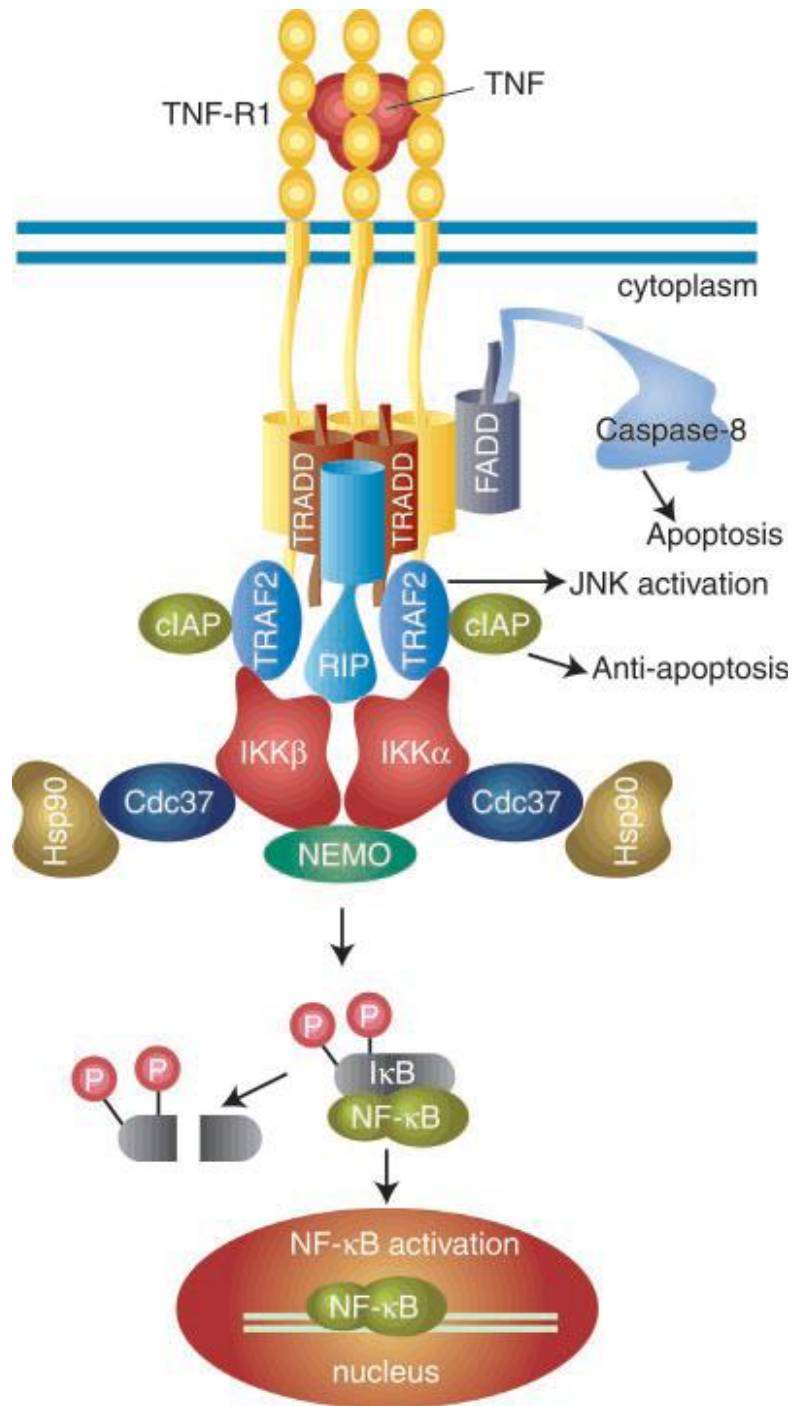
**Figure 1.14** LPS signaling pathways (Guha and Mackman 2001)

#### 1.10.2.2 Mechanism of Inflammation by TNF- $\alpha$

TNF- $\alpha$  is a cytokine primarily produced by monocytes and macrophages and is involved mainly in acute inflammatory responses. Over-production of TNF- $\alpha$  has been implicated in numerous human diseases including sepsis, diabetes, cancer, osteoporosis, rheumatoid arthritis, inflammatory bowel disease and multiple sclerosis (Chen and Goeddel

2002). TNF- $\alpha$  is a homotrimer comprised of 157 amino acids in its active form (Tang and others 1996; Chen and Goeddel 2002). TNF- $\alpha$  receptors occur on nearly all types of cells (Fiers 1991). TNF- $\alpha$  signaling can occur through two separate TNF- $\alpha$  receptors, TNF-R1 and TNF-R2, however, most signaling occurs through TNF-R1 (Chen and Goeddel 2002).

The binding of TNF- $\alpha$  to TNF-R1 can activate several signaling pathways (Figure 1.15). One pathway occurs through the TNF-receptor death domain (TRADD) protein, which recruits the TNF-R-associated factor 2 (TRAF2) and fas-associated death domain (FADD). FADD recruits caspase-8, which ultimately causes apoptosis and cell death (Chen and Goeddel 2002). The main result of TNF-R1 signaling results in the activation of two transcription factors: c-jun and NF- $\kappa$ B (Chen and Goeddel 2002). TRAF2 activates mitogen activated protein kinase kinase kinase (MAPKKK), which ultimately activates c-jun. I $\kappa$ B kinase (IKK) is recruited to TNF-R1 and is responsible for activating NF- $\kappa$ B. (Further details on the activation of NF- $\kappa$ B can be found in the NF- $\kappa$ B section of this literature review). By activating the NF- $\kappa$ B pathway, TNF- $\alpha$  also activates cyclooxygenase-2 (COX-2) expression and interleukin-8 (IL-8) production in HT-29 cells (Gross and others 1995; Crofford 1997).



**Figure 1.15** TNF- $\alpha$  signal transduction pathway (Chen and Goeddel 2002)

### ***1.10.3 Markers of Inflammation***

GT catechins themselves may not be effective antioxidants *in vivo* due to their low plasma and intracellular concentrations. Therefore, it is more likely that the health benefits observed from GT consumption are due to cell signaling events (Williams and others 2004). Polyphenols show promise in protecting against oxidative stress by modulating inflammatory responses in the cyclooxygenase (COX) and NF- $\kappa$ B pathways, which are of interest in this study, as well as in the lipoxygenase (LOX) and nitric oxide (NO) pathways (Santangelo and others 2007).

#### ***1.10.3.1 IL-8***

Interleukin-8 (IL-8) is a 8.4 kDa protein consisting of 72 amino acid residues (Rollins 1997). It is the prototypic chemokine from the CXC family of cytokines (Rollins 1997). IL-8 is produced by an array of cell types including monocytes, T lymphocytes, neutrophils, fibroblasts, endothelial cells and epithelial cells (Rollins 1997). The main function of IL-8 is to attract and activate neutrophils during an inflammatory response (Rollins 1997). IL-8 is also known to chemoattract T lymphocytes (Larsen and others 1989) and to stimulate the release of histamine from basophils (White and others 1989).

IL-8 can be produced through different pathways as its gene promoter contains multiple binding sites. The main pathway that produces IL-8 is the NF- $\kappa$ B inflammatory pathway. NF- $\kappa$ B is mainly responsible for IL-8 synthesis in HT-29 cells stimulated by LPS (10 ng/ml) (Lenoir and others 2008). IL-8 can also be produced through AP-1 and NF-IL-6 pathways (Mukaida and others 1994).

After IL-8 is produced it can activate multiple intracellular signaling pathways, which are mediated through the binding of IL-8 with two G protein-coupled cell surface receptors: CXCR1 and CXCR2 (Holmes and others 1991; Murphy and Tiffany 1991). IL-8 expression and signaling has been found in cancer cells, endothelial cells, infiltrating neutrophils and tumor-associated macrophages, indicating that IL-8 may have a significant function in the progression of cancer (Waugh and Wilson 2008). Specifically, IL-8 has been shown to increase the aggressiveness of colon cancer by promoting tumor growth and angiogenesis (Sparmann and Bar-Sagi 2004).

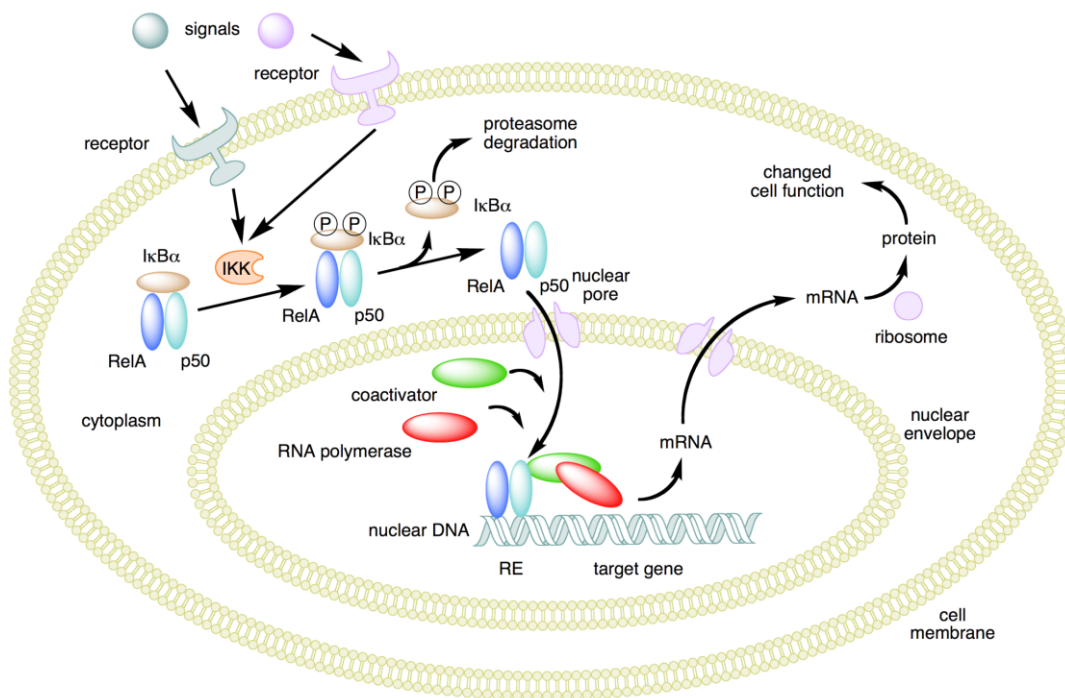
#### *1.10.3.2 NF- $\kappa$ B*

Nuclear factor- $\kappa$ B (NF- $\kappa$ B) is a collective name for a group of redox-sensitive dimeric transcription factors that are found in virtually all cell types (Karin and Ben-Neriah 2000). There are currently five mammalian NF- $\kappa$ B transcription factors that are members of the Rel family of DNA binding proteins that recognize a similar DNA sequence motif (Baldwin 1996; Karin and Ben-Neriah 2000). These members are c-Rel, NF- $\kappa$ B1 (p50/p105), NF- $\kappa$ B2 (p52/p100), RelA (p65), and RelB (Baldwin 1996).

NF- $\kappa$ B is suspected to play an important role in immune, inflammatory, stress, proliferative and apoptotic responses to a wide array of cellular stimuli (Karin and Ben-Neriah 2000). NF- $\kappa$ B is responsible for altering the transcription of a variety of genes that encode pro-inflammatory cytokines (*e.g.*, TNF- $\alpha$ , IL-1, IL-2, IL-6), chemokines (*e.g.*, IL-8), adhesion molecules and growth factors (Nam 2006).

When not activated, NF- $\kappa$ B is sequestered in the cytoplasm of a cell bound with inhibitory proteins (I $\kappa$ B $\alpha$  and I $\kappa$ B $\beta$ ) from the I $\kappa$ B family. The I $\kappa$ B proteins prevent NF- $\kappa$ B from localizing to the nucleus by hiding its nuclear localization signal. When a cell is exposed to an inflammatory stimulus, such as ROS, inflammatory cytokines (*e.g.*, TNF- $\alpha$ , IL-1, IL-2, IL-6), LPS, viruses, viral proteins, bacteria, mitogens, double stranded RNA or UV light, the I $\kappa$ B proteins are phosphorylated by I $\kappa$ B kinase (IKK), ubiquitinated and proteolytically degraded (Karin and Ben-Neriah 2000). This allows NF- $\kappa$ B to translocate to the nucleus, bind to the DNA and alter the transcription and expression of numerous genes involved in inflammatory responses (*e.g.* COX-2 and IL-8) (Figure 1.16) (Karin and Ben-Neriah 2000; Higdon and Frei 2003). The NF- $\kappa$ B signaling pathway is eventually terminated when NF- $\kappa$ B is resequenced in the cytoplasm, which requires I $\kappa$ B $\alpha$  synthesis.





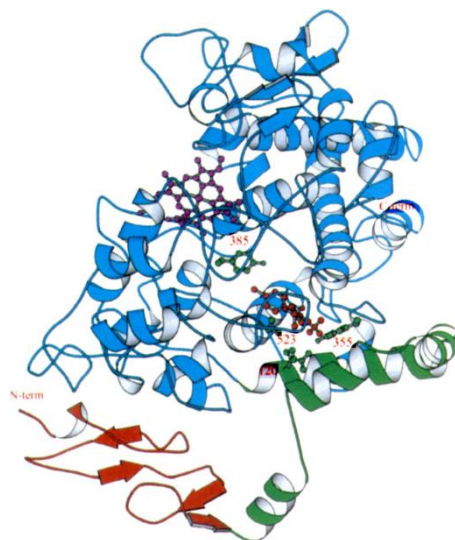
**Figure 1.16** Mechanism of NF- $\kappa$ B (Victor and others 2004)

Molecules that suppress NF- $\kappa$ B activation have the potential to reduce inflammation in cells and may be useful in the treatment of inflammatory related diseases. Polyphenols, like EGCG, are believed to have such potential. For example, EGCG (10-80  $\mu$ M) inhibited the TNF- $\alpha$  and LPS mediated activation of NF- $\kappa$ B in human epidermoid carcinoma (A431) cells (Ahmad and others 2000). Both the cytoplasmic and nuclear NF- $\kappa$ B levels were reduced in a dose dependent manner upon EGCG treatment through the inactivation of IKK, which prevented the degradation of I $\kappa$ B $\alpha$ , therefore, preventing NF- $\kappa$ B gene expression in the nucleus (Ahmad and others 2000). EGCG has also been shown to inhibit IL-1 $\beta$  mediated activation of NF- $\kappa$ B in respiratory epithelial (A549) cells (Wheeler and others 2004). Additionally, EGCG inhibited the phosphorylation of the p65 subunit of NF- $\kappa$ B, which

resulted in the prevention of IL-8 gene expression in A549 cells (Chen and others 2002). GT polyphenols, like EGCG, appear to suppress the formation of numerous inflammatory molecules through inactivation of the NF- $\kappa$ B pathway. However, the interaction between polyphenols and multiple intracellular signaling pathways is unpredictable and not fully understood at this time (Santangelo and others 2007). Therefore, more research needs to be conducted to determine whether the consumption of EGCG has therapeutic effects in the prevention of chronic inflammatory disorders.

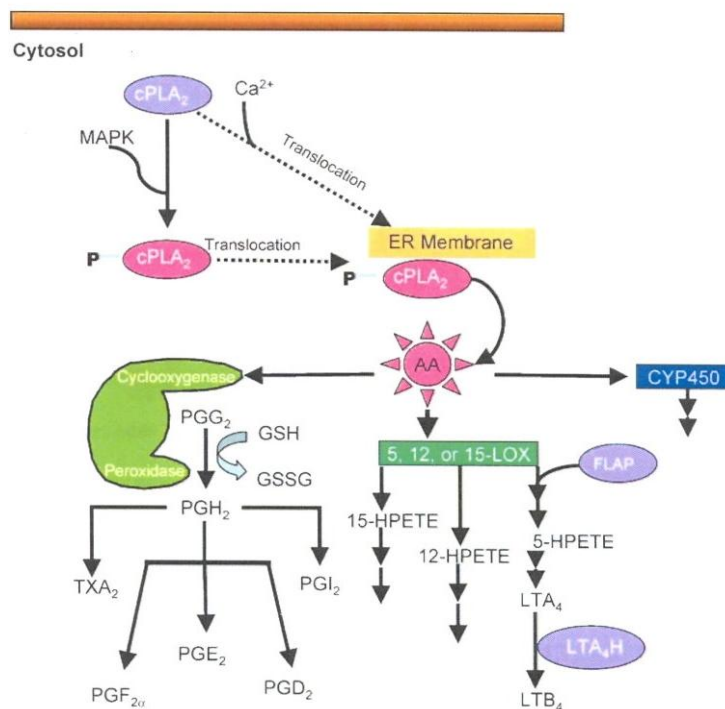
#### *1.10.3.3 COX-2*

Cyclooxygenase-2 (COX-2) is a pro-inflammatory membrane-bound protein that is a member of the COX family of enzymes (Figure 1.17). The other family members include COX-1 and COX-3. COX-1 is constitutively expressed in almost all tissues and is considered a housekeeping gene (Crofford 1997). COX-1 is responsible for the production of prostaglandins involved in many homeostatic functions such as preserving the gastric mucosa integrity, assisting normal platelet function and controlling renal blood flow. COX-3 is most abundantly found in human heart and cerebral cortex tissues and is selectively inhibited by acetaminophen and some non-steroidal anti-inflammatory drugs (NSAIDs) (Chandrasekharan and others 2002). COX-2 expression is highly inducible but tightly regulated. Under basal conditions in most tissues almost no COX-2 is expressed, however, during inflammation there is a dramatic increase in COX-2 production (Crofford 1997). COX-2 is produced mainly by macrophages, mast cells and fibroblasts (Santangelo and others 2007).



**Figure 1.17** Crystal structure of COX-2 (Kurumbail and others 1996)

COX-2 is involved in the oxidation of arachidonic acid (AA), a 20 carbon, polyunsaturated, omega-6 fatty acid. The oxidation of AA begins with the cleavage of AA from the phospholipid membrane by phospholipase A<sub>2</sub> (PLA<sub>2</sub>) (Smith and others 2000). AA is converted to prostaglandin (PG) G<sub>2</sub>, which is subsequently converted to PGH<sub>2</sub> in the presence of glutathione by the peroxidase activity of the COX enzymes (Hou and others 2004). PGH<sub>2</sub> can then be metabolized to a variety of eicosanoid molecules such as PGD<sub>2</sub>, PGF<sub>2α</sub>, PGI<sub>2</sub>, PGE<sub>2</sub> and thromboxane (TX) A<sub>2</sub> (Figure 1.18) (Dubois and others 1998). AA can also be metabolized by the LOX pathway. This involves the oxidation of AA by lipoxygenase-5 to 5-hydroperoxy-6,8,11,14-eicosatetraenoic acid (5-HPETE), which is then dehydrated to form leukotriene A<sub>4</sub> (Fordhutchinson and others 1994).



**Figure 1.18** Arachidonic acid oxidation pathway (Hou and others 2004)

COX-2 is linked with colon cancer inflammation because it was originally found in colon cancer tissue (Eberhart and others 1994). Therefore, compounds that inhibit COX-2 activation may be helpful in the prevention of inflammation-related chronic diseases like cancer and CVD. For example, non-steroidal anti-inflammatory drugs (NSAIDs), compounds known to inhibit COX-2 activity, have been linked to reduced colon cancer risk and tumor regression (Zha and others 2004). Other compounds have also demonstrated their ability to inhibit COX-2 inflammation. For example, an *in vitro* study found that EGCG significantly reduced COX-2 mRNA and protein over-expression in human colon cancer (HT-29) cells. Inhibition of NF- $\kappa$ B activation and a reduction in COX-2 promoter activity were found to be the mechanisms for reduced COX-2 expression (Peng and others 2006). In

contrast, EGCG has also been shown to have pro-inflammatory effects. For example, EGCG was found to up-regulate COX-2 expression levels in non-stimulated RAW 264.7 macrophage cells through activation of the extracellular signal-regulated protein kinase (ERK) and protein-tyrosine phosphatase pathways (Park and others 2001).

#### 1.10.3.4 PGE<sub>2</sub>

Prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) is an eicosanoid product of AA metabolism. It is produced when PGH<sub>2</sub> is isomerized to PGE<sub>2</sub> by a PGE<sub>2</sub> synthase (PGES) (Murakami and others 2003). Prostaglandins have a variety of functions including the regulation of smooth muscle contractions, blood platelets, neuronal activity and inflammatory responses (Narumiya and others 1999). When cells are activated, PGE<sub>2</sub> is synthesized *de novo* and released into the extracellular space. PGE<sub>2</sub> is typically found at a concentration of 3-12 pg/ml in the plasma and has a half life of 30 seconds in the circulatory system (Fitzpatrick and others 1980). PGE<sub>2</sub> is quickly metabolized to 13,14-dihydro-15-keto PGE<sub>2</sub> *in vivo* (Hamberg and Samuelss.B 1971; Granstrom and others 1980).

PGE<sub>2</sub> is produced as a result of stimulation with LPS and TNF- $\alpha$  and is also closely linked with colon cancer as it is the main product of the COX-2 enzyme during AA metabolism. PGE<sub>2</sub> is known to help promote cancer cell survival, growth, migration, invasion and angiogenesis (Chell and others 2006). Also, treatment with GT or EGCG can cause the production of PGE<sub>2</sub>. For example, treatment with EGCG in RAW 264.7 macrophage cells caused an increase in the production of PGE<sub>2</sub> (Park and others 2001).

#### *1.10.3.5 H<sub>2</sub>O<sub>2</sub>*

Hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) is a reactive oxygen species (ROS) formed from the incomplete reduction of oxygen (D'Autreaux and Toledano 2007). H<sub>2</sub>O<sub>2</sub> can be formed when EGCG is used as a treatment in cell culture studies (Long and others 2000; Hong and others 2002). Additionally, H<sub>2</sub>O<sub>2</sub> is also known to be a signaling molecule produced during inflammatory responses (Schreck and others 1991). For example, H<sub>2</sub>O<sub>2</sub> stimulated PLA<sub>2</sub> mediated AA release in intestinal epithelial cells (INT-407 cells), which caused an increase in pro-inflammatory PG and TX synthesis (Gustafson and others 1991). Similarly, in HeLa cells, H<sub>2</sub>O<sub>2</sub> stimulated the NF-κB pathway (Meyer and others 1993). Therefore, if cells are treated with EGCG, the formation of H<sub>2</sub>O<sub>2</sub> may cause a pro-inflammatory response to occur.

### **1.11 The Effect of Green Tea and EGCG Consumption on Chronic Diseases**

#### *1.11.1 Cancer*

Cancer is the second leading cause of death in the United States, with approximately 1.5 million new cases diagnosed in 2010 (American Cancer Society 2010). Approximately 50% of new cancer cases and 35% of cancer deaths in the U.S. have been attributed to poor diet (Williams and others 1999). Epidemiological studies have shown that people that consume diets rich in fruits and vegetables have a reduced risk of cancer incidence (Block and others 1992). This indicates that plant based diets, including GT consumption, may contribute to a reduced risk of cancer.

GT has been established as a potent antioxidant. Mechanisms for its anti-carcinogenic properties have been proposed, which include induction of phase II enzymes,

inhibition of TNF- $\alpha$  expression and release, cell cycle arrest and induction of apoptosis (Pham-Huy and others 2008). Specifically, EGCG has been shown to have anticarcinogenic effects *in vitro* by inhibiting the chymotrypsin-like activity of proteasomes, which contributes to the prevention of tumor growth (Nam and others 2001). However, epidemiological and clinical trial studies are mixed as to GT's potential as a chemopreventative agent (Pham-Huy and others 2008).

No association was found between GT consumption and reduced risk of cancer incidence (Nagano and others 2001; Nagao and others 2007) or mortality (Kuriyama and others 2006) when examining all cancers at once. However, prospective cohort studies have found an inverse relationship for GT consumption and cancer incidence for oral and gastric cancer in Japanese women (Sasazuki and others 2004; Ide and others 2007). Additionally, case-cohort studies have reported inverse relationships between GT consumption and cancer incidence for gastric, pancreatic and breast cancers (Ji and others 1996; Ji and others 1997; Wu and others 2003).

Most of these studies used an Asian population of which Japan was the most common country studied. Therefore, it is important to conduct epidemiological and clinical trial studies using other populations to further explore the relationship between GT consumption and cancer risk and mortality.

#### *1.11.1.1 Colon Cancer*

Colon cancer is of particular interest to this study because markers of inflammation associated with colon cancer incidence were measured and a colon cancer cell line was used

for experiments. Colon cancer was responsible for the third highest amount of new cancer cases diagnosed (142,570) and cancer deaths reported (51,370) for both men and women in the U.S. in 2010 (American Cancer Society 2010). Colon cancer incidence has been linked to a variety of factors including inflammation and diet (Marshall 2009).

Epidemiological studies do not provide conclusive evidence that the consumption of GT prevents colon cancer incidence. A prospective cohort study of 69,710 Chinese subjects found that the consumption of GT reduced the risk of colorectal cancer in Chinese women (Yang and others 2007). Additionally, a prospective cohort study of 14,001 elderly Japanese subjects found a protective effect of GT against colorectal cancer, indicating that GT consumption is beneficial to the elderly population (Suzuki and others 2009). On the other hand, the Singapore Chinese Health Study, a prospective cohort study of more than 60,000 people, showed that the consumption of GT had a non-statistically significant increase in risk of colorectal cancer compared to non GT drinkers (Sun and others 2007). A pooled analysis of two prospective cohort studies of more than 65,000 Japanese subjects found no link between GT consumption and decreased risk of colon cancer incidence (Suzuki and others 2005). Additionally, a comparative case-reference study (with 21,128 non-cancer patients and 1,706 cancer cases, of which 362 cases were colon cancer) in Japan found no correlation between GT consumption and colon cancer risk (Inoue and others 1998).

### ***1.11.2 Cardiovascular Disease***

Cardiovascular disease (CVD) is the leading cause of death in the U.S. and was responsible for 35.2% of all deaths in 2005. There are currently 80,700,000, or



approximately 1 in 3 people, with CVD in the U.S. CVD can be defined as all diseases that affect the circulatory system, which includes high blood pressure, stroke, coronary heart disease (myocardial infarction and angina pectoris) and heart failure (Rosamond and others 2008).

Epidemiological evidence most strongly supports a positive association between GT consumption and reduced mortality from CVD (Kuriyama 2008). For example, a prospective cohort study in Japan found an inverse relationship between GT consumption and mortality due to CVD and all causes; with a stronger relationship found in women, especially for cerebral infarction related deaths (Kuriyama and others 2006). A case-control study of Chinese men and women found that the consumption of GT had a dose dependent protective effect against atherosclerosis in Chinese men, however, no inverse relationship was found in Chinese women (Wang and others 2010b).

Over half of the randomized control trials that examined the relationship between GT consumption and CVD risk profiles found an inverse association (Kuriyama 2008). For example, a dietary intervention study that added 2 cups of GT (250 mg total catechins) to a controlled diet in 24 female subjects for 42 days found a significant decrease in LDL cholesterol as well as a significant increase in plasma total antioxidant level (Erba and others 2005). Another study with 30 subjects also found an increase in plasma total antioxidant activity 8 hours after GT extract (193.3 mg EGCG) consumption (Henning and others 2004). Additionally, subjects that consumed GT (583 mg catechins per day) for 12 weeks had a significant decrease in serum LDL cholesterol, body weight and blood pressure compared to subjects that consumed GT with only 96 mg catechins per day (Nagao and others 2007).

### ***1.11.3 Diabetes***

Diabetes mellitus is a serious disease that affects 25.8 million children and adults, or 8.3% of the population, in the U.S. alone. Diabetes mellitus was the seventh leading cause of death in the U.S. in 2006 (American Diabetes Association 2011). Diabetes is a group of disorders that commonly present themselves as hyperglycemia and glucose intolerance that can be attributed to insulin resistance, insulin deficiency or both. Type-1 diabetes is associated with insulin deficiency due to a lack of insulin producing cells in the pancreas. This type of diabetes only affects about 5-10% of all people with diabetes. Type-2 diabetes is associated with insulin resistance and relative insulin deficiency. It is estimated that 90-95% of the population with diabetes mellitus have type-2 diabetes. Type-2 diabetes is associated with obesity, diet and physical inactivity (International Diabetes Federation 2010). Diet is one way to manage the risk of type-2 diabetes and the consumption of GT is a dietary change that may be helpful in preventing the onset of type-2 diabetes.

Several cell culture and animal studies have explored the effect of GT on glucose metabolism and diabetes. GT increased insulin sensitivity in rat adipocytes by enhancing insulin-stimulated glucose uptake. EGCG had the greatest effect of any of the tea components (Anderson and Polansky 2002). Additionally, in adipocytes treated with low doses of EGCG (5-10  $\mu\text{M}$ ), genes associated with increased insulin sensitivity were induced (Sakurai and others 2009). An animal study involving normal and alloxan diabetic rats found that the consumption of GT polyphenols (500 mg/kg by weight) significantly increased glucose tolerance in normal rats and significantly reduced the serum glucose level in the

diabetic rats (Sabu and others 2002). Additionally, GT extract alleviated hyperglycemia, a common symptom of diabetes, in rats (Ramadan and others 2009).

Human epidemiological studies on the relationship between GT consumption and the incidence of type-2 diabetes are inconclusive. There are studies that show a protective effect from consuming GT, while others show no effect. For example, a protective effect of GT in relation to type-2 diabetes was shown in a retrospective cohort study of 17,413 Japanese adults (Iso and others 2006). This study found that adults that consumed greater than 6 cups of GT/day (1440 ml/day) had a 33% reduction in their risk of diabetes incidence. There was an issue with follow-up response rate, however, with only 6% of the original subjects reporting after 5 years.

In contrast, many studies did not find a link between GT consumption and a reduced risk of type-2 diabetes. In a prospective cohort study, called the Singapore Chinese Health Study, the association between the consumption of coffee, BT and GT with type-2 diabetes incidence was investigated. This study of 36,908 Chinese subjects found that there was no association between the consumption of GT and a reduced incidence of type-2 diabetes at all levels of GT consumption (Odegaard and others 2008). Additionally, a study by Oba and others (2010) also found no association between the consumption of GT and type-2 diabetes incidence in Japanese men and women (Oba and others 2010).

There have been few clinical intervention studies on the relationship between GT consumption and type-2 diabetes risk factors to date. A study of 55 type-2 diabetes patients that consumed GT (900 ml water with 9 g GT) for 4 weeks did not find a relationship between GT consumption and reduced risk of type-2 diabetes as blood glucose and insulin

resistance levels did not change over the course of the study (Ryu and others 2006). Another study of overweight and obese males that consumed EGCG (400 mg, twice daily) for 8 weeks, found no reduction in insulin sensitivity, insulin secretion or glucose tolerance (Brown and others 2009).

## **1.12 SUMMARY AND CONCLUSIONS**

Research shows that GT and probiotic bacteria have beneficial health effects individually, however, there is limited evidence on the effect of GT on probiotic bacteria growth. Therefore, it is important to conduct studies to determine whether GT could act as a prebiotic in the gut and increase the growth rate or total growth of probiotic bacteria in the colon, thereby conferring a greater health benefit on the host.

There have been numerous *in vitro*, animal, epidemiological and clinical intervention studies investigating the effect of GT and EGCG on health. While there is strong evidence from *in vitro* and animal studies that GT or EGCG have a beneficial effect on health, findings from human studies have been inconclusive. Therefore, it is necessary to further understand the mechanisms by which GT and EGCG exert their health effects in order to elucidate whether the consumption of GT can be recommended to prevent the incidence of chronic diseases like colon cancer.

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**CHAPTER 2: Effects of Green Tea and EGCG on the Growth Rate and Total Growth  
of Probiotic Bacteria**

## 2.1 ABSTRACT

Green tea (GT) and probiotic bacteria have been reported to have beneficial health effects individually. The purpose of this study was to determine whether GT or epigallocatechin-3-gallate (EGCG), the main flavonoid component of GT, served as prebiotics, as measured by increasing cell growth rate and cell density of the probiotic bacteria strains *Lactobacillus acidophilus* NCFM (*L. acidophilus*) and *Lactobacillus gasseri* ATCC 33323 (*L. gasseri*). Aqueous GT and EGCG extracts were prepared and matched for total phenol content. Growth rate was determined using a 96-well microtiter plate assay. Cell density was assessed via optical density at 600 nm. Plate counts (CFU/ml) were determined by a plate reader. GT (1, 2, 10, 20, 30 and 40%) and EGCG (1, 2 and 10%) appeared to enhance the maximum specific growth rate (MSGR) of *L. acidophilus* relative to the control. GT (4, 10, 20, 30, and 40%) and EGCG (1, 2, 4, 10, and 20%) also appeared to enhance the MSGR of *L. gasseri* relative to the control. GT (10 and 30%) and EGCG (1%) increased the apparent cell density of both *L. acidophilus* and *L. gasseri* relative to the control. GT (1 and 30%) and EGCG (1%) did not significantly increase cell plate counts for *L. acidophilus* and GT (1%) and EGCG (1%) did not significantly increase cell plate counts for *L. gasseri* relative to the controls. In summary, GT and EGCG appeared to increase the growth rate and cell density of *L. acidophilus* and *L. gasseri* when estimated via optical density measurements. Plate count experiments did not confirm a positive effect on cell growth for either strain. Therefore, this study does not support the hypothesis that GT or EGCG function as prebiotics for *L. acidophilus* NCFM or *L. gasseri* ATCC 33323.

## 2.2 INTRODUCTION

Tea is one of the most popular beverages worldwide, second only to water (Chen and others 2008). Green tea (GT) and epigallocatechin 3-gallate (EGCG), the main phenolic component of GT, have been shown to have antioxidant, anti-carcinogenic, anti-mutagenic and anti-inflammatory activities (Fujiki 1999). Lactic acid bacteria have also been shown to have anti-carcinogenic, anti-mutagenic and anti-inflammatory activities, as well as the ability to reduce blood pressure, reduce the symptoms of lactose intolerance, decrease the incidence and duration of diarrhea and sustain mucosal lining integrity (Klaenhammer 2000). Probiotic bacteria, a type of lactic acid bacteria, confer a health benefit on the host when consumed in high enough quantities (FAO/WHO 2001). GT and probiotic bacteria have each been reported to have beneficial health effects, however, there is little research on whether consuming GT and probiotic bacteria together could maximize potential health benefits. The few *in vitro* and animal studies that have been conducted show that GT increases or maintains the concentration of probiotic bacteria (Ishihara and others 2001; Molan and others 2009; Shah and others 2010). Therefore, it is important to continue to study whether GT or EGCG has a positive effect on probiotic bacteria growth and to determine how GT and EGCG are eliciting this result if a positive effect is observed.

The purpose of this experiment is to determine the effect of varying concentrations of GT or EGCG on the growth rate and total growth of the probiotic bacteria strains *Lactobacillus acidophilus* NCFM (*L. acidophilus*) and *Lactobacillus gasseri* ATCC 33323 (*L. gasseri*). Growth rate will be determined through optical density measurements and total growth will be measured through plate count experiments. We predict that GT or EGCG will

increase the growth rate and total growth of *L. acidophilus* NCFM and *L. gasseri* ATCC 33323 at one or more of the concentrations tested (1, 10, 30%).

## **2.3 MATERIALS AND METHODS**

### ***2.3.1 Preparation of Green Tea and EGCG***

Sencha GT (Maeda-en; Irvine, CA) was prepared by steeping 1 g of loose-leaf tea in 100 ml deionized (DI) water for 5 minutes. Liquid was decanted away from loose GT leaves. GT solution was centrifuged at 130 g for 2 minutes. Supernatant (GT) was removed and aliquoted. EGCG (>95%, Sigma; St. Louis, MO) was prepared in the same manner as GT at a 1.8 mM concentration to match the EGCG phenol content of GT (Nutrient Data Laboratory U.S. Department of Agriculture 2007). The total phenol content of the GT and EGCG treatments was measured via the Folin-Ciocalteu method described previously by Parejo and others (Parejo and others 2002).

### ***2.3.2 Preparation of Media***

MRS Lactobacilli Broth (Difco Laboratories Inc.; Detroit, MI) was prepared according to manufacturer's instructions. Semi-Defined Medium (SDM) was prepared as previously described (Kimmel and Roberts 1998). Media was aliquoted and autoclaved before use.

### **2.3.3 Microtiter Plate Experiment**

#### **2.3.3.1 Preparation of 1, 2, 4, 10, 20, 30 and 40% GT and EGCG Treatments**

Frozen cultures of *Lactobacillus acidophilus* NCFM (propagated from frozen stock) and *Lactobacillus gasseri* ATCC 33323 (ATCC; Manassas, VA) were propagated in MRS broth and incubated overnight at 37°C before use. For experiments, a 1% inoculation (0.1 ml) using the bacteria grown overnight was made into SDM (amounts varied depending on GT and EGCG concentration). Then, the corresponding amount of GT or EGCG was added to make the 1, 2, 4, 10, 20, 30 and 40% GT and EGCG treatments.

#### **2.3.3.2 Microtiter Plate Protocol**

Cell growth was monitored in 96 well plates at OD<sub>600nm</sub> using a BMB Lab Tech FLUOstar Optima (Offenburg, Germany) plate reader. The cultures were grown in 200 µL of media for 22 hours at 37°C.

### **2.3.4 Growth Rate Curve Experiments**

#### **2.3.4.1 Preparation of 1, 10, and 30% GT and EGCG Treatments**

Frozen initial starter cultures of *L. acidophilus* NCFM and *L. gasseri* ATCC 33323 were inoculated into 10 ml of MRS broth and incubated overnight at 37°C before use. For experiments, a 1% inoculation (0.1 ml) using the bacteria grown overnight was made into SDM (amounts varied depending on GT and EGCG concentration) in a test tube. The corresponding amount of GT or EGCG was then added to the test tube to make the 1, 10 and 30% GT and EGCG treatments (i.e. for a 10% GT treatment, 1 ml of GT was added to 8.9 ml

of SDM). A final volume of 10 ml was reached in each test tube. All conditions were run in triplicate.

#### *2.3.4.2 Growth Rate Curve Experiment Protocol*

After test tubes were inoculated, the broth cultures were incubated at 37°C in between readings. Readings were taken at OD<sub>600 nm</sub> using a Spectronic® Genesys™ 20 spectrophotometer (Spectronic Instruments, Inc.; Rochester, NY) at varying time points (typically over a 12 hour period).

#### *2.3.5 Plating Experiments*

The 1, 10 and 30% GT and EGCG treatments in SDM with *L. acidophilus* and *L. gasseri* were prepared as described above. The test tubes containing the cultures were stored at 37°C in between plate count data collection using a Whitley Automatic Spiral Plater (Don Whitley Scientific Ltd.; West Yorkshire, England). Plate count data was collected over a 30-40 hour period. After all the treatments were plated, the MRS plates were stored inverted in an anaerobic chamber for 24 hours. Plate counts (CFU/ml) were determined using a ProtoCOL colony counter (Microbiology International; Frederick, MD).

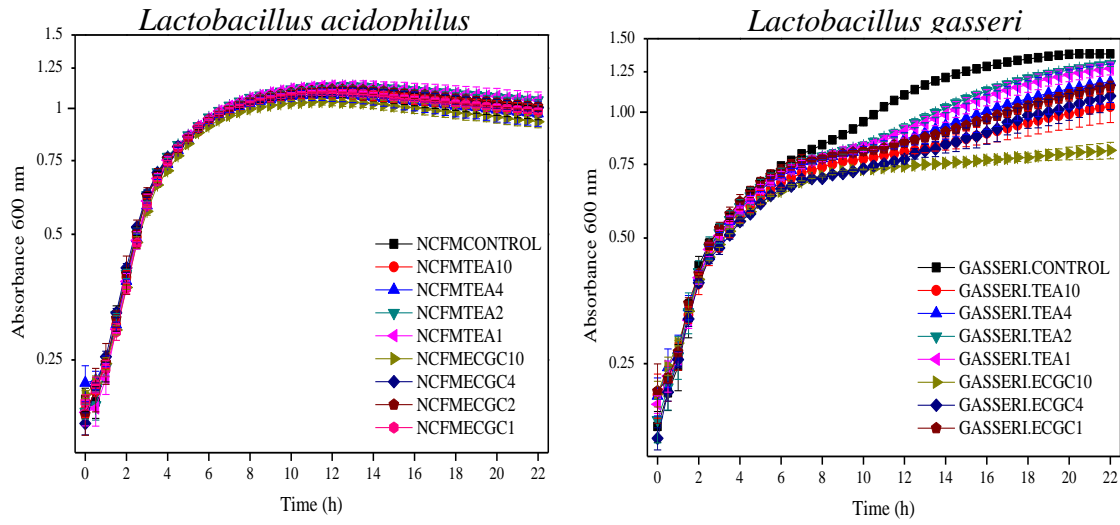


## 2.4 RESULTS AND DISCUSSION

### 2.4.1 Microtiter Plate Experiment

The microtiter plate experiment was used as a way to screen numerous combinations of GT and EGCG with *L. acidophilus* and *L. gasseri*. It served a way to collect preliminary data to determine which concentrations of GT and EGCG to use in future experiments.

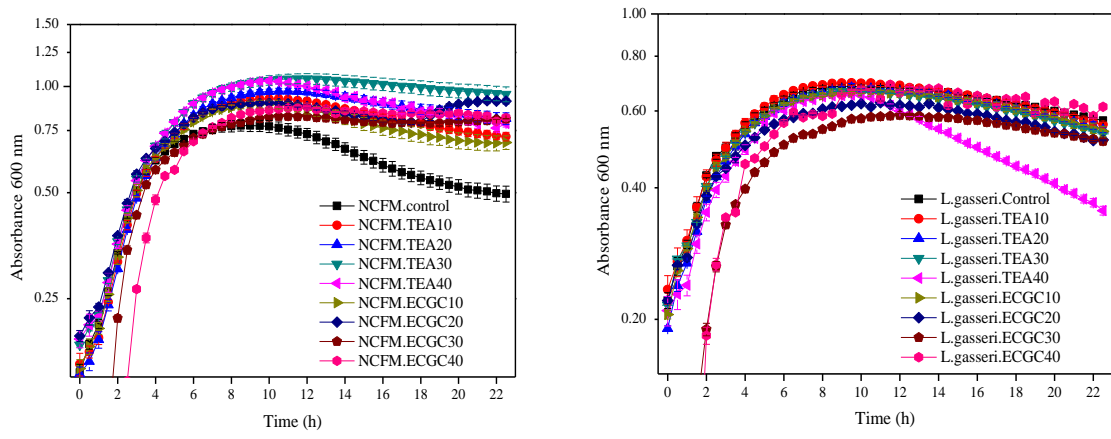
Results of the microtiter plate experiment showed that GT (1, 2, 10, 20, 30 and 40%) and EGCG (1, 2 and 10%) enhanced the apparent maximum specific growth rate (MSGR) of *L. acidophilus* relative to the control (Figure 2.1, Table 2.1). Additionally, GT (4, 10, 20, 30, and 40%) and EGCG (1, 2, 4, 10, and 20%) enhanced the apparent MSGR of *L. gasseri* relative to the control (Figure 2.2, Table 2.2). Based on these results, the concentrations of 1, 10 and 30% GT and EGCG were chosen for future experiments as these cover a large range of concentrations and would provide the best opportunity to see any effect that GT or EGCG may have on probiotic bacteria growth rate and total growth.



**Figure 2.1** Microtiter plate growth rate curves for *Lactobacillus acidophilus* NCFM and *Lactobacillus gasseri* ATCC 33323 with varying concentrations of GT and EGCG (low doses)

**Table 2.1** Maximum specific growth rates of *Lactobacillus acidophilus* NCFM and *Lactobacillus gasseri* ATCC 33323 with varying concentration of GT and EGCG (low doses)

<b>Strain</b>	<b>Media</b>	<b>Maximum Specific Growth Rate (<math>\mu \text{ h}^{-1}</math>)</b>	<b>Standard Deviation</b>
<i>L. acidophilus</i> NCFM	SDM	0.193	0.009
	SDM + 10% Tea	0.188	0.007
	SDM + 4% Tea	0.192	0.001
	SDM + 2% Tea	0.200	0.003
	SDM + 1% Tea	0.211	0.013
	SDM + 10% ECGC	0.189	0.010
	SDM + 4% ECGC	0.188	0.006
	SDM + 2% ECGC	0.196	0.021
	SDM + 1% ECGC	0.212	0.003
<i>L. gasseri</i> ATCC 33323	SDM	0.105	0.008
	SDM + 10% Tea	0.105	0.004
	SDM + 4% Tea	0.111	0.006
	SDM + 2% Tea	0.106	0.009
	SDM + 1% Tea	0.102	0.007
	SDM + 10% ECGC	0.089	0.003
	SDM + 4% ECGC	0.100	0.009
	SDM + 2% ECGC	0.112	0.020
	SDM + 1% ECGC	0.110	0.010



**Figure 2.2** Microtiter plate growth rate curves for *Lactobacillus acidophilus* NCFM and *Lactobacillus gasseri* ATCC 33323 with varying concentrations of GT and EGCG (high doses)

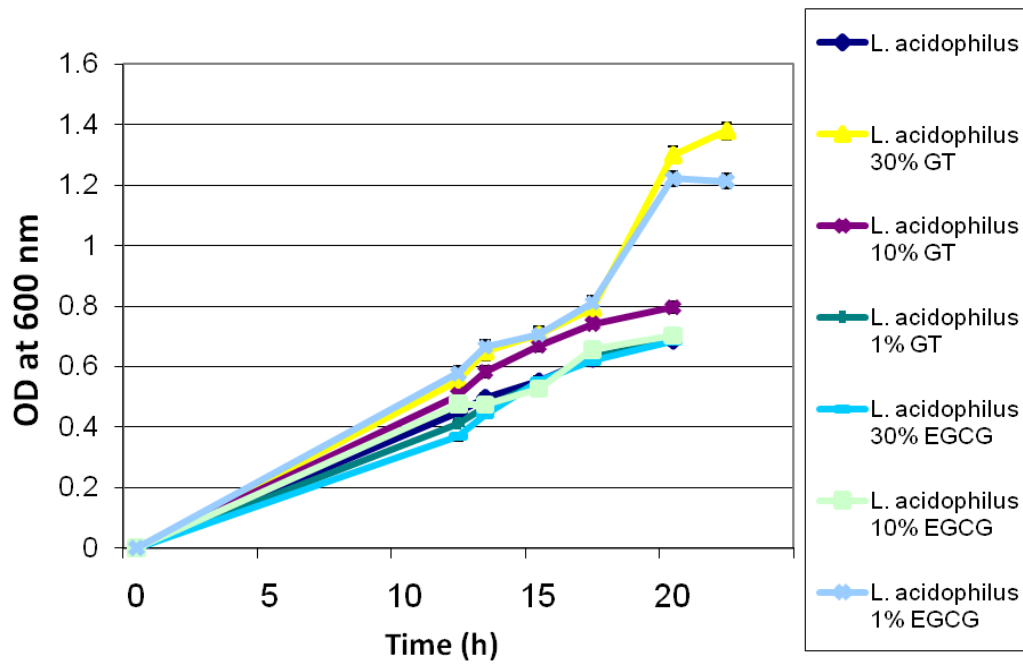
**Table 2.2** Maximum specific growth rates of *Lactobacillus acidophilus* NCFM and *Lactobacillus gasseri* ATCC 33323 with varying concentration of GT and EGCG (high doses)

Strain	Media	Maximum Specific Growth Rate ( $\mu \text{ h}^{-1}$ )	Standard Deviation
<i>L. acidophilus</i> NCFM	SDM	0.175	0.008
	SDM + 10% Tea	0.189	0.006
	SDM + 20% Tea	0.207	0.018
	SDM + 30% Tea	0.189	0.007
	SDM + 40% Tea	0.189	0.009
	SDM + 10% EGCG	0.183	0.007
	SDM + 20% EGCG	0.168	0.010
	SDM + 30% EGCG	0.089	0.007
	SDM + 40% EGCG	0.106	0.012
<i>L. gasseri</i> ATCC 33323	SDM	0.079	0.006
	SDM + 10% Tea	0.091	0.005
	SDM + 20% Tea	0.100	0.008
	SDM + 30% Tea	0.085	0.007
	SDM + 40% Tea	0.088	0.007
	SDM + 10% EGCG	0.088	0.007
	SDM + 20% EGCG	0.082	0.001
	SDM + 30% EGCG	0.034	0.001
	SDM + 40% EGCG	0.047	0.005

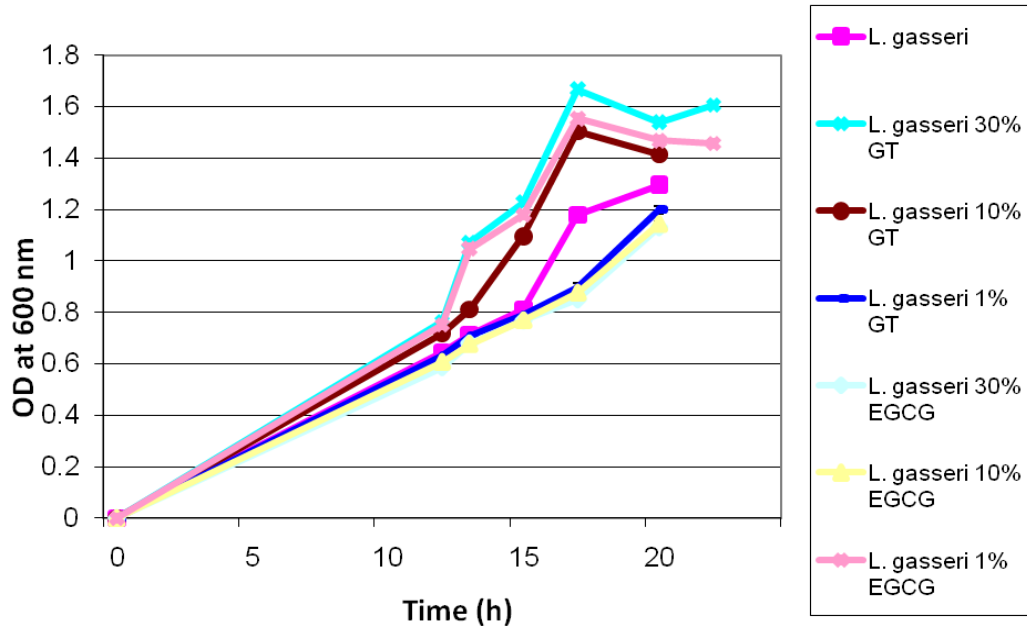
#### 2.4.2 Growth Rate Curve Experiment

The purpose of the growth rate curve experiment was to determine whether GT or EGCG (1, 10, 30%) enhanced the growth rate or total growth of the probiotic bacteria *L. acidophilus* and *L. gasseri* in SDM. The growth rate curve experiments showed that GT (10 and 30%) and EGCG (1%) increased the apparent cell density of *L. acidophilus* NCFM (Figure 2.3) and *L. gasseri* ATCC 33323 (Figure 2.4) compared to the controls during the 12-24 time period of growth (*L. acidophilus* and *L. gasseri* alone). Previous studies have shown that GT or EGCG may act as a prebiotic causing an increase in probiotic bacteria growth

(Molan and others 2009). Additionally, it is possible that the GT or EGCG had an effect on growth due to hormesis.



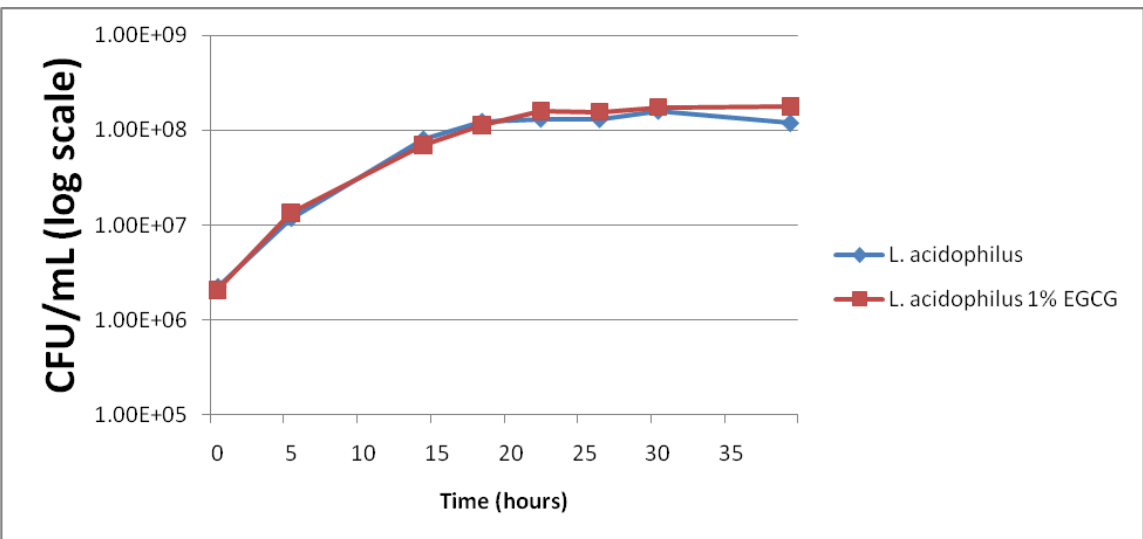
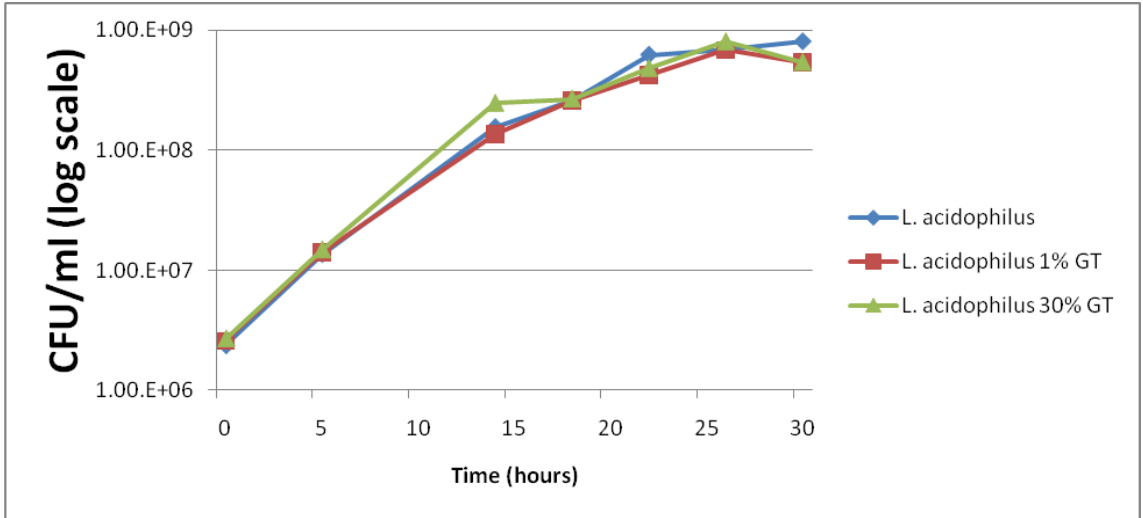
**Figure 2.3** Growth rate curves of *L. acidophilus* NCFM in 30%, 10% and 1% GT and EGCG in SDM



**Figure 2.4** Growth rate curves of *L. gasseri* ATCC 33323 in 30%, 10% and 1% GT and EGCG in SDM

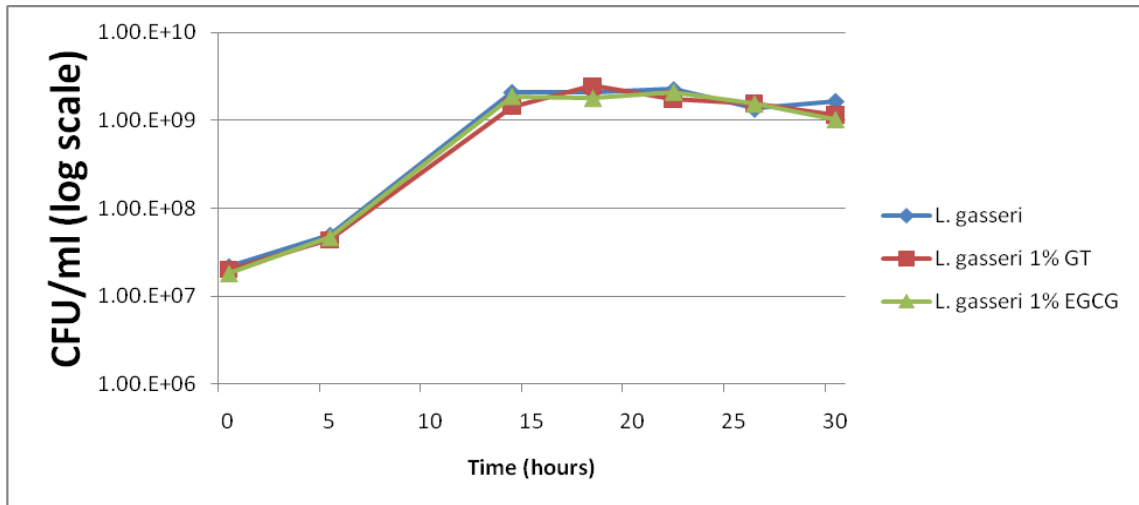
### 2.4.3 Plate Count Experiments

Plate count experiments were conducted to confirm the results of the growth rate curve experiments. These experiments showed that GT (1, 30%) and EGCG (1%) did not significantly increase the bacterial plate counts of *L. acidophilus* (Figure 2.5). In the same manner, GT (1%) and EGCG (1%) did not significantly increase the bacterial plate counts of *L. gasseri* (Figure 2.6). Significance was defined as a one log difference in CFU/ml. It was determined that GT and EGCG did not have a significant effect on the growth of *L. acidophilus* or *L. gasseri* bacteria as there was not a log difference in CFU/ml between the controls and treatment groups.



**Figure 2.5** Plate Counts of *Lactobacillus acidophilus* NCFM GT (1%, 30%) and EGCG (1% treatments)





**Figure 2.6** Plate Counts of *Lactobacillus gasseri* ATCC 33323 with GT (1%) and EGCG (1% treatments)

## 2.5 CONCLUSIONS

Preliminary data based on OD measurements indicate that growth was increased by GT and EGCG. Plate count data did not confirm this finding, however, as there was no significant difference (as indicated by a one log difference) between the growth of bacteria treated with GT or EGCG and the control. This experiment showed that GT and EGCG did not influence the total growth of the probiotic bacteria *L. acidophilus* NCFM and *L. gasseri* ATCC 33323 and therefore, does not support the hypothesis that GT or EGCG act as prebiotics for the probiotic bacteria *L. acidophilus* NCFM or *L. gasseri* ATCC 33323.

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**CHAPTER 3: The Effects of Green Tea and EGCG on H<sub>2</sub>O<sub>2</sub>, COX-2 and PGE<sub>2</sub>  
Production in LPS-Induced, Differentiated Human Colon Cancer (HT-29) Cells**

### 3.1 ABSTRACT

Green tea (GT) and epigallocatechin-3-gallate (EGCG) have demonstrated anti-inflammatory and chemopreventative effects in colon cancer. EGCG has also been reported to cause the formation of hydrogen peroxide ( $H_2O_2$ ), a pro-inflammatory species, *in vitro*.

The purpose of this study was to examine the effects of GT and EGCG on the production of prostaglandin  $E_2$  ( $PGE_2$ ) and the expression and activity of cyclooxygenase-2 (COX-2) in lipopolysaccharide (LPS)-induced, differentiated human colon cancer (HT-29) cells.

Differentiated cells were co-treated with LPS 055:B5 (1 ng/ml) and three concentrations of GT or EGCG (0.0045, 0.009 and 0.045 mg/ml) for 24 hours. ELISA and  $H_2O_2$  assay (FOX-2 method) results indicated that increasing concentrations of GT and EGCG showed a trend toward increased  $PGE_2$  and  $H_2O_2$  formation. The highest GT and EGCG treatments (0.045 mg/ml) were significantly different from the control ( $p < 0.05$ ). Replacing GT and EGCG treatments with corresponding amounts of  $H_2O_2$  showed a similar trend compared the GT/EGCG  $PGE_2$  ELISA results, suggesting that  $H_2O_2$  may have been involved in the observed increases in  $PGE_2$ . Treatments containing catalase did not show decreased  $PGE_2$  and COX-2 formation, indicating that the observed increases in  $PGE_2$  were not exclusively due to a  $H_2O_2$ -mediated inflammatory response. Additionally, differences in COX-2 expression and activity were not observed, indicating that another mechanism besides COX-2-mediated inflammation caused the increase in  $PGE_2$  production. This study highlights the need to study the complex interactions of reactive oxygen species and inflammatory responses in order to fully understand the effects of foods on health.

### 3.2 INTRODUCTION

Cancer is the second leading cause of death in the United States (American Cancer Society 2010). Of all types of cancer, colon cancer was responsible for the third highest amount of new cases diagnosed and deaths in the U.S. in 2010 (American Cancer Society 2010). Chronic inflammation and diet have been linked with colon cancer incidence, which indicates that modifications to the diet may reduce the risk of colon cancer (Marshall 2009).

Green tea (GT) and epigallocatechin-3-gallate (EGCG), the major phenolic component of GT, have been shown to possess anti-inflammatory properties (Butt and Sultan 2009). However, EGCG has also been associated with the production of hydrogen peroxide ( $H_2O_2$ ), a pro-inflammatory reactive oxygen species, *in vitro* (Long and others 2000; Hong and others 2001; Hong and others 2002). Therefore, it is still unclear whether the consumption of GT or EGCG elicits a protective effect against colon cancer incidence through reducing chronic inflammation or whether they may increase inflammation via oxidative stress.

A variety of inflammatory markers are associated with colon cancer incidence. For this study two markers of inflammation, cyclooxygenase-2 (COX-2) and prostaglandin  $E_2$  ( $PGE_2$ ), were chosen for their strong correlation with colon cancer. COX-2 was initially discovered in colon cancer tissue and is the rate limiting step in the oxidation of arachidonic acid (AA) (Eberhart and others 1994). Additionally, compounds that inhibit COX-2 activity, like non-steroidal anti-inflammatory drugs (NSAIDs), have been linked to decreased colon cancer risk (Zha and others 2004).  $PGE_2$  is also strongly correlated with colon cancer because it is the main product of COX-2 enzyme activity in AA oxidation and has been

found to be elevated in colon cancer tissue samples (Rigas and others 1993). It is known to promote cancer cell survival, growth, migration, invasion and angiogenesis (Chell and others 2006). As a result, compounds that suppress the expression of COX-2 and PGE<sub>2</sub> have the potential to decrease the risk of colon cancer incidence, while compounds that increase them, may increase the risk of incidence.

This study aimed to simulate the environment of the human colon to make the results of these cell culture experiments more relevant to humans. In order to more closely approximate the actual environment of the human colon lumen, differentiated human colon cancer (HT-29) cells were used in this study. Differentiated cells vary from undifferentiated cells in their level of sensitivity to various stimuli, such as cytotoxic agents, inducers of apoptosis and inhibitors of proliferation (Kovarikova and others 2004). Additionally, differentiated HT-29 cells showed greater sensitivity to TNF- $\alpha$  and had increased expression of COX-2 compared to undifferentiated cells (Kovarikova and others 2004). Sodium butyrate (NaBt) was used to differentiate the cells because butyrate is naturally present in the colon at millimolar concentrations and is a natural byproduct of carbohydrate fermentation (Cummings 1981).

Many studies have explored the anti-inflammatory effects of GT and EGCG *in vitro* using numerous compounds to induce inflammation. One study examined the effect of EGCG on the secretion of interleukin-8 (IL-8), macrophage inflammatory protein (MIP)-3 $\alpha$  and PGE<sub>2</sub> in TNF- $\alpha$  stimulated HT-29 cells and determined that EGCG dose-dependently decreased the secretion and gene expression of the inflammatory markers (Porath and others 2005). To the author's knowledge, there have not been any studies on the effect of GT or

EGCG on the inflammatory markers of COX-2 and PGE<sub>2</sub> in LPS-induced, differentiated HT-29 cells. Therefore, the purpose of this study was to observe the effect of GT and EGCG on LPS-induced inflammation in differentiated HT-29 cells by measuring the production and expression of PGE<sub>2</sub> and COX-2. Reduced expression of COX-2 or PGE<sub>2</sub> would indicate that GT or EGCG possesses anti-inflammatory properties and, therefore, may be helpful in reducing the risk of colon cancer incidence.

Here we report that GT and EGCG increased PGE<sub>2</sub> production in HT-29 human colon cancer cells. We explore multiple possible mechanisms for the observed increase in PGE<sub>2</sub> production that include: H<sub>2</sub>O<sub>2</sub>-mediated inflammation, PGE<sub>2</sub> synthase activity differences with COX isoforms and PGE<sub>2</sub> ELISA cross-reactivity.

### **3.3 MATERIALS AND METHODS**

#### ***3.3.1 Cell Culture***

Human colon cancer, HT-29 (HTB), cells were obtained from American Type Culture Collection (ATCC; Manassas, VA) and maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen; Carlsbad, CA) with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin-glutamine at 37°C in an atmosphere of 5% CO<sub>2</sub>.

#### ***3.3.2 Preparation of Green Tea and EGCG***

Sencha GT (Maeda-en; Irvine, CA) was prepared by steeping 1 g of loose-leaf tea in 100 ml boiling deionized (DI) water for five minutes. After steeping the liquid was decanted away from loose GT leaves and centrifuged at 130 g for 2 minutes. Supernatant (GT) was

removed, aliquoted for later use, and stored at -80°C. EGCG (>95%, Sigma; St. Louis, MO) was prepared in the same manner as GT. The total phenol content of the GT and EGCG treatments was measured via the Folin-Ciocalteu method described previously by Parejo and others (Parejo and others 2002). (See Appendix C for protocol).

### ***3.3.3 Differentiation and Treatment of Cells***

HT-29 cells were plated at a concentration of  $4 \times 10^5$  cells/ml in 12 well plates and incubated at 37°C and 5% CO<sub>2</sub>. Once confluent, cells were exposed to sodium butyrate (NaBt, 5 mM) for 24 hours to induce differentiation, which was confirmed by alkaline phosphatase (ALP) assay (Biovision; Mountain View, CA). Cells were treated with GT and EGCG (0.0045, 0.009, 0.045 mg/ml), with or without LPS (O55:B5, 1 ng/ml, Sigma; St. Louis, MO) and incubated at 37°C and 5% CO<sub>2</sub>. After 24 hours cells were harvested for media and cell lysate samples using a protein extraction reagent and protease inhibitors.

### ***3.3.4 Cell Viability***

Cell viability was determined using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenoltetrazolium) assay based on the method developed by Mosmann (Mosmann 1983). Twenty-four hours after LPS mediated inflammation was induced, 16 µl of MTT solution (7.8 mg/ml) was added to each well. After a 2 hour incubation at 37°C and 5% CO<sub>2</sub> to allow for formazan crystal formation, the media was removed from each well. The formazan crystals were re-suspended in acidified iso-propanol (0.5 ml). Absorbance was measured at 620 nm using a plate reader (Thermo Electron Corporation; Vantaa, Finland).



### **3.3.5 PGE<sub>2</sub> ELISA**

The PGE<sub>2</sub> monoclonal EIA enzyme linked immuno-sorbent assay (ELISA) kit was purchased from the Cayman Chemical Company (Ann Arbor, MI). The concentration of PGE<sub>2</sub> in the harvested media samples was determined by following the manufacturer's instructions.

### **3.3.6 H<sub>2</sub>O<sub>2</sub> Assay**

The formation of H<sub>2</sub>O<sub>2</sub> was measured using a modified version of the FOX-2 reagent method previously described by Nakagawa (Nakagawa and others 2004). Immediately after treatment with GT and EGCG, 1 ml of FOX-2 reagent (250 μM ammonium iron(II) sulfate, 25 mM H<sub>2</sub>SO<sub>4</sub>, 100 mM sorbitol and 125 μM xylenol orange (Sigma)) was added to 0.1 ml of each sample, vortexed and incubated at room temperature for 20 minutes. Following incubation, the absorbance was measured at 595 nm using a spectrophotometer (Spectronic Instruments, Inc.; Rochester, NY). A standard curve with H<sub>2</sub>O<sub>2</sub> (Sigma) concentrations of 0, 1, 5, 10, 25, 50 and 100 μM was used to determine the unknown H<sub>2</sub>O<sub>2</sub> concentration in each sample.

### **3.3.7 COX-2 Western Blot Analysis**

Cell lysate protein concentration was determined by the BCA protein assay using bovine serum albumin as the standard (Pierce; Rockford, IL). Cell lysate samples were matched for protein content, prepared with SDS-loading buffer and boiled for 10 minutes.

SDS-PAGE was run on Invitrogen equipment and 12% Tris-Glycine gels. Proteins were transferred to a PVDF membrane.

COX-2 was detected by Western Blot analysis. Polyclonal COX-2 (murine, host:rabbit) primary antibody was purchased from Cayman Chemical Company. Anti-rabbit IgG horseradish peroxidase conjugate secondary antibody was purchased from Cell Signaling Technology, Inc. (Danvers, MA). The PVDF membrane was washed in a tris buffered saline with tween (0.05%) (TBS-T) solution then blocked in a TBS-T solution with 5% non-fat dry milk (NFDM) for 1.5 hours. The membrane was incubated with the primary COX-2 antibody (1:100, 2% NFDM in TBS-T) overnight at 4°C and incubated in secondary antibody (1:1000, 2% NFDM in TBS-T) for 1 hour at room temperature. The membrane was visualized with Super Signal West Femto Maximum Sensitivity Substrate (Pierce). Beta actin (1:1000, Cell Signaling, Inc.) was used as a control.

### ***3.3.8 COX-2 Activity Assay***

The COX Activity Assay was purchased from Cayman Chemical Company. The COX-2 activity level for the cell lysate samples was determined following the manufacturer's instructions.

### ***3.3.9 Statistical Analysis***

Results are representative of three independent experiments with all treatments run in triplicate. Values are reported as means  $\pm$  standard error (SE). Statistical analyses were performed using SAS software (Cary, NC). Significant differences ( $p < 0.05$ ) between

treatment means were determined by conducting analysis of variance (ANOVA) analyses with Tukey's adjustment. Treatments labeled with the same letter are not statistically different. Treatments labeled with an asterisk (\*) are statistically different from a given reference.

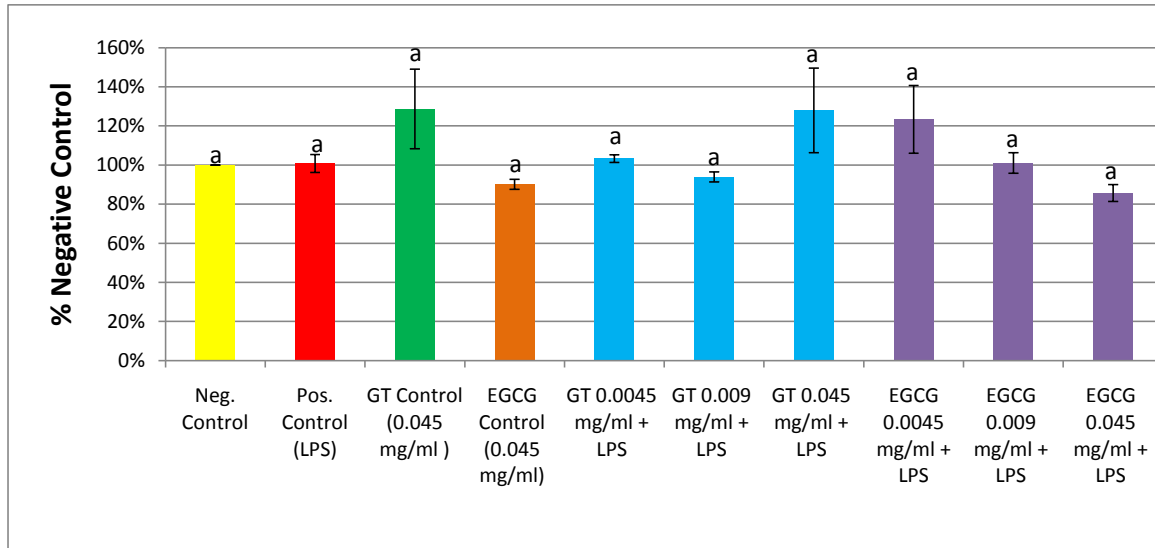
### **3.4 RESULTS AND DISCUSSION**

#### ***3.4.1 Cell Viability Assay***

The results of the cell viability (MTT) assay are shown in Figure 3.1. Cell viability is reported as a percent of the negative control. The ten proposed treatments for the differentiated HT-29 cells were:

- 1.) Negative Control (cells + NaBt (5 mM))
- 2.) Positive Control (cells + NaBt (5 mM) + LPS O55:B5 (1 ng/ml))
- 3.) GT Control (cells + NaBt (5 mM) + 0.045 mg/ml GT)
- 4.) EGCG Control (cells + NaBt (5 mM) + 0.045 mg/ml EGCG)
- 5.) 0.0045 mg/ml GT (cells + NaBt (5 mM) + 0.0045 mg/ml GT + LPS)
- 6.) 0.009 mg/ml GT (cells + NaBt (5 mM) + 0.009 mg/ml GT + LPS)
- 7.) 0.045 mg/ml GT (cells + NaBt (5 mM) + 0.045 mg/ml GT + LPS)
- 8.) 0.0045 mg/ml EGCG (cells + NaBt (5 mM) + 0.0045 mg/ml EGCG + LPS)
- 9.) 0.009 mg/ml EGCG (cells + NaBt (5 mM) + 0.009 mg/ml EGCG + LPS)
- 10.) 0.045 mg/ml EGCG (cells + NaBt (5 mM) + 0.045 mg/ml EGCG + LPS)

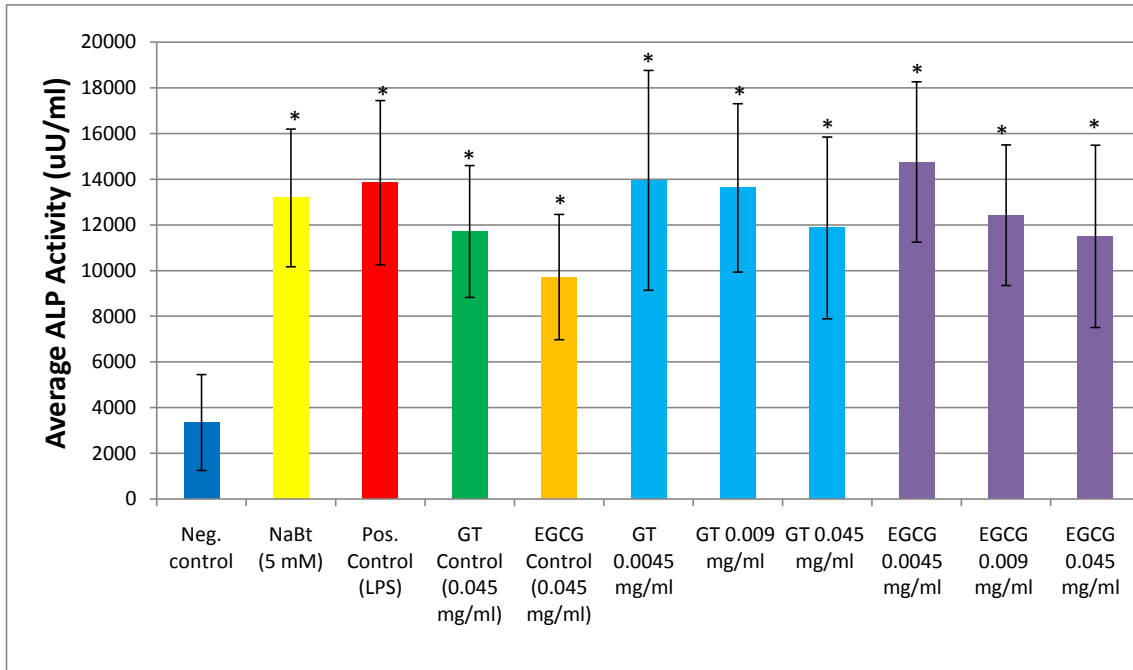
While there appeared to be some variability between treatments, statistical analysis did not identify any significant differences between treatment means and the negative control mean, indicating that the proposed treatments were not toxic to the cells.



**Figure 3.1** Cell viability (MTT) assay: Relative concentrations of cells in differentiated HT-29 cells. Treatments with the same letters are not statistically different.

### 3.4.2 ALP Assay

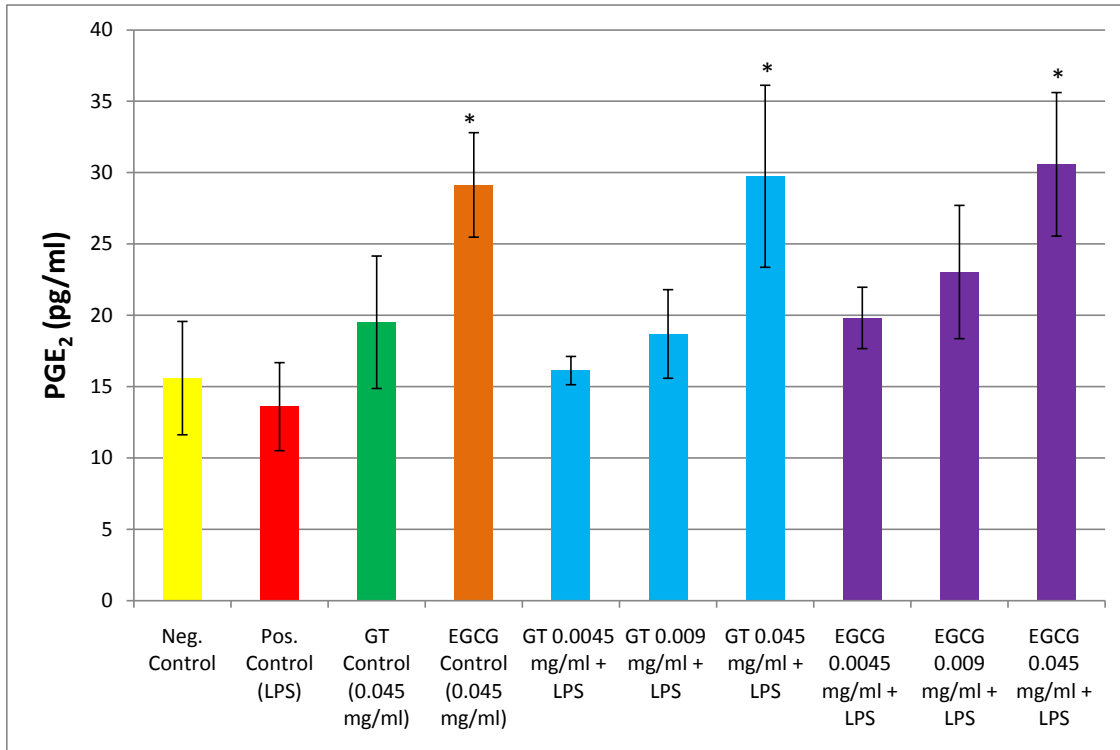
Differentiated cells exhibit different characteristics than undifferentiated cells such as increased ALP enzyme activity (Heerdt and others 1994). Therefore, the purpose of the ALP assay was to determine whether cells treated with NaBt (5 mM) were differentiated compared to cells not treated with NaBt (negative control) after 24 hours (Figure 3.2). The results of the ALP assay demonstrate that all cells treated with NaBt for 24 hours had significantly higher ALP enzyme activity compared to the negative control ( $p < 0.05$ ), indicating the cells were differentiated. Although not significant, there is a trend of lower ALP activity correlating with increasing concentrations of GT and EGCG treatments. There is previous evidence of EGCG inhibiting ALP activity in rat femoral tissue, which may explain the lower ALP activity values observed in this study (Yamaguchi and Ma 2001).



**Figure 3.2** Alkaline phosphatase activity in differentiated HT-29 cells. The ‘\*’ symbol indicates a significant difference ( $p < 0.05$ ) from the negative control

### 3.4.3 PGE<sub>2</sub> ELISA

PGE<sub>2</sub> ELISA experiments were used to measure the concentration of PGE<sub>2</sub> in the media samples collected 24 hours after stimulation by LPS (Figure 3.3). Stimulation with LPS alone (Positive Control) did not cause a strong PGE<sub>2</sub> inflammatory response in the differentiated HT-29 cells. Increasing concentrations of GT and EGCG caused an increase in PGE<sub>2</sub> concentration with the highest concentration of GT and EGCG (0.045 mg/ml) with LPS and EGCG alone (EGCG Control) causing significantly increased concentrations of PGE<sub>2</sub> compared to the negative control.



**Figure 3.3** PGE<sub>2</sub> concentrations in differentiated HT-29 cells. The ‘\*’ symbol indicates a significant difference ( $p < 0.05$ ) from the negative control

The absence of an inflammatory response in the HT-29 cells following stimulation by LPS may have been due to a lack of LPS receptors on the cell surface of HT-29 cells. Intestinal epithelial cells are constantly in a state of controlled inflammation because they are continuously exposed to gram negative bacteria and LPS in the lumen of the colon (Vamadevan and others 2010). Therefore, the cells may have developed a tolerance to the exposure by decreasing the number of receptors on their surface. Previous work has shown that the cell surface expression of myeloid differentiation (MD)-2, a critical protein receptor for the recognition of LPS, is much lower in HT-29 cells (12% positive cells) than in other cells like monocytes (95% positive cells) (Lenoir and others 2008). This may explain why

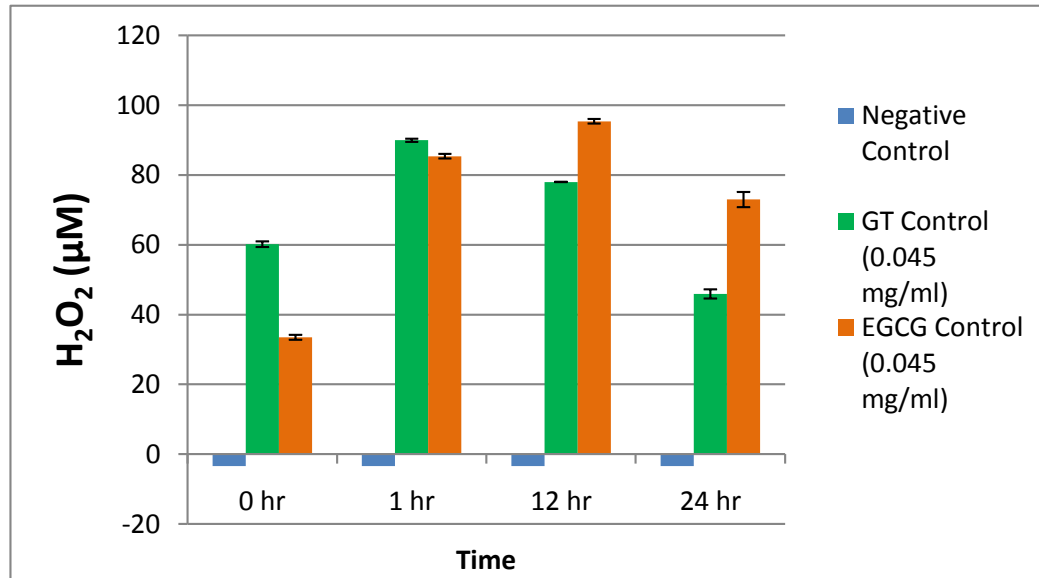
the HT-29 cells in this experiment did not have a strong inflammatory response when exposed to LPS. Other serotypes (O127:B8, O111:B4) and concentrations (10 ng/ml, 100 ng/ml, 1 ug/ml, 10 ug/ml and 100 ug/ml) of LPS were also tested but no significant inflammatory response resulted (data not shown).

Based on data emphasizing the anti-inflammatory effects of green tea components, the observed increased production of PGE<sub>2</sub> by the HT-29 cells in response to the GT and EGCG treatments was unexpected. Therefore, a new hypothesis to explain the formation of PGE<sub>2</sub> after treatment with GT and EGCG was formulated. EGCG has previously been shown to produce H<sub>2</sub>O<sub>2</sub> upon addition to cell culture media (Long and others 2000; Hong and others 2001; Hong and others 2002). Additionally, H<sub>2</sub>O<sub>2</sub> is a known signaling molecule in inflammatory responses and has been shown to mediate the release of arachidonic acid (AA) in other *in vitro* studies (Gustafson and others 1991; Schreck and others 1991). The release of AA stimulates the AA oxidation pathway, which increases the activation of COX-2 and the production of PGE<sub>2</sub>. Therefore, a new hypothesis was developed: H<sub>2</sub>O<sub>2</sub> is generated in the culture media as a result of the GT and EGCG treatments and is responsible for the observed increased production of PGE<sub>2</sub>, which also indicates an increase in COX-2 expression or activity.

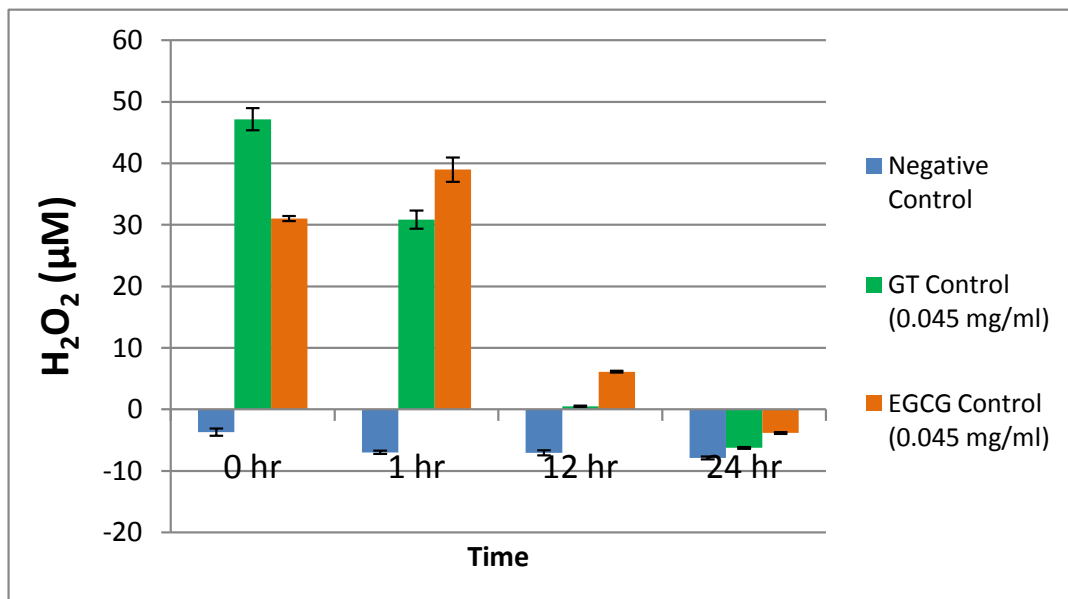
#### **3.4.4 H<sub>2</sub>O<sub>2</sub> Assay**

The cell culture media was tested for the presence of H<sub>2</sub>O<sub>2</sub> after treatment with GT and EGCG to determine whether these treatments were the causes of H<sub>2</sub>O<sub>2</sub> formation. The

maximum H<sub>2</sub>O<sub>2</sub> production in response GT and EGCG treatments was determined in cell culture media both with and without cells over 24 hours (Figure 3.4 and Figure 3.5).



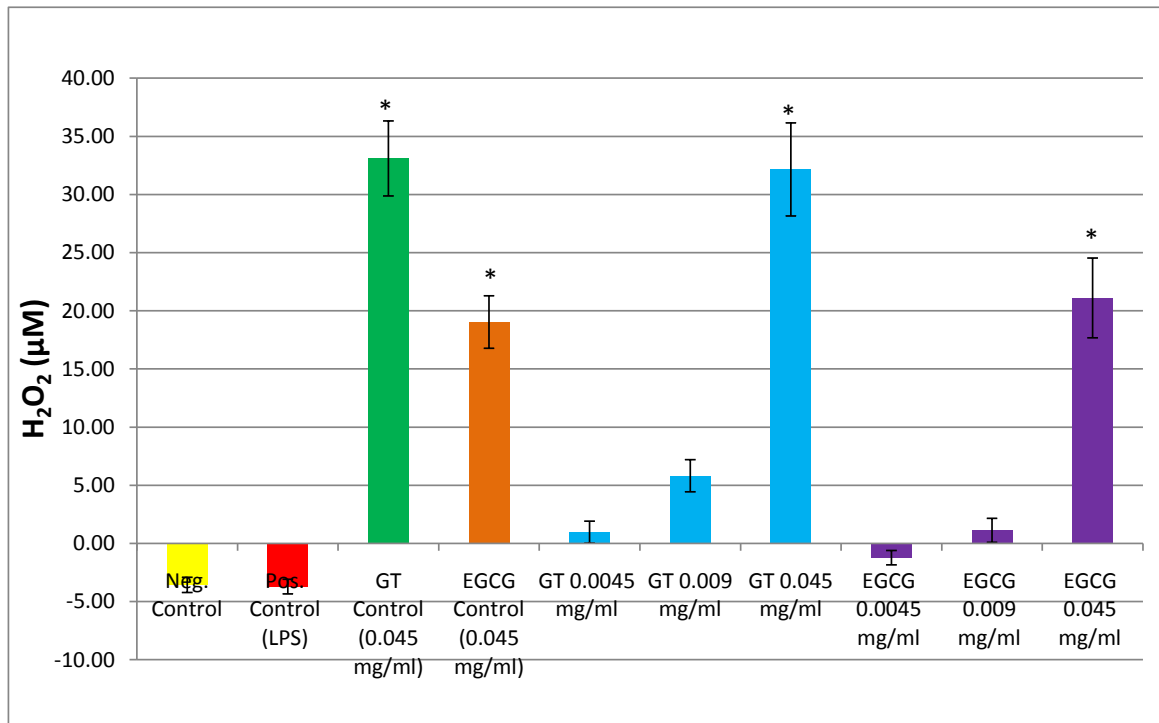
**Figure 3.4** H<sub>2</sub>O<sub>2</sub> production in DMEM without cells over 24 hours



**Figure 3.5** H<sub>2</sub>O<sub>2</sub> production in DMEM with HT-29 cells over 24 hours



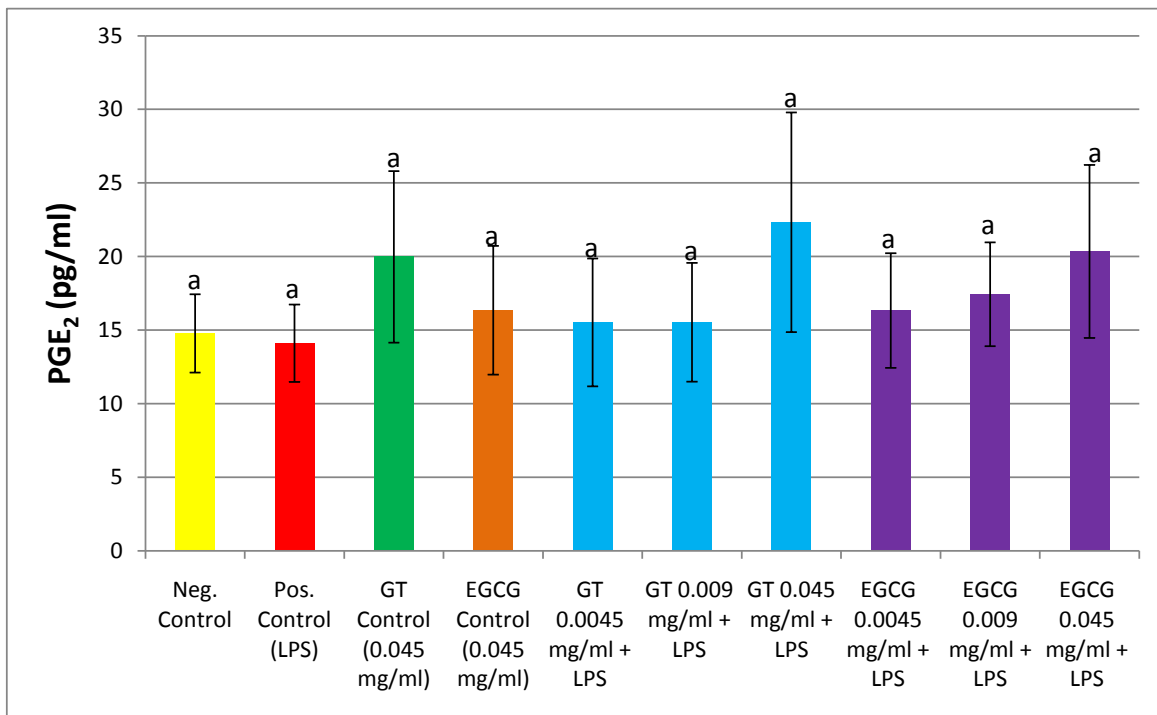
In cell culture media with HT-29 cells (Figure 3.5) a maximum H<sub>2</sub>O<sub>2</sub> response occurred immediately after GT or EGCG treatment (hour 0). Therefore, H<sub>2</sub>O<sub>2</sub> production at hour 0 was measured in the HT-29 cells after treatment with GT and EGCG (Figure 3.6). Results of the H<sub>2</sub>O<sub>2</sub> assay indicate that increasing concentrations of GT and EGCG caused increased H<sub>2</sub>O<sub>2</sub> production. Only the highest concentration (0.045 mg/ml) of GT and EGCG produced significantly more H<sub>2</sub>O<sub>2</sub> compared to the negative control (p<0.05). The data reported here support previous research that has shown that EGCG produces H<sub>2</sub>O<sub>2</sub> in cell culture media.



**Figure 3.6** H<sub>2</sub>O<sub>2</sub> production at hour 0 in differentiated HT-29 cells. ‘\*’ symbol indicates a significant difference (p<0.05) from the negative control.

### 3.4.5 PGE<sub>2</sub> ELISA with H<sub>2</sub>O<sub>2</sub> Treatments

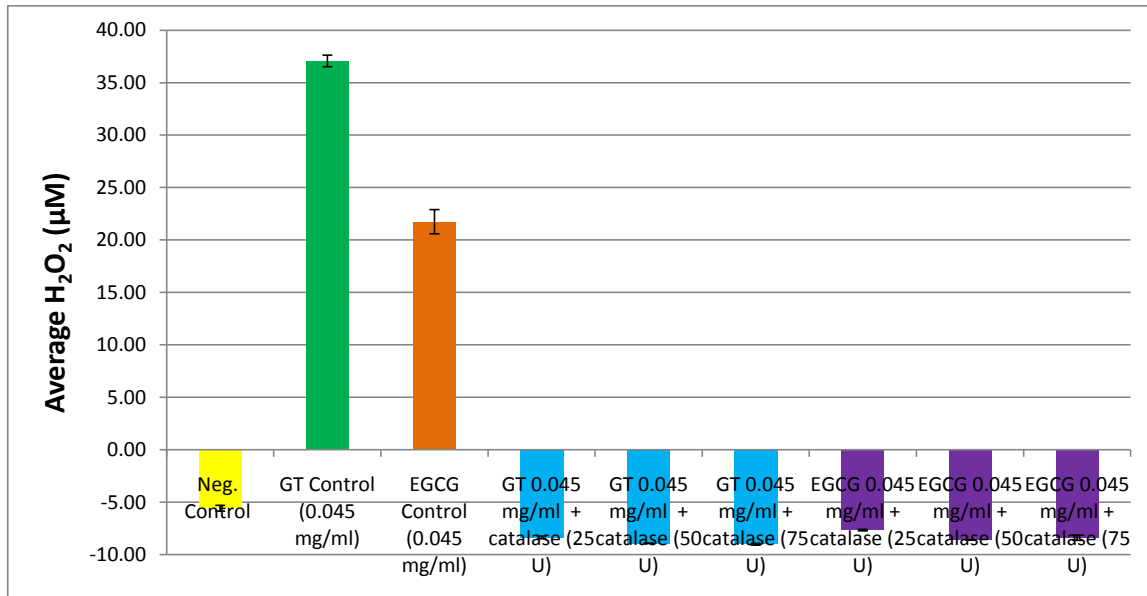
To confirm that H<sub>2</sub>O<sub>2</sub> was responsible for the observed increase in PGE<sub>2</sub> concentration, GT and EGCG treatments were replaced with corresponding amounts of H<sub>2</sub>O<sub>2</sub> that were determined from the H<sub>2</sub>O<sub>2</sub> assay (i.e., the GT Control treatment received 33 μM of H<sub>2</sub>O<sub>2</sub> instead of 0.045 mg/ml GT). The results of the PGE<sub>2</sub> ELISA with H<sub>2</sub>O<sub>2</sub> in place of the GT and EGCG treatments (Figure 3.7) show a similar trend to the GT and EGCG treated PGE<sub>2</sub> ELISA (Figure 3.3): increasing concentrations of H<sub>2</sub>O<sub>2</sub> produced higher amounts of H<sub>2</sub>O<sub>2</sub>. However, none of the treatments were statistically significant compared to the negative control, indicating that the observed increase in PGE<sub>2</sub> may not have been mediated by an increase in H<sub>2</sub>O<sub>2</sub> production.



**Figure 3.7** PGE<sub>2</sub> concentrations in differentiated HT-29 cells treated with corresponding amounts of H<sub>2</sub>O<sub>2</sub> in place of GT and EGCG.

### 3.4.6 Catalase Controls

A concentration of catalase that completely abolished  $H_2O_2$  when added to the GT and EGCG treatments was determined using the  $H_2O_2$  assay (Figure 3.8). All catalase concentrations measured (25, 50 and 75 U) showed similar decreases in  $H_2O_2$ , therefore, the lowest concentration (25 U) of catalase was chosen for use in future experiments.

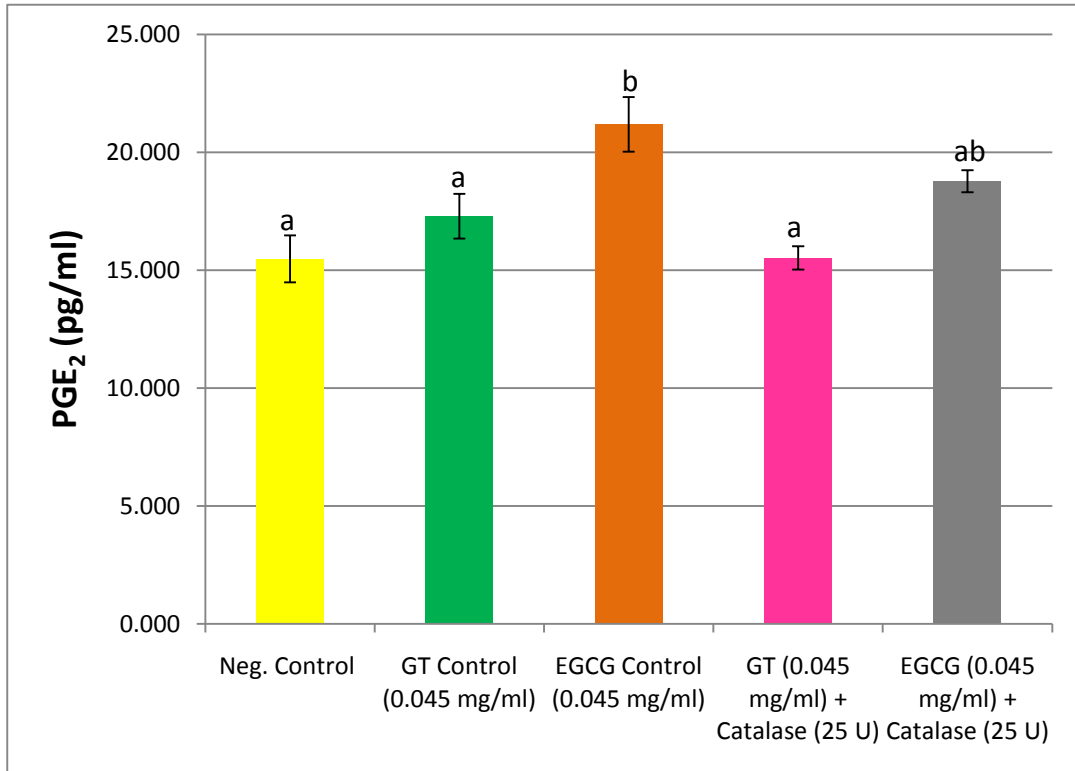


**Figure 3.8**  $H_2O_2$  production in GT and EGCG treatments with varying levels of catalase (25, 50, 75 U)

To measure the effect of catalase on  $H_2O_2$  formation, catalase (25 U) was added to the highest concentrations of GT and EGCG treatments. The five proposed treatments for the catalase control experiments were as follows:

- 1.) Negative Control (cells + NaBt (5 mM))
- 2.) GT Control (cells + NaBt (5 mM) + 0.045 mg/ml GT)
- 3.) EGCG Control (cells + NaBt (5 mM) + 0.045 mg/ml EGCG)
- 4.) 0.045 mg/ml GT (cells + NaBt (5 mM) + 0.045 mg/ml GT + catalase (25 U))
- 5.) 0.045 mg/ml EGCG (cells + NaBt (5 mM) + 0.045 mg/ml GT + catalase (25 U))

The catalase treatments were analyzed by PGE<sub>2</sub> ELISA and COX-2 Western blot to further investigate whether H<sub>2</sub>O<sub>2</sub> production from the GT and EGCG treatments was responsible for the production of PGE<sub>2</sub> (Figure 3.9 and Figure 3.10). Although there is a trend of treatments containing GT or EGCG with catalase (25 U) having lower PGE<sub>2</sub> concentrations than the GT or EGCG control, these differences are not statistically significant (Figure 3.9). Additionally, there are not significant differences in COX-2 expression levels between the GT and EGCG controls and the catalase treated treatments (Figure 3.10). Considering the combined results of the PGE<sub>2</sub> ELISA with H<sub>2</sub>O<sub>2</sub> treatments (Figure 3.7) and the data from Figure 3.9, there does not appear to be strong evidence for H<sub>2</sub>O<sub>2</sub>-mediated PGE<sub>2</sub> inflammation.



**Figure 3.9** PGE<sub>2</sub> ELISA data for catalase control treatments. Treatments with the same letters are not statistically different.

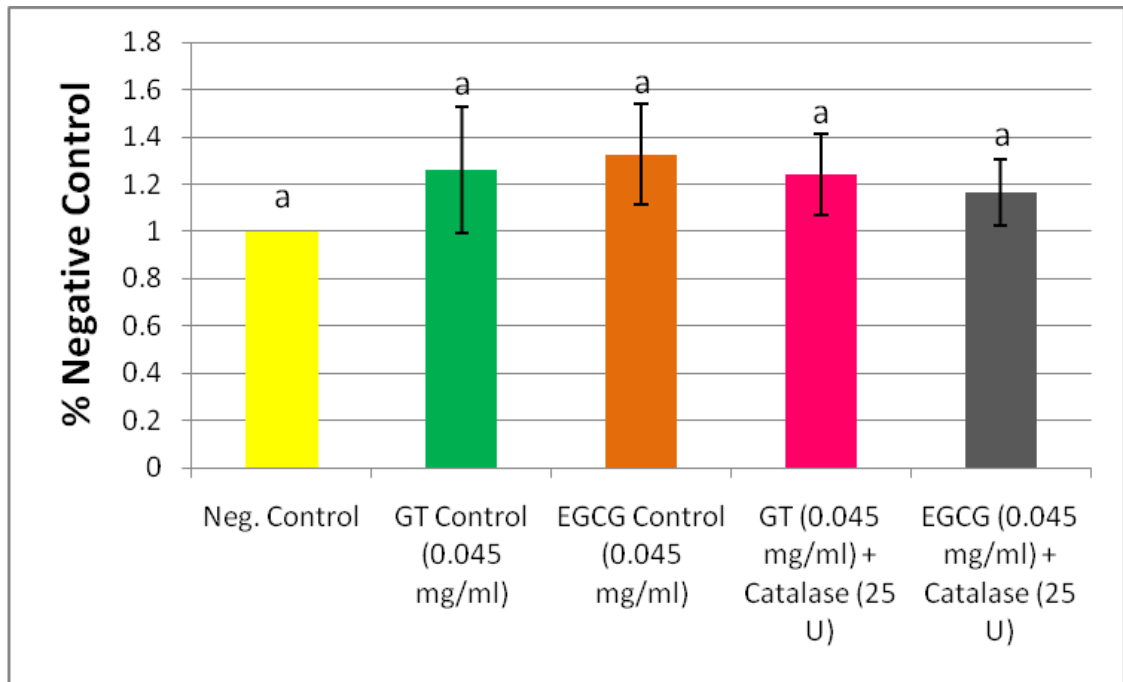
## COX-2

72 kDa



## Beta Actin

43 kDa



**Figure 3.10** COX-2 and beta actin Western blot results with COX-2 expression for catalase control treatments. Western bands correlate to treatments on the graph in same position. Treatments with the same letters are not statistically different.

The results of our experiment are similar to others reported in the literature. One such study reported that the addition of catalase did not completely abolish the cytotoxic effects of EGCG observed, suggesting that the effect of EGCG on tumor cells lines cannot solely be attributed to  $H_2O_2$  production (Yamamoto and others 2004). Another study reported that the addition of catalase did not prevent the inhibitory effects of EGCG on activator protein-1 (AP-1) activity in mouse epidermal H-ras transformed cells, indicating that oxidative stress is

not the sole way that EGCG signalling occurs (Chung and others 1999). Further research is needed to determine how EGCG elicits an effect on cell signaling.

### **3.4.7 COX-2**

#### *3.4.7.1 COX-2 Western Blot*

The presence of COX-2 is highly correlated with inflammation and colon cancer, therefore, we expected to see an increase in COX-2 expression in the LPS-stimulated HT-29 cells (Eberhart and others 1994). Figure 3.11 shows representative COX-2 and corresponding beta actin Western blots, as well as COX-2 expression levels as a percent of the positive control adjusted for beta actin, after HT-29 cells were differentiated with NaBt (5 mM) and treated with GT, EGCG and LPS (O55:B5, 1 ng/ml). Statistical analysis did not indicate that any of the treatments resulted in significantly different COX-2 expression levels compared to the negative control.

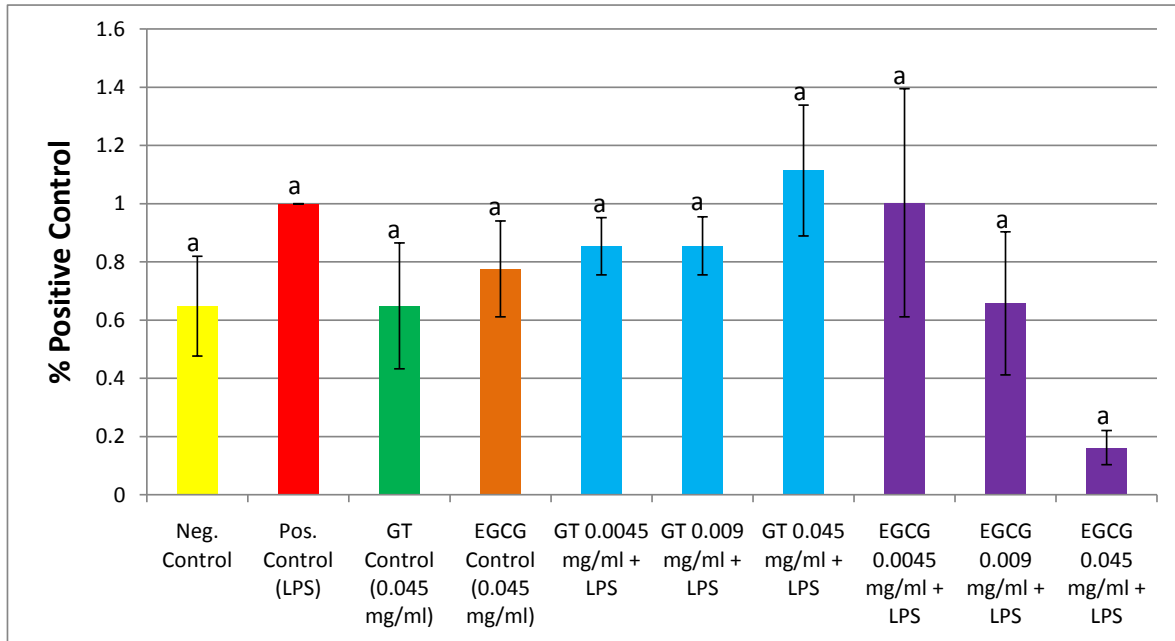
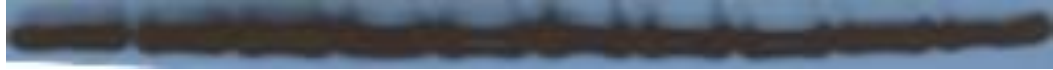
## COX-2

72 kDa



## Beta Actin

43 kDa



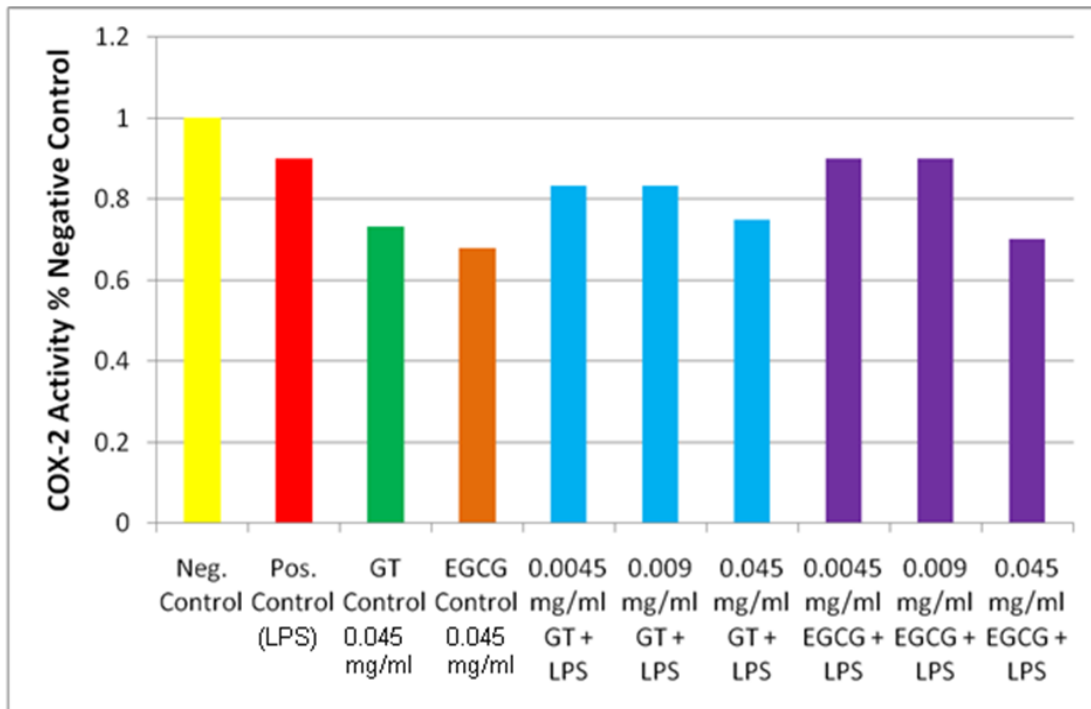
**Figure 3.11** Representative COX-2 and beta actin Western blot images with relative COX-2 expression levels. Western bands correlate to treatments on the graph in same position. Treatments with the same letters are not statistically different.

### 3.4.7.2 COX-2 Activity Assay

As a result of the COX-2 Western not indicating a significant difference in COX-2 expression for any of the treatments, a COX-2 Activity Assay was conducted to determine whether the differences in PGE<sub>2</sub> concentration were due to the activity of the COX-2 enzyme and not its expression levels. Figure 3.12 reports the preliminary results of the COX-2 Activity Assay. The results of the COX-2 Activity Assay indicate that all of the treatments



had less COX-2 activity compared to the negative control. This suggests that any differences in PGE<sub>2</sub> concentration were probably not a result of the differences in COX-2 activity. Further COX-2 activity analyses are necessary to determine if any of the differences observed are significant.



**Figure 3.12** Preliminary results of the COX-2 activity assay.

Even though COX-2 is the rate limiting step in the oxidation of AA to PGE<sub>2</sub>, there are a variety of possible reasons for not observing a difference in COX-2 expression or activity in the samples measured, but still observing an increase in PGE<sub>2</sub> concentration. One possible explanation is the activity of the enzymes directly responsible for the production of PGE<sub>2</sub>, the PGE<sub>2</sub> synthases. PGH<sub>2</sub>, the immediate product of COX enzyme activity, is isomerized to PGE<sub>2</sub> by a PGE<sub>2</sub> synthase (Murakami and others 2003). The COX enzymes, COX-1 and COX-2, are highly correlated with the downstream PGES enzymes cytosolic PGE<sub>2</sub> synthase

(cPGES) and membrane bound PGE<sub>2</sub> synthase (mPGES-1) respectively. However, another isoform of PGE<sub>2</sub> synthase, mPGES-2, has recently been isolated from the bovine heart (Watanabe and others 1999). A high concentration of mPGES-2 has been detected in the intestine where the concentration of mPGES-1 is relatively low, indicating that mPGES-2 may play a major role in PGE<sub>2</sub> synthesis in this tissue (Murakami and others 2003). Additionally, mPGES-2 has been shown to promote the formation of PGE<sub>2</sub> with both COX-1, the constitutively expressed COX isoform, and COX-2, the inducible isoform; thereby playing a role in homeostasis and disease states of the cell (Murakami and others 2003). A possible explanation for the lack of COX-2 enzyme expression and activity with increased production of PGE<sub>2</sub> could be a result of the activity of the mPGES-2 and COX-1 enzymes. Since mPGES-2 is active during both normal and stress responses of a cells, it could have been active without COX-2 expression and activity in the HT-29 cells. The amount of PGE<sub>2</sub> production observed was not very large and a small amount of mPGES-2 activity with the PGH<sub>2</sub> produced from constitutive levels of COX-1 may have been enough to cause the elevation in PGE<sub>2</sub> concentration. Future work should focus on the activity and expression of the PGE<sub>2</sub> synthase enzymes and how EGCG or H<sub>2</sub>O<sub>2</sub> caused their increased activity to produce more PGE<sub>2</sub>. Additionally, the expression and activity level of COX-1 should be investigated.

A second explanation for the observed increase in PGE<sub>2</sub> is cross-reactivity in the PGE<sub>2</sub> ELISA kits. If there was another compound present in the measured sample that the PGE<sub>2</sub> ELISA kit reacted to besides PGE<sub>2</sub>, this may have shown a false increase in reported PGE<sub>2</sub>. Cayman Chemical, the supplier of the PGE<sub>2</sub> ELISA kits, provided cross-reactivity

data for the PGE<sub>2</sub> ELISA. The kits have 100% cross-reactivity with PGE<sub>2</sub> and PGE<sub>2</sub> ethanolamide, 43% with PGE<sub>3</sub>, 18.7% with PGE<sub>1</sub> and 2.5% with 8-iso-PGE<sub>2</sub> (Cayman Chemical Company 2011). The kit is 100% cross-reactive with the desired target PGE<sub>2</sub>, so if there was any PGE<sub>2</sub> present in the sample, the kit should have detected it. Additionally, PGE<sub>2</sub> ethanolamide, although 100% reactive with the PGE<sub>2</sub> ELISA kit, has not been shown to be present in any tissues or intact cells (Ross and others 2002). Also, PGE<sub>1</sub> is virtually undetectable in human cells, therefore, it was probably not present in the sample tested (Cawello and others 1997). The most likely compound that could have cross-reacted with the PGE<sub>2</sub> ELISA kit is PGE<sub>3</sub>, an edema promoting, pro-inflammatory product of eicosapentaenoic acid metabolism (Hawkes and others 1992). PGE<sub>3</sub> comprises 2.4% of all COX products in human ocular tissue (Kulkarni and Srinivasan 1986). This indicates that a very small amount of the compounds produced from the COX enzymes in this experiment could have been PGE<sub>3</sub>, which would have been detected since PGE<sub>3</sub> has a cross-reactivity of 43% with the Cayman PGE<sub>2</sub> ELISA kit. Based on the cross-reactivity data it is not likely that cross-reactivity occurred, however, it is possible.

Our study produced interesting results that we did not initially expect. There is significant literature on the anti-inflammatory properties of GT and EGCG (Park and Surh 2004), however, our study contributes to the growing amount of literature on the pro-oxidant effects that GT and EGCG can possess (Hou and others 2004). Other studies have also shown that a compound can have varying effects on inflammation depending on which markers of inflammation are measured and when the measurements are taken. For example, ibuprofen, a non-steroidal anti-inflammatory drug (NSAID) typically consumed to reduce symptoms

associated with inflammation, was shown to have a pro-inflammatory effect in ultra-marathon runners that consumed it the day before and the day of a 160 km race compared to non-ibuprofen users (McAnulty and others 2007). This study highlights the importance of consuming anti-inflammatory compounds at the correct time relative to when the anti-inflammatory effects are desired. There is still much research that needs to be done to fully understand how dietary compounds affect inflammatory signaling pathways in humans. Only then will it be possible to make educated recommendations as to when and how much people should consume compounds with potential anti-inflammatory benefits.

### **3.5 CONCLUSIONS**

We report significant increases in PGE<sub>2</sub> and H<sub>2</sub>O<sub>2</sub> production in differentiated HT-29 cells treated with non-toxic concentrations of GT and EGCG. Replacing the original GT and EGCG treatments with corresponding concentrations of H<sub>2</sub>O<sub>2</sub> produced a similar, but non-significant trend toward increased PGE<sub>2</sub> production. PGE<sub>2</sub> ELISA and COX-2 Western blot experiments containing catalase as a means to eliminate H<sub>2</sub>O<sub>2</sub> did not provide strong evidence for an H<sub>2</sub>O<sub>2</sub>-mediated PGE<sub>2</sub> response. GT and EGCG treatments had no significant effects on COX-2 expression and activity. A proposed explanation for these results may be the expression and activity of the mPGES-2 enzyme. This enzyme is constitutively expressed and metabolizes both COX-1 and COX-2 enzyme products, indicating that it may have been responsible for the observed increases in PGE<sub>2</sub> since COX-1 is also constitutively expressed. Future work will investigate the expression and activity levels of COX-1 and the

PGE<sub>2</sub> synthase enzymes to further examine the potential pro-inflammatory effects of GT and EGCG.

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**CHAPTER 4: Effects of Green Tea and EGCG on IL-8 and NF- $\kappa$ B Inflammatory Responses in LPS-Induced, Differentiated Human Colon Cancer (HT-29) Cells**

#### 4.1 ABSTRACT

Consumption of green tea (GT) is associated with reduced colon cancer incidence. Colon cancer has been linked to chronic inflammation and diet. Epigallocatechin-3-gallate (EGCG), the main flavonoid component of GT, as well as GT itself, have demonstrated anti-inflammatory and cancer preventive effects in numerous *in vitro* and *in vivo* systems. Interleukin-8 (IL-8) is a pro-inflammatory cytokine produced as a result of the activation of nuclear factor- $\kappa$ B (NF- $\kappa$ B). The purpose of this research was to determine the effects of GT or EGCG on IL-8 formation and NF- $\kappa$ B expression in lipopolysaccharide (LPS)-induced, differentiated human colon cancer (HT-29) cells. Aqueous GT and EGCG extracts were prepared and matched for total phenol content by the Folin-Ciocalteu method. Cell viability was determined by the MTT assay. Differentiation was induced by sodium butyrate (5 mM) and confirmed by the alkaline phosphatase (ALP) assay. Cells were co-treated with LPS 055:B5 (1 ng/ml) and three concentrations of GT or EGCG (0.0045, 0.009 and 0.045 mg/ml) for 24 hours. IL-8 concentration was measured by ELISA. NF- $\kappa$ B concentration was examined by Western blot. Neither GT or EGCG were toxic at the levels used. Increasing concentrations of GT and EGCG showed trends toward decreased IL-8 formation, however, only the highest EGCG treatment (0.045 mg/ml) was significant ( $p < 0.05$ ). NF- $\kappa$ B Western blot results were inconclusive. This experiment demonstrated that EGCG, but not GT, had anti-inflammatory effects on IL-8 in this cell line when matched for total phenol content, suggesting potential benefits relative to human colon cancer.

## 4.2 INTRODUCTION

Cancer is the second leading cause of death in the United States (American Cancer Society 2010). Colon cancer was the third leading cause of U.S. cancer incidence and mortality in 2010 (American Cancer Society 2010). Chronic inflammation and diet have been linked with colon cancer incidence, which indicates that modifications to the diet may reduce the risk of colon cancer (Marshall 2009). Green tea (GT) and the major phenolic component of GT, epigallocatechin-3-gallate (EGCG), have been shown to possess anti-inflammatory properties, and have been associated with the prevention of diseases associated with inflammation and oxidative stress (Butt and Sultan 2009). These reports suggest that consumption of GT or EGCG may be protective effect against colon cancer.

A variety of inflammatory markers are associated with colon cancer incidence. We chose to examine two inflammatory markers, interleukin-8 (IL-8) and nuclear factor- $\kappa$ B (NF- $\kappa$ B), because of their strong correlation to colon cancer. IL-8 was chosen because it is known to increase the aggressiveness of colon cancer by promoting tumor growth and angiogenesis (Sparmann and Bar-Sagi 2004). NF- $\kappa$ B was measured because it is the main regulator of IL-8 expression and is constitutively expressed in many tumor cells (Aggarwal and others 2006). As a result, compounds that suppress the expression of NF- $\kappa$ B and IL-8 may have chemopreventive effects.

Many studies have explored the anti-inflammatory effects of GT and EGCG against an array of inflammatory inducers *in vitro*. GT and EGCG have been reported to suppress IL-8 or NF- $\kappa$ B activation in a variety of different cell lines including human mast cells stimulated with phorbol myristate acetate (PMA) (Shin and others 2007), human synovial

fibroblasts treated with interleukin-1 $\beta$  (IL-1 $\beta$ ) (Huang and others 2010), HT-29 cells induced with tumor necrosis factor-alpha (TNF- $\alpha$ ) (Porath and others 2005) and Caco-2 cells stimulated with IL-1 $\beta$  (Netsch and others 2006). One study explored the effect of EGCG in lipopolysaccharide (LPS)-stimulated HT-29 cells but reported conflicting results: EGCG increased the activation of NF- $\kappa$ B luciferase activity but decreased the phosphorylation of I $\kappa$ B- $\alpha$ , indicating that EGCG has the potential to either activate or inactivate NF- $\kappa$ B (Jeong and others 2004). To the author's knowledge, there have not been any studies on the effect of GT or EGCG on the inflammatory markers of IL-8 and NF- $\kappa$ B in LPS-induced, differentiated HT-29 cells. It is important to differentiate the HT-29 cells so that they take on the typical characteristics of normal colon epithelial cells, therefore, more closely approximating the natural environment of the colonic lumen. Therefore, the purpose of this study was to observe the effect of GT and EGCG on LPS-induced inflammation in differentiated HT-29 cells by measuring the production and expression of IL-8 and NF- $\kappa$ B. Reduced expression of IL-8 or NF- $\kappa$ B would indicate that GT or EGCG possesses anti-inflammatory properties and, therefore, may be helpful in reducing the risk of colon cancer incidence.

## **4.3 MATERIALS AND METHODS**

### ***4.3.1 Cell Culture***

Human colon cancer (HT-29) cells (ATCC; Manassas, VA) were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen; Carlsbad, CA) with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin-glutamine at 37°C in an atmosphere of

5% CO<sub>2</sub>. Cells were maintained in culture for up to 30 passages and were passaged using Trypsin (0.25%)-EDTA (0.53 mM) (ATCC) approximately every 2-3 days.

#### ***4.3.2 Preparation of Green Tea and EGCG***

Sencha GT (Maeda-san; Irvine, CA) was prepared by steeping 1 g of loose-leaf tea leaves in 100 ml deionized (DI) boiling water for 5 minutes and decanting the resulting solution. The GT solution was centrifuged at 130 g for 2 minutes. The supernatant (GT) was removed, aliquoted, and frozen at -80°C. EGCG (>95%, Sigma) was prepared at a 1.8 mM concentration in the same manner as GT. The total phenol content of the GT and EGCG treatments was measured via the Folin-Ciocalteu method described previously by Parejo and others (Parejo and others 2002) (See Appendix C for protocol).

#### ***4.3.3 Cell Differentiation***

The trypan blue assay was used to determine the live cell count. HT-29 cells were plated at a concentration of 400,000 cells/ml in 12 well plates and incubated at 37°C and 5% CO<sub>2</sub>. Once confluent, cells were exposed to sodium butyrate (NaBt) (5 mM, Sigma; St. Louis, MO) for 24 hours to induce differentiation. Cell differentiation was confirmed by the alkaline phosphatase (ALP) assay (Biovision; Mountain View, CA).

#### ***4.3.4 Cell Treatments***

Cells were treated with varying concentrations of GT and EGCG (0.0045, 0.009, 0.045 mg/ml) for two hours prior to inducing inflammation with LPS (O55:B5, 1 ng/ml, Sigma). Cells were then incubated at 37°C and 5% CO<sub>2</sub>.

#### ***4.3.5 Harvesting Cells: Media, Cytosolic Proteins and Nuclear Proteins***

Twenty-four hours after LPS stimulation, cells were harvested for media samples. Supernatant of each well was removed and saved for analysis by ELISA. Five minutes after LPS stimulation, cells were harvested for cytosolic and nuclear proteins. The nuclear extraction protocol was adapted from a previous study (La Ferla and others 2004). Media was removed from each well. Cells were washed with cold phosphate buffered saline (PBS) two times then scraped with cell lifters. The cell solution was transferred to a 2 ml eppendorf tube and centrifuged at 1000 rpm for 5 minutes. The cell pellet was resuspended in 300 µl of Buffer A (10 mM HEPES, pH 7.9, 1.5 mM MgCl<sub>2</sub>, 10 mM KCl, protease inhibitor cocktail (10 µL per 1 ml)) and incubated on ice for 10 minutes. Next, 10% Igepal (25 µl) was added to the cells. The cell solution was vortexed vigorously for 20 seconds and centrifuged at 7,000 rpm for 30 seconds. The cytosolic supernatant was collected and stored at -80°C for protein and Western blot analysis. The nuclear pellet was resuspended in 80 µL Buffer B (20 mM HEPES, pH 7.9, 420 mM NaCl, 1.5 mM MgCl<sub>2</sub>, 0.2 mM EDTA, 25% glycerol, protease inhibitor cocktail (10 µL per 1 ml)). Cells were incubated on ice while shaking for 20 minutes, then centrifuged at 16,000 rpm for 15 minutes at 4°C. The supernatant containing nuclear proteins was collected and stored at -80°C for protein and Western blot analysis.

#### **4.3.6 Cell Viability Assay**

Cell viability was determined by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenoltetrazolium) assay based on the method developed by Mosmann (Mosmann 1983). Twenty-four hours after LPS mediated inflammation was induced, 16 µl of MTT (Molecular Probes; Eugene, OR) solution (7.8 mg/ml) was added to each well. After a 2 hour incubation at 37°C and 5% CO<sub>2</sub> to allow for formazan crystal formation, the media was removed from each well. The formazan crystals were resuspended in acidified iso-propanol (0.5 ml). Absorbance was measured at 620 nm using a Multiskan® EX plate reader (Thermo Electron Corporation; Vantaa, Finland).

#### **4.3.7 ALP Assay**

Sodium butyrate (5 mM) mediated differentiation was confirmed after 24 hours by alkaline phosphatase (ALP) assay (Biovision; Mountain View, CA) following the manufacturer's instructions.

#### **4.3.8 IL-8 ELISA**

A human interleukin-8 (IL-8) Enzyme Linked Immuno-Sorbent Assay (ELISA) kit was purchased from Abcam (Cambridge, MA). The concentration of IL-8 in the media samples was determined by following the manufacturer's instructions.



#### **4.3.9 NF- $\kappa$ B Western Blot**

Cytosolic and nuclear cell lysate protein concentrations were determined by the bicinchoninic acid (BCA) protein assay using bovine serum albumin (BSA) as the standard (Pierce; Rockford, IL). Cell lysate samples were matched for protein content, prepared with 5X SDS-loading buffer and heated at 100°C for 10 minutes to denature the proteins. SDS-PAGE was run on Invitrogen (Carlsbad, CA) equipment and 12% Tris-Glycine gels. Proteins were transferred to a PVDF membrane.

NF- $\kappa$ B expression was detected by Western blot analysis. Primary phospho-NF- $\kappa$ B p65 (Ser536) rabbit antibody and anti-rabbit IgG horseradish peroxidase conjugate secondary antibody were purchased from Cell Signaling Technology, Inc. (Danvers, MA). The PVDF membrane was blocked in a Tris Buffered Saline with Tween-80 (TBS-T) solution with 3% BSA for 1.5 hours. The membrane was incubated with the primary NF- $\kappa$ B antibody (1:100, 2% BSA in TBS-T) overnight at 4°C, then incubated in secondary antibody (1:1000, 2% BSA in TBS-T) for one hour at room temperature. The membrane was visualized with Super Signal West Femto Maximum Sensitivity Substrate (Pierce). Beta actin (1:1000, Cell Signaling, Inc.) was used as a control using the cytosolic cell lysate samples.

#### **4.3.10 Statistical Analysis**

Results are representative of three independent experiments with all treatments run in triplicate except when otherwise noted. Values are reported as means  $\pm$  standard error (SE). Statistical analyses were performed using SAS<sup>®</sup> (Cary, NC) software. Analysis of variance (ANOVA) analyses using the Proc Mixed and Proc GLM procedures with Tukey's

adjustment were conducted to determine significant differences ( $p < 0.05$ ) between means. Treatments labeled with the same letter are not statistically different. Treatments labeled with an asterisk (\*) are statistically different from a given reference.

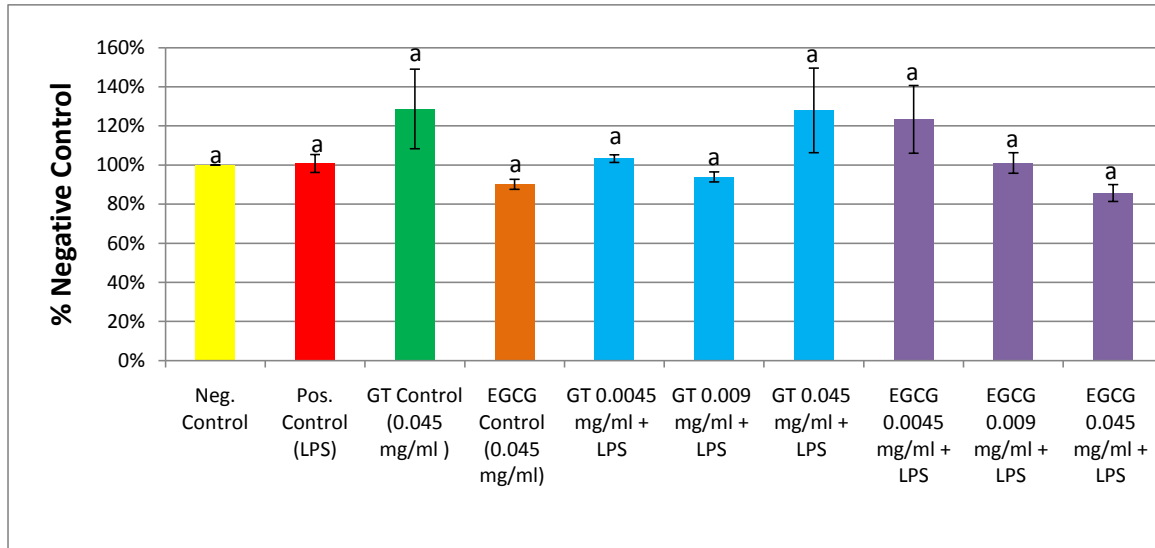
## **4.4 RESULTS AND DISCUSSION**

### **4.4.1 Cell Viability Assay**

Figure 4.1 represents the results of the cell viability (MTT) assay. The graph shows cell viability as a percent of the negative control. The ten proposed treatments in this study were:

- 1.) Negative Control (cells + NaBt (5 mM))
- 2.) Positive Control (cells + NaBt (5 mM) + LPS O55:B5 (1 ng/ml))
- 3.) GT Control (cells + NaBt (5 mM) + 0.045 mg/ml GT)
- 4.) EGCG Control (cells + NaBt (5 mM) + 0.045 mg/ml EGCG)
- 5.) 0.0045 mg/ml GT (cells + NaBt (5 mM) + 0.0045 mg/ml GT + LPS)
- 6.) 0.009 mg/ml GT (cells + NaBt (5 mM) + 0.009 mg/ml GT + LPS)
- 7.) 0.045 mg/ml GT (cells + NaBt (5 mM) + 0.045 mg/ml GT + LPS)
- 8.) 0.0045 mg/ml EGCG (cells + NaBt (5 mM) + 0.0045 mg/ml EGCG + LPS)
- 9.) 0.009 mg/ml EGCG (cells + NaBt (5 mM) + 0.009 mg/ml EGCG + LPS)
- 10.) 0.045 mg/ml EGCG (cells + NaBt (5 mM) + 0.045 mg/ml EGCG + LPS)

While there is some variability between treatments, statistical analysis did not signify any significant differences ( $p < 0.05$ ) between treatment means, indicating that the proposed treatments were not toxic to the cells.

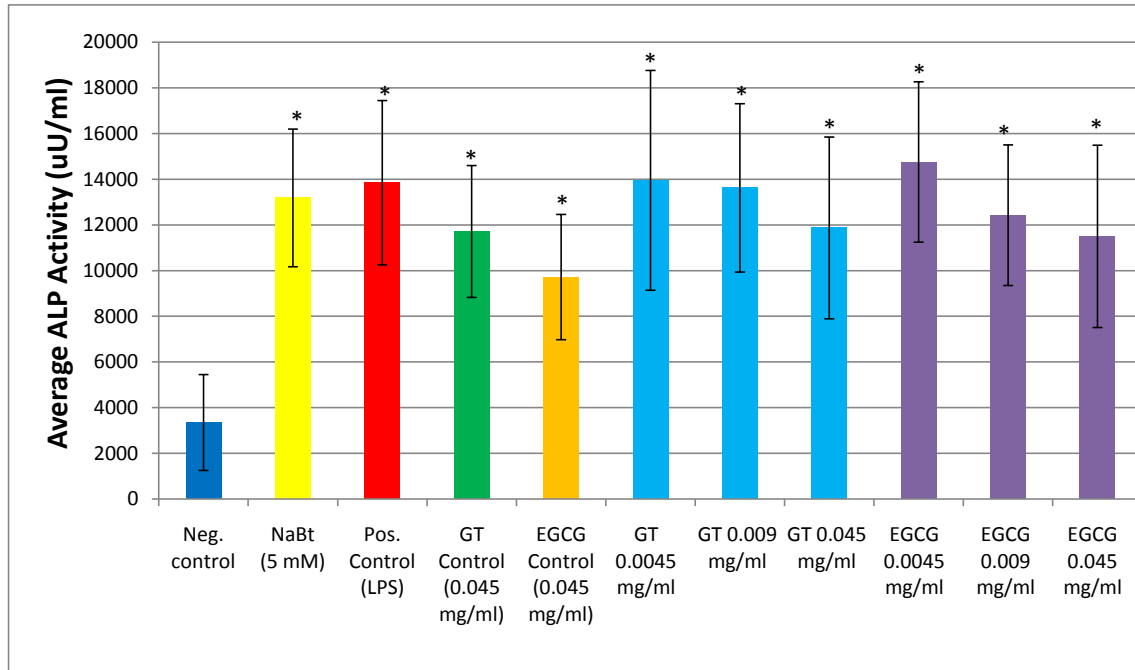


**Figure 4.1** Results of the cell viability (MTT) assay. Treatments with the same letter are not significantly different ( $p < 0.05$ ).

#### 4.4.2 ALP Assay

Figure 4.2 shows the results of the ALP assay. The purpose of this assay was to determine whether cells treated with NaBt (5 mM) for 24 hours were differentiated compared to cells not receiving NaBt (negative control). Differentiated cells exhibit different characteristics than undifferentiated cells, such as increased ALP enzyme activity (Heerdt and others 1994). The results of the ALP assay indicate that all cells treated with NaBt for 24 hours had significantly higher ALP enzyme activity compared to the negative control, indicating the cells had become differentiated.

While not significant, there also is a trend of increasing concentrations of GT and EGCG resulting in lower ALP activity rates. This may be a result of EGCG inhibiting ALP activity since there has been evidence of EGCG inhibiting ALP activity in rat femoral tissue (Yamaguchi and Ma 2001).

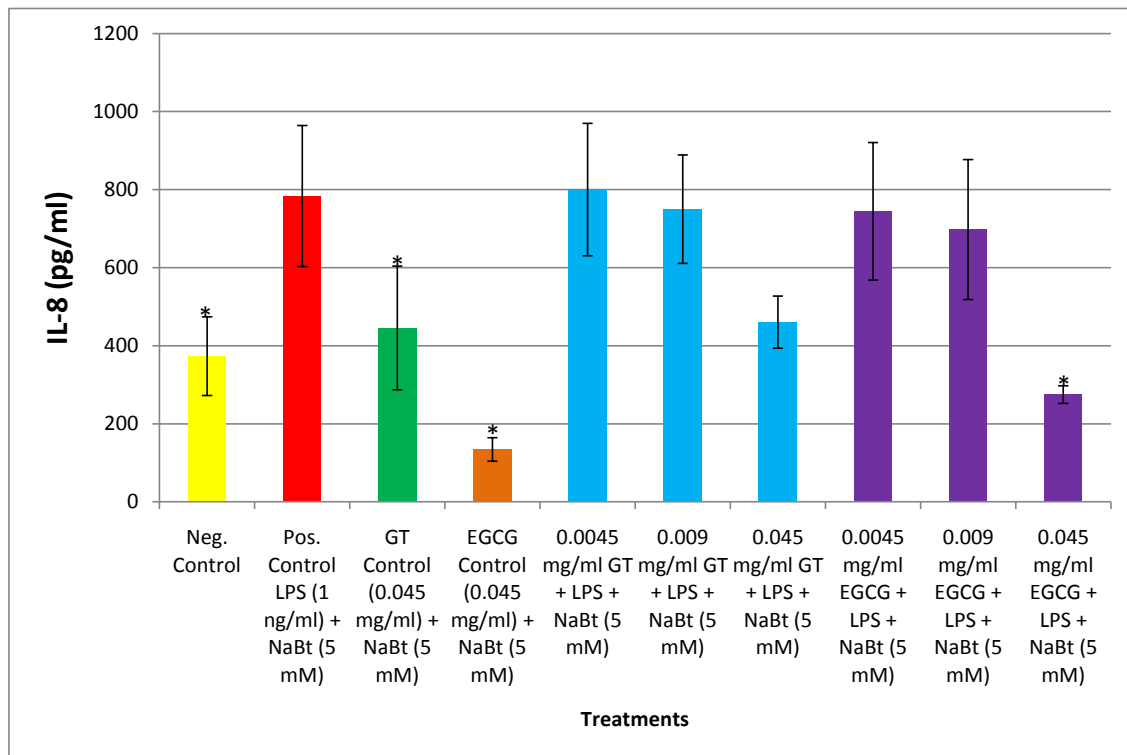


**Figure 4.2** Results of the ALP assay. Treatments marked with ‘\*’ are significantly ( $p < 0.05$ ) different from the negative control.

#### 4.4.3 IL-8 ELISA

The IL-8 ELISA was run to determine the concentration of IL-8 present in the media after HT-29 cells were stimulated with LPS for 24 hours. The results of the IL-8 ELISA are shown in Figure 4.3. This graph shows that the concentration of LPS (O55:B5, 1 ng/ml) was adequate to produce a significant inflammatory response (positive control vs. negative control). Additionally, the results indicate a trend of increasing concentrations of GT and EGCG causing in a reduction in IL-8 formation. However, only the highest concentration of EGCG (0.045 mg/ml) with LPS was significantly different from the positive control. The highest amount of EGCG alone (EGCG control) decreased the concentration of IL-8 substantially compared to the negative control. This indicates that EGCG is able to reduce constitutive amounts of IL-8 that were already expressed in HT-29 cells prior to LPS

stimulation. The results of this experiment are similar to the results of previous studies that found EGCG to decrease the expression of IL-8 in respiratory epithelial cells through the inhibition of the NF- $\kappa$ B pathway (Wheeler and others 2004). Whether EGCG inhibited IL-8 formation through the NF- $\kappa$ B pathway in this study is discussed in the next section.



**Figure 4.3** Results of the IL-8 ELISA. Treatments marked with ‘\*’ are significantly ( $p < 0.05$ ) different from the positive control.

Overall, the results of the IL-8 ELISA show that EGCG has significant anti-inflammatory properties in relation to IL-8 formation in HT-29 cells. IL-8 is related to increased colon cancer aggressiveness (Sparmann and Bar-Sagi 2004), therefore, this data suggests that EGCG, but not GT, may be helpful in the attenuation of colon cancer.

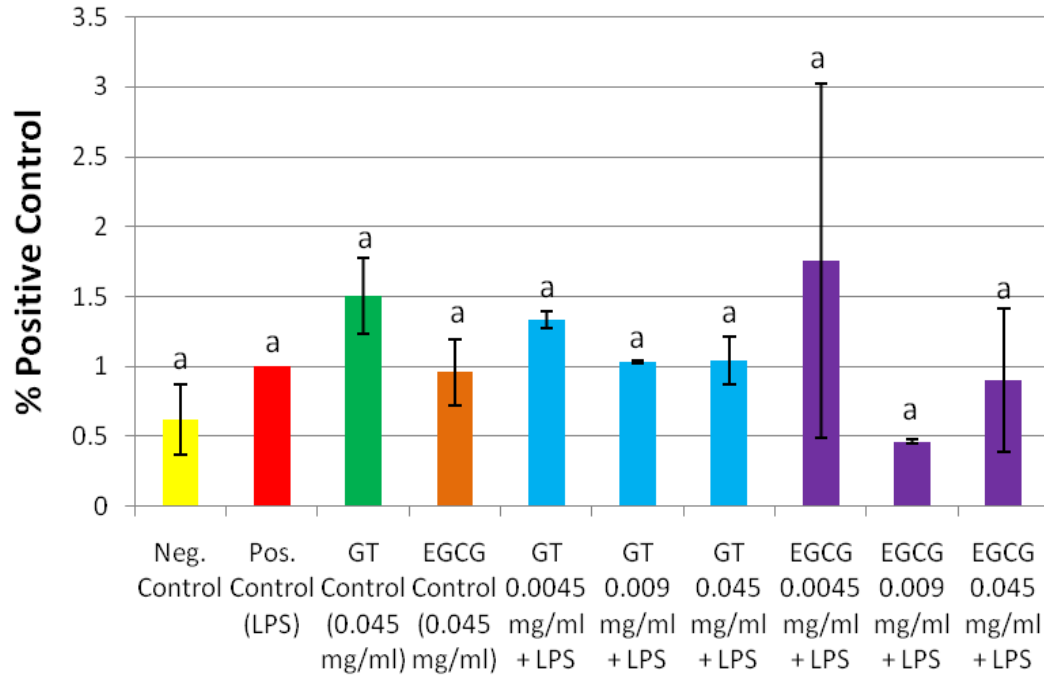
#### ***4.4.4 NF- $\kappa$ B Western Blot***

In the literature, LPS stimulation of the NF- $\kappa$ B pathway in HT-29 cells has been shown to be mainly responsible for IL-8 production (Lenoir and others 2008). Therefore, this study measured NF- $\kappa$ B expression to validate whether the NF- $\kappa$ B pathway was responsible for the observed IL-8 production. Figure 4.4 shows representative NF- $\kappa$ B and corresponding beta actin Western blots and NF- $\kappa$ B expression levels as a percent of the positive control adjusted for beta actin after HT-29 cells were differentiated with NaBt (5 mM) and treated with GT, EGCG and LPS (O55:B5, 1 ng/ml). The results of the NF- $\kappa$ B Western blot did not indicate a significant difference in NF- $\kappa$ B expression.

## NF-κB



## Beta Actin



**Figure 4.4** NF-κB and beta actin Western blot results. Western bands correlate to treatments on the graph in same position. Graph represents average of two independent experiments with  $\pm$  SE. Treatments with the same letter are not significantly different ( $p < 0.05$ ).

There are many possible limitations of the NF-κB Western blot, which may explain why we did not observe significant results. The first possibility is that maximum expression of NF-κB had not yet occurred at the time point used in this study (5 minutes after stimulation with LPS). The five minute time point was chosen because TNF- $\alpha$  was initially used as an inducer of inflammation in this study and it was known to produce the greatest amount of inflammation after five minutes of TNF- $\alpha$  (20 ng/ml) stimulation in HeLa and NIH/3T3 cells (Cell Signaling Technology 2010). However, some *in vitro* studies that used

LPS as a stimulator of inflammation detected NF- $\kappa$ B expression after longer exposures to LPS (1-3 hours) (La Ferla and others 2004; Barish and others 2010), indicating that the maximum NF- $\kappa$ B response may have occurred later.

Another possible limitation in measuring NF- $\kappa$ B expression is the inhibition of NF- $\kappa$ B expression by NaBt. A previous study found that HT-29 cells treated with TNF- $\alpha$  and NaBt (4 mM for 24 hours) had suppressed levels of NF- $\kappa$ B expression (Yin and others 2001). This suppression was due to inhibition of I $\kappa$ B- $\alpha$  degradation, which did not allow NF- $\kappa$ B to translocate to the nucleus and begin an inflammatory cascade. It is possible that the suppression of NF- $\kappa$ B levels by NaBt did not allow for sufficient activation of NF- $\kappa$ B in this study.

Additionally, IL-8 production may not have been completely due to NF- $\kappa$ B activation. NF- $\kappa$ B was chosen for this study because it is the main transcription factor that causes IL-8 production (Lenoir and others 2008). However, LPS is known to activate other inflammatory transcription factors that can also cause IL-8 production like activator protein-1 (AP-1) and NF-IL-6 (Mukaida and others 1994; Guha and Mackman 2001). Therefore, AP-1 and NF-IL-6 expression should also be explored to further elucidate how IL-8 was activated by LPS in HT-29 cells (Mukaida and others 1994).

The limitations of the NF- $\kappa$ B Western blot highlight the complexity of inflammatory signaling pathways. The previous study by Jeong (2004) (referenced in the introduction) showed how EGCG had conflicting effects on the NF- $\kappa$ B pathway (Jeong and others 2004). There is still much research that needs to be done to more fully understand how dietary compounds affect inflammatory signaling pathways in humans.



## 4.5 CONCLUSIONS

GT and EGCG showed similar trends toward the inhibition of LPS-induced IL-8 formation in differentiated HT-29 cells. Only the highest concentration of EGCG (0.045 mg/ml) was significant, however. This indicates that EGCG has a protective effect against IL-8 inflammation in HT-29 cells, suggesting potential protective effects against human colon cancer. At the concentration of LPS (O55:B5, 1 ng/ml) and measuring time point (5 minutes) used in this study, it appears that the NF- $\kappa$ B pathway was not involved in the stimulation or inhibition of IL-8 formation in differentiated HT-29 cells. Future work will focus on determining which pathway was responsible for the anti-inflammatory effects of GT and EGCG on IL-8 production in LPS-induced differentiated HT-29 cells.

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## **APPENDICES**

**APPENDIX A: Twelve Month Stability Study of EGCG Aliquots Frozen at -80°C**

## **A.1 ABSTRACT**

Large batches of epigallocatechin-3-gallate (EGCG) are often made at one time. Therefore, it is necessary to store aliquots of EGCG in a -80°C freezer until further use. The purpose of this study was to determine whether the phenol content of the EGCG aliquots remained constant after storage at -80°C. EGCG was prepared and stored in a -80°C freezer for one year. Total phenol content was determined by the Folin-Ciocalteu method after 0, 1, 3, 6, and 12 months of storage. The phenol content of the EGCG aliquots remained constant after 6 months of storage. After 12 months of storage the phenol content of the Month 12 sample ( $789 \pm 16$  GAE) was significantly ( $p < 0.05$ ) less than the Month 0 sample ( $881 \pm 31$  GAE). It is recommended that EGCG samples at -80°C be discarded after 6 months.

## **A.2 INTRODUCTION**

It is impractical to prepare a single treatment amount of GT or EGCG prior to every cell culture experiment. Therefore, large batches of EGCG are made a one time, aliquoted and stored for future use. A previous storage study determined that the total phenol content of green tea (GT) and black tea (BT) was more stable at -80°C compared to -20°C after 3 months (Summers 2009). Additionally, then phenol content of GT was higher after 3 months of storage at -80°C compared to the freshly prepared GT (Summers 2009). It would be beneficial to know whether the phenol content of these samples is stable over longer periods of time and whether an isolated phenolic compound, like EGCG, behaves differently than GT or BT. The purpose of this study was to determine the stability of EGCG stored at -80°C over a longer period of time: 1 year.

## **A.3 MATERIALS AND METHODS**

### ***A.3.1 Experimental Methods Summary***

EGCG was prepared as described in previous chapters of this thesis. The EGCG aliquots were stored in a -80°C freezer for one year taking samples at 0, 1, 3, 6, and 12 month time-points throughout the study. The stability of the EGCG aliquots at -80°C was determined by the total phenol content of the sample, which was measured by the Folin Ciocalteu method (see Appendix C for protocol).

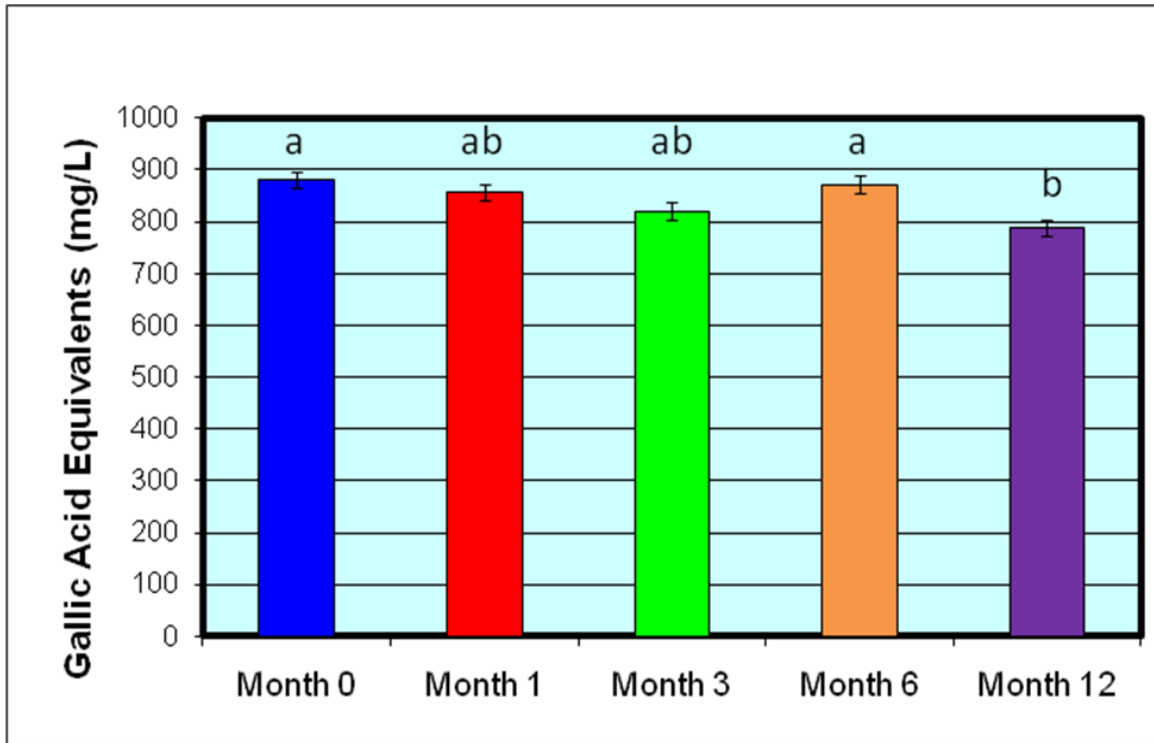
### ***A.3.2 Experimental Design and Statistical Analysis***

The total phenol assay was completed at months 0, 1, 3, 6, and 12 of stability study. For every assay each EGCG aliquot was run in triplicate. Data was analyzed by the Proc GLM procedure and Tukey's test using SAS® software (SAS Inc, Cary, NC). Values in all figures are reported as means  $\pm$  standard error with a probability (p-value) less than 0.05 considered statistically significant (\*). Treatments labeled with different letters are statistically different.

## **A.4 RESULTS AND DISCUSSION**

Average gallic acid equivalents (GAE, mg/L) are reported in Figure 3.1 for aliquots of EGCG measured after incubation at -80°C for 0, 1, 3, 6 and 12 months. The measurements taken after 1, 3 and 6 months of storage at -80°C were not statistically different from the initial GAE content. After 12 months of storage at -80°C, the GAE

concentration of EGCG at Month 12 ( $789 \pm 16$  GAE) was statistically different than the initial concentration of EGCG at Month 0 ( $881 \pm 31$  GAE).



**Figure A.1** Phenol content of EGCG aliquots after 0, 1, 3, 6 and 12 months of incubation at  $-80^{\circ}\text{C}$

## A.5 CONCLUSIONS

The phenolic concentration (measured as GAE) of the EGCG solution was significantly lower after storage for one year at  $-80^{\circ}\text{C}$ , but no significant decrease was noted after six months. This indicates that it is safe to store EGCG aliquots at  $-80^{\circ}\text{C}$  for up to six months.



## **A.6 LITERATURE CITED**

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**APPENDIX B: The Effects of Green Tea and EGCG on Inflammation in LPS- and  
TNF- $\alpha$ - Induced Partially Differentiated Human Colon Cancer (HT-29) Cells**

## **B.1 ABSTRACT**

Colon cancer incidence has been linked to inflammation and lifestyle factors, including diet. Green tea (GT) and epigallocatechin-3-gallate (EGCG), the main flavonoid component of GT, have been reported to have anti-inflammatory and cancer preventive effects in numerous model systems. Populations that consume GT have been found to have a reduced risk of colon cancer incidence, which is the reason for exploring the anti-inflammatory effects of GT in this study. The purpose of this research was to determine the effects of GT or EGCG on inflammation in partially differentiated human colon cancer (HT-29) cells. Aqueous GT and EGCG extracts were prepared and matched for total phenol content by the Folin-Ciocalteu method. Cell viability was determined by the MTT assay. Partial differentiation was induced by sodium butyrate (5 mM, 2 hrs) and confirmed by the alkaline phosphatase (ALP) assay. Inflammation was induced in the HT-29 cells by LPS 0127:B8 (1 ng/ml) and TNF- $\alpha$  (100 pg/ml). Interleukin 8 (IL-8) and prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) concentration were measured by ELISA. Hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) production was measured using the FOX-2 reagent method. Results of the IL-8 ELISA indicate that increasing levels (0.0045, 0.009 and 0.045 mg/ml) of GT and EGCG cause a dose dependent decrease in IL-8 concentration after co-treatment with LPS and TNF- $\alpha$  with the highest concentration of GT and EGCG being significant ( $p < 0.05$ ) in cells stimulated with LPS. Results of the PGE<sub>2</sub> ELISA indicate that increasing concentrations of GT and EGCG cause a dose dependent increase in PGE<sub>2</sub> formation. Hydrogen peroxide formation was proposed as the mechanism for PGE<sub>2</sub> production since increasing concentrations of GT and EGCG caused a dose dependent increase in H<sub>2</sub>O<sub>2</sub> concentration. In conclusion, GT and EGCG

demonstrated both anti-inflammatory (IL-8) and pro-inflammatory (PGE<sub>2</sub>) effects in partially differentiated HT-29 cells. This study highlights the complexity of inflammatory responses and the importance of assessing multiple endpoints in order to fully understand the effects of foods on health. Additionally, it shows that partially differentiated HT-29 cells respond in a similar manner to induced inflammation as fully differentiated cells.

## **B.2 INTRODUCTION**

In the United States cancer is the second leading cause of death (American Cancer Society 2010). Colon cancer incidence has been linked with inflammation and diet, which indicates that modifications to the diet may reduce the risk of colon cancer (Marshall 2009). Green tea (GT) and the major phenolic component of GT, epigallocatechin-3-gallate (EGCG), have been shown to possess anti-inflammatory properties (Butt and Sultan 2009). Additionally, the GT and EGCG have been associated with the prevention of diseases associated with inflammation and oxidative stress (Butt and Sultan 2009) and the consumption of green tea (GT) has been shown to reduce colon cancer incidence (Yang and others 2007). Therefore, the consumption of GT or EGCG may elicit a protective effect against colon cancer incidence.

There are a variety of markers of inflammation that are associated with colon cancer incidence including interleukin-8 (IL-8), prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) (Zhu and others 2002; Sparmann and Bar-Sagi 2004; Chell and others 2006). As a result, compounds that suppress the expression of IL-8, PGE<sub>2</sub> and H<sub>2</sub>O<sub>2</sub> have the potential to decrease the risk of cancer incidence.

The purpose of this experiment was to determine the effect of GT and EGCG on IL-8, PGE<sub>2</sub> and H<sub>2</sub>O<sub>2</sub> inflammatory responses in partially differentiated human colon cancer (HT-29) cells induced by lipopolysaccharide (LPS) and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ). Partially differentiated cells were examined in this study to understand more about the differentiation process that occurs in crypts of the human intestine. Results of this experiment were intended to be presented with the fully differentiated data in chapters 3 and 4 to determine whether partially differentiated cells respond differently to inflammation than fully differentiated cells. However, different LPS serotypes were used for each study making a comparison of the data not possible.

### **B.3 MATERIALS AND METHODS**

#### ***B.3.1 Cell Culture***

Human colon cancer, HT-29 (HTB), cells were obtained from American Type Culture Collection (ATCC) (Manassas, VA). The HT-29 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen; Carlsbad, CA) with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin-glutamine at 37°C in an atmosphere of 5% CO<sub>2</sub>.

#### ***B.3.2 Preparation of Green Tea and EGCG***

Sencha green tea (GT) (Maeda-en; Irvine, CA) was prepared by steeping 1 g of loose-leaf tea in 100 ml deionized (DI) water for 5 minutes. Epigallocatechin-3-gallate (EGCG) (Sigma; St. Louis, MO) was prepared at a 1.8 mM concentration to match the EGCG phenol

content of GT (Nutrient Data Laboratory U.S. Department of Agriculture 2007). The total phenol content of the GT and EGCG treatments was measured via the Folin-Ciocalteu method described previously by Parejo and others (Parejo and others 2002).

### ***B.3.3 Differentiation and Treatment of Cells***

Using the trypan blue assay to determine the live cell count, the HT-29 cells (400,000 cells/ml) were plated in 12 well plates and incubated at 37°C and 5% CO<sub>2</sub>. Once confluent, cells were exposed to sodium butyrate (NaBt, 5 mM) for 2 hours to partially induce differentiation. Differentiation was confirmed by alkaline phosphatase (ALP) assay (Biovision; Mountain View, CA). Cells were treated with varying concentrations of GT and EGCG (0.0045, 0.009, 0.045 mg/ml) for 2 hours prior to inducing inflammation with LPS (O127:B8, 1 ng/ml, Sigma, St. Louis, MO) or TNF- $\alpha$  (100 pg/ml, Sigma). After 24 hours cells were harvested for media samples.

### ***B.3.4 Cell Viability Assay***

Cell viability was determined using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenoltetrazolium) assay based on the method developed by Mosmann (Mosmann 1983). Twenty-four hours after LPS mediated inflammation was induced, 16  $\mu$ l of MTT solution (7.8 mg/ml) was added to each well. After a 2 hour incubation at 37°C, 5% CO<sub>2</sub> to allow for formazan crystal formation, the media was removed from each well. The formazan crystals were resuspended in acidified iso-propanol (0.5 ml). Absorbance was measured at 620 nm using a Multiskan® EX plate reader (Thermo Electron Corporation; Vantaa, Finland).

### ***B.3.5 IL-8 ELISA***

A human IL-8 ELISA kit was purchased from Abcam (Cambridge, MA). The concentration of IL-8 in the media samples was determined by following the manufacturer's instructions.

### ***B.3.6 PGE<sub>2</sub> ELISA***

The PGE<sub>2</sub> monoclonal EIA ELISA kit was purchased from the Cayman Chemical Company (Ann Arbor, MI). The concentration of PGE<sub>2</sub> in the harvested media samples was determined by following the manufacturer's instructions.

### ***B.3.7 H<sub>2</sub>O<sub>2</sub> Assay***

The formation of H<sub>2</sub>O<sub>2</sub> was measured using a modified version of the FOX-2 reagent method previously described by (Nakagawa and others 2004). Immediately after treatment with GT and EGCG, 1 ml of FOX-2 reagent (250 µM ammonium iron(II) sulfate, 25 mM H<sub>2</sub>SO<sub>4</sub>, 100 mM sorbitol and 125 µM xylenol orange (Sigma)) was added to 0.1 ml of each sample, vortexed and incubated at room temperature for 20 minutes. Following incubation, the absorbance was measured at 595 nm using a Spectronic® Genesys™ 2 spectrophotometer (Spectronic Instruments, Inc.; Rochester, NY).

### ***B.3.8 Statistical Analysis***

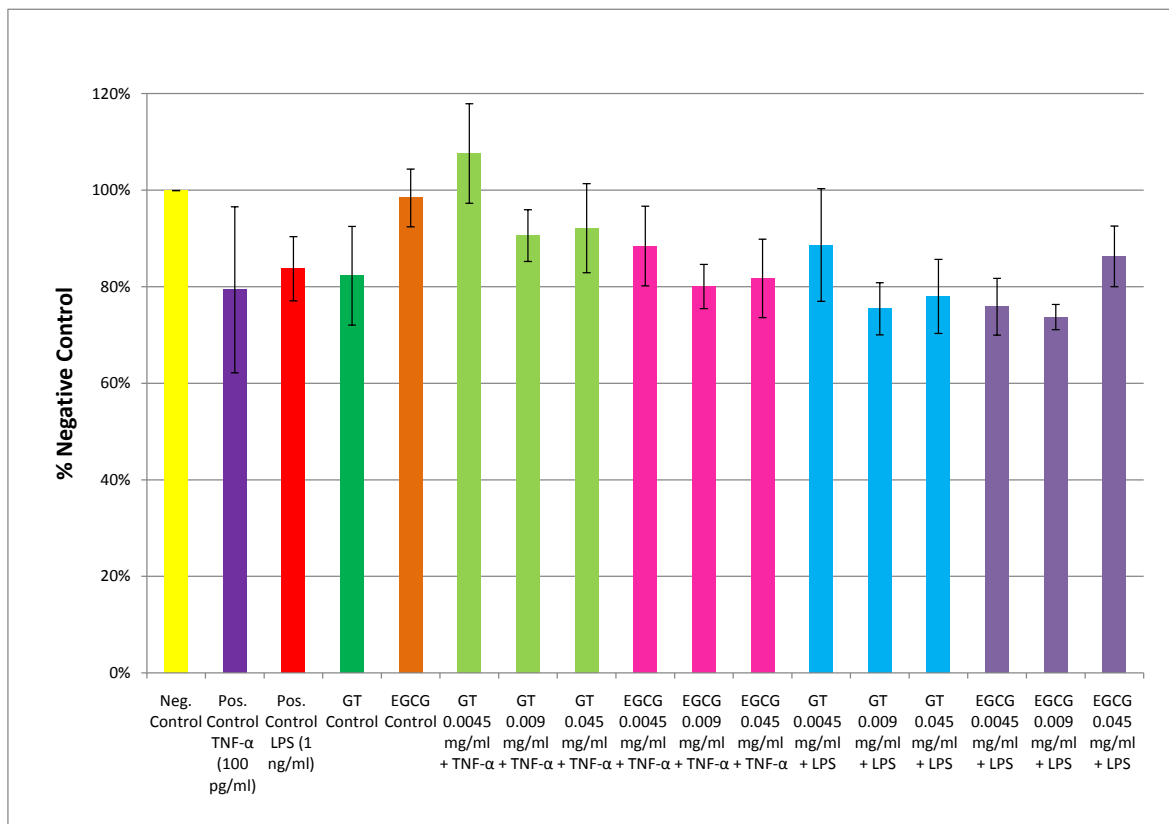
All treatments were run in triplicate. Values are reported as means ± standard error. Data was analyzed by the Proc Mixed and Proc GLM procedure with Tukey's adjustment

using SAS<sup>®</sup> software (SAS Inc, Cary, NC). A probability (p-value) less than 0.05 was considered significant (\*).

## B.4 RESULTS AND DISCUSSION

### B.4.1 Cell Viability Assay

The results of the MTT assay indicate that the treatments of NaBt, LPS, TNF- $\alpha$ , GT and EGCG had no significant effects on cell viability (Figure B.1).

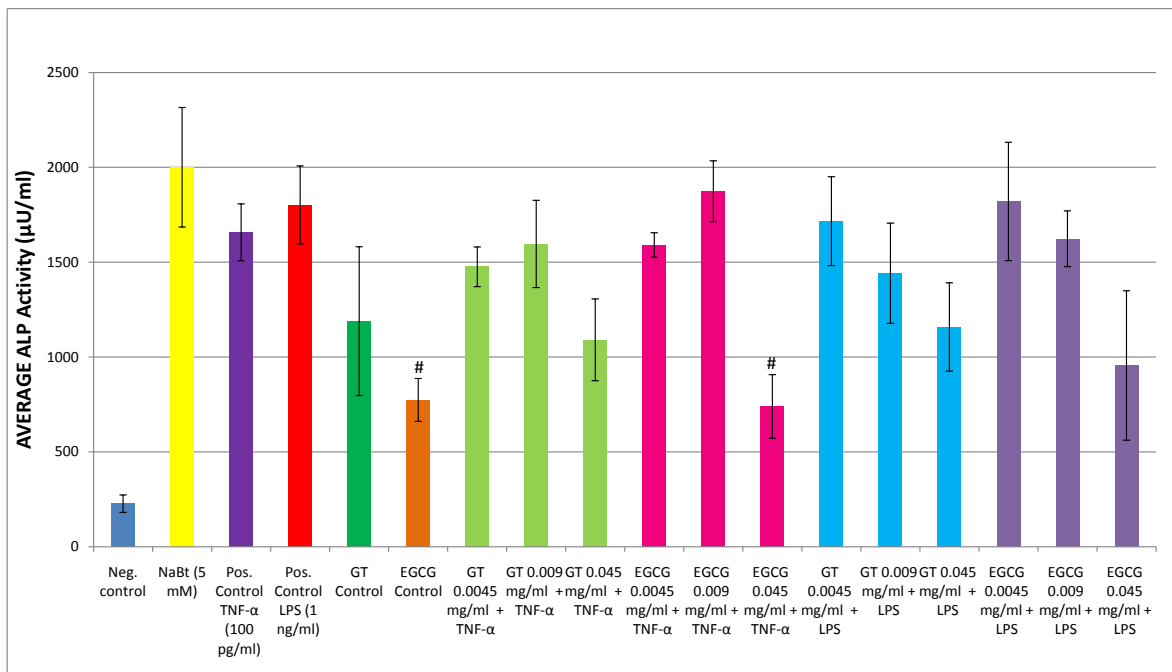


**Figure B.1** Results of MTT Assay with LPS and TNF- $\alpha$ -stimulated partially differentiated HT-29 stimulated cells



### B.4.2 ALP Assay

The results of the ALP assay are shown in Figure B.2. Although there were trends toward lower ALP values for cells treated with higher levels of GT or EGCG, only cells treated with 0.05 mg/ml EGCG alone and with TNF- $\alpha$  showed significantly lower ALP values relative to the negative control, indicating an inhibition of the differentiation process. There is previous evidence of EGCG inhibiting ALP activity, which may explain the lower ALP activity values observed in this study (Yamaguchi and Ma 2001).

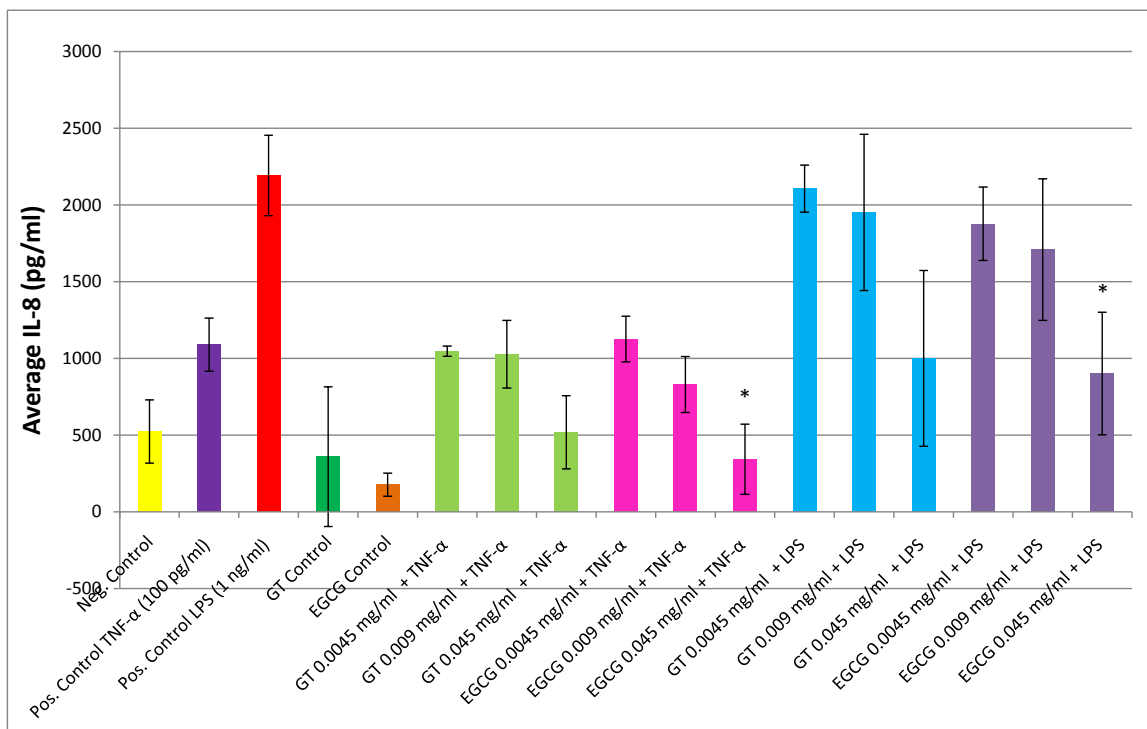


**Figure B.2** Results of ALP Assay for LPS (1 ng/ml) and TNF- $\alpha$  (100 pg/ml)-stimulated partially differentiated HT-29 cells. ‘#’ signifies the only treatments not significantly different from the Neg. Control.

### B.4.3 IL-8 ELISA

IL-8 ELISA experiments were used to measure the concentration of IL-8 in the media samples collected 24 hours after stimulation by LPS and TNF- $\alpha$  and treatment with GT and

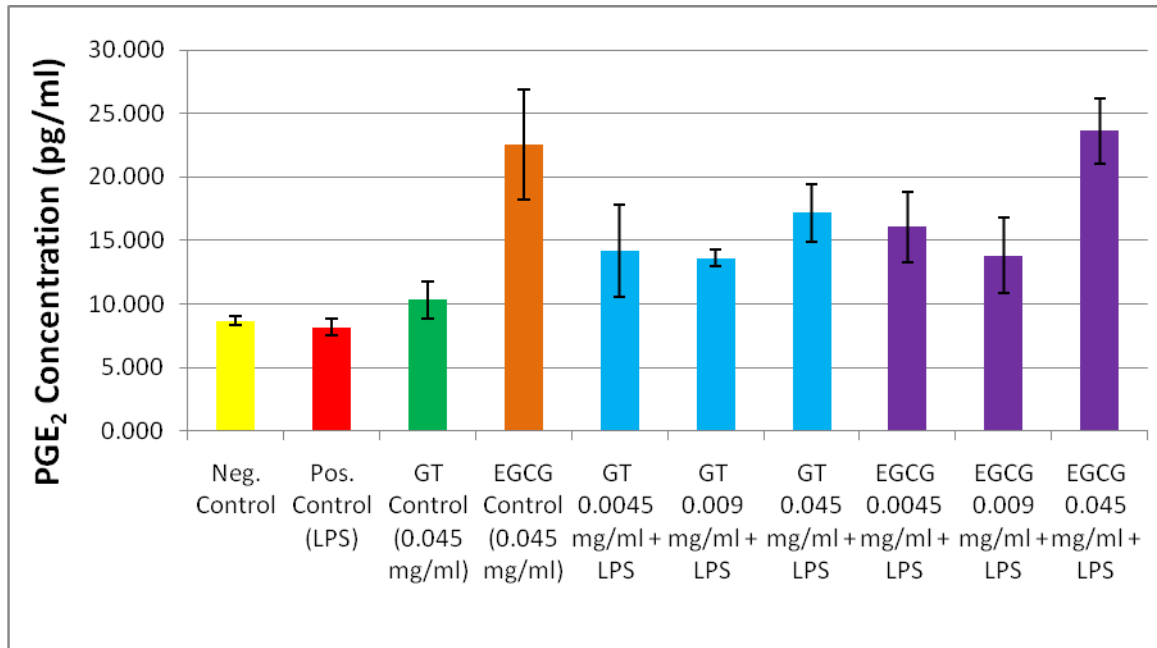
EGCG (Figure B.3). Relative to the negative control, LPS and TNF- $\alpha$  significantly induced inflammation as indicated by an increase in IL-8 levels. Among cells induced with LPS and TNF- $\alpha$ , only those also treated with the highest concentration (0.05 mg/ml) of EGCG showed significant decreases in IL-8 levels compared to the corresponding positive control. While not significant, there was also trend among GT treatments of the highest concentration of GT reducing the production of IL-8 for both LPS- and TNF- $\alpha$ -induced cells. These results indicate that EGCG is more effective than GT at reducing IL-8 inflammation in HT-29 cells. Since IL-8 is correlated with increased risk of colon cancer incidence, it may be beneficial to consume EGCG.



**Figure B.3** Results of IL-8 ELISA for LPS and TNF- $\alpha$ -stimulated partially differentiated HT-29 cells. ‘\*’ signifies the treatments were significantly different from the corresponding positive control.

#### B.4.4 PGE<sub>2</sub> ELISA

The results of the PGE<sub>2</sub> ELISA are reported in Figure B.4. Similar to the results for fully differentiated cells, LPS did not strongly induce inflammation in terms of PGE<sub>2</sub> production in HT-29 cells. Additionally, the GT and EGCG treatments appeared to increase PGE<sub>2</sub> production as their concentration increased. Please see the Results and Discussion section of Chapter 3 for further discussion about the implications of PGE<sub>2</sub> production increases.

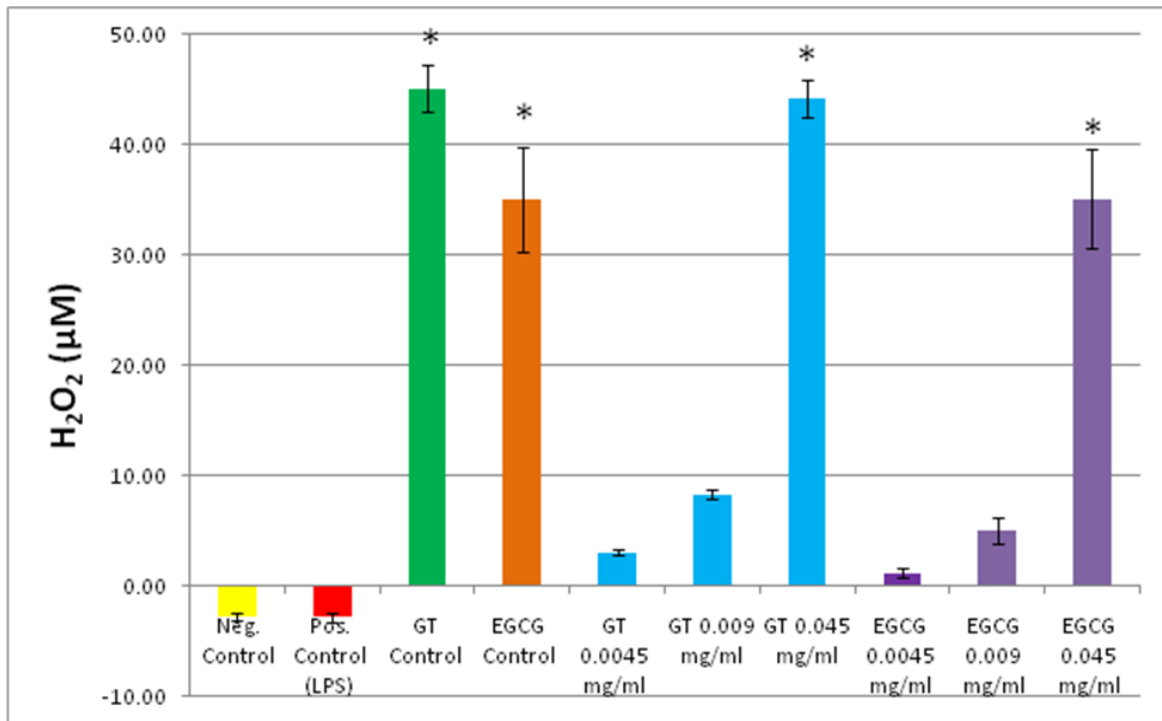


**Figure B.4** Results of PGE<sub>2</sub> ELISA (one replicate reported)

#### B.4.5 H<sub>2</sub>O<sub>2</sub> Assay

A H<sub>2</sub>O<sub>2</sub> detection assay was run to determine whether the observed increases in PGE<sub>2</sub> were due to H<sub>2</sub>O<sub>2</sub> formation. The results of the H<sub>2</sub>O<sub>2</sub> assay are reported in Figure B.5. The results indicate that as the concentration of GT or EGCG increased so did the concentration

of H<sub>2</sub>O<sub>2</sub>. Additionally, the highest concentration (0.05 mg/ml) of GT and EGCG produced a significantly greater amount of H<sub>2</sub>O<sub>2</sub> compared to the negative control. This evidence supports the hypothesis that GT and EGCG treatments were causing a H<sub>2</sub>O<sub>2</sub>-mediated increase in PGE<sub>2</sub> production.



**Figure B.5** Results of H<sub>2</sub>O<sub>2</sub> Assay-0hr for partially differentiated HT-29 cells. ‘\*’ signifies that treatments were significantly different from the negative control.

## B.5 CONCLUSIONS

Overall, the results of the experiments with partially differentiated cells were similar to those with fully differentiated cells (see Chapters 3 and 4 for results). All cell treatment combinations were differentiated after treatment with NaBt for 24 hours except for the highest concentration of EGCG alone and with TNF- $\alpha$ , indicating the EGCG may play a role in inhibiting NaBt mediated cell differentiation. Additionally, EGCG but not GT reduced the

production of IL-8 in LPS and TNF- $\alpha$  induced HT-29 cells. This indicates that it may be more beneficial to consume EGCG as a pure compound as opposed to in a food, like GT. In contrast, both GT and EGCG showed trends of increasing the production of PGE<sub>2</sub> and H<sub>2</sub>O<sub>2</sub> in HT-29 cells. These differences exemplify the complexity of the inflammatory response and how treatments can cause a pro- or anti-inflammatory response depending on what time-point and what marker of inflammation is being measured. Further research is necessary to better understand the effects of foods and food components on health.

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## **APPENDIX C: Detailed Materials and Methods for Chapter 2**

## C.1 Materials

Loose-leaf Sencha GT (Product Code #04859) was purchased from Maeda-san (Irvine, CA). Epigallocatechin-3-gallate (EGCG, >95%), gallic acid, Folin-Ciocalteu phenol reagent, anhydrous sodium carbonate, and Tween-80 were purchased from Sigma-Aldrich, Incorporated (St. Louis, MO). Ethanol (190 proof), glucose, ammonium citrate, sodium acetate, magnesium sulfate, magnesium sulfate\*H<sub>2</sub>O and dipotassium phosphate were purchased from Fisher Scientific (Pittsburg, PA). The MRS Lactobacilli Broth, agar and yeast nitrogen base were purchased from Difco (Difco Laboratories Inc.; Detroit, MI). Casitone was purchased from Bacto (Mt. Pritchard, Australia). The culture of *Lactobacillus acidophilus* NCFM was propagated from frozen stocks and *Lactobacillus gasseri* ATCC 33323 was purchased from American Type Culture Collection (ATCC; Manassas, VA). The laminar flow hood used was a 1300 Series A2, Class II, Type A2 Biological Safety Cabinet bought from Thermo Scientific (Marietta, OH). Absorbance measurements were taken with a Spectronic® Genesys™ 2 spectrophotometer (Spectronic Instruments, Inc.; Rochester, NY). The microtiter plate experiment was conducted using a BMG Lab Tech (Offenburg, Germany) FLUOstar Optima plate reader. Optical density measurements were taken with a Spectronic® Genesys™ 20 spectrophotometer (Spectronic Instruments, Inc.; Rochester, NY). Cells were plated using a Whitley Automatic Spiral Plater (Don Whitley Scientific Ltd.; West Yorkshire, England). Plate counts (CFU/ml) were determined using a ProtoCOL colony counter (Microbiology International; Frederick, MD).



## **C.2 Preparation of Green Tea Aliquots**

Autoclaved water (100 ml) was brought to a boil in an Erlenmeyer flask. Japanese Sencha GT (1.007 g) was added to the boiling water. The GT was removed from the heat source and let steep for 5 minutes. In a sterile laminar flow hood the supernatant was distributed equally into two 50 ml Falcon® centrifuge tubes. The tubes were centrifuged at 130 G for 2 minutes. In the laminar flow hood the top two-thirds of the supernatant were used to make aliquots for future experiments. The GT aliquots were stored in a -80°C freezer.

## **C.3 Preparation of 1.8 mM EGCG Aliquots**

Epigallocatechin-3-gallate (>95%) was prepared in the same manner as GT at a 1.8 mM concentration to match the EGCG phenol content of GT (Nutrient Data Laboratory U.S. Department of Agriculture 2007). Autoclaved water (100 ml) was brought to a boil in an Erlenmeyer flask. EGCG (0.041 g) was added to the boiling water. The EGCG was removed from the heat source and steeped for five minutes. In a sterile cell culture hood the EGCG solution was aliquoted. The EGCG aliquots were stored in a -80°C freezer.

## **C.4 Total Phenol Assay**

The total phenol assay was performed using the Folin-Ciocalteu method as described by Parejo (Parejo and others 2002). It was necessary to determine the total phenol content of GT and EGCG so that their phenol contents could be matched.

#### ***C.4.1 Preparation of the 20% Sodium Carbonate Solution***

Anhydrous sodium carbonate (200 g) was weighed and added to deionized (DI) water (800 ml). The solution was heated with gentle stirring on hot plate until the solution began to boil. The solution was boiled until all the sodium carbonate was dissolved. The solution was removed from the heat and allowed to cool to room temperature. Then, the solution was seeded with 3 heaping tablespoons of anhydrous sodium carbonate. The solution was stored at room temperature, filtered through a Whatman® 4 filter and transferred to a 1L volumetric flask. The solution was brought to a 1L volume with additional DI water.

#### ***C.4.2 Preparation of the Phenol Stock Solution***

Gallic acid (0.25 g) was added to a 50 ml volumetric flask. Ethanol (5 ml) was added to the flask and the flask was swirled gently. The solution was brought to 50 ml volume with DI water and mixed well.

#### ***C.4.3 Preparation of the Calibration (Standard) Curve***

Eight clean test tubes were labeled as follows: 0, 0.1, 0.2, 0.3, 0.5, 1.0, 1.5, 2.0. Sixteen clean test tubes were labeled as follows (in duplicate): 0, 50, 100, 150, 250, 500, 750, 1000. Phenol stock solution was pipetted in the amount indicated (0, 0.1, 0.2...2.0 ml) into correspondingly labeled tubes. DI water was added to each tube to make a final volume of 10 ml in each tube (i.e. 10 ml DI water was added to the tube labeled “0” and 9.9 ml DI water was added to the tube labeled “0.1”). Of the standard solutions 0.1 ml was pipetted into each of the correspondingly labeled tubes (i.e. the tube labeled 0.1 was pipetted into the tube

labeled “50” and the tube labeled 0.2 was pipetted into the tube labeled “100”). DI water (7.9 ml) was added to each tube and mixed by vortexing. The Folin-Ciocalteu reagent (0.5 ml) was added to each tube and a timer was started. After 30 seconds, but before 8 minutes, 20% sodium carbonate (1.5 ml) was added to each test tube and mixed by vortexing. All test tubes were covered in foil and incubated in the dark for 2 hours at room temperature. After 2 hours the absorbance of each tube was read at 765 nm using a Spectronic® Genesys™ 2 spectrophotometer (Spectronic Instruments, Inc.; Rochester, NY). A standard curve was plotted using the gallic acid concentration (milliequivalents) versus the absorbance at 765 nm.

#### ***C.4.4 Preparation of Samples***

First, 0.1 ml of GT or EGCG was pipetted into a test tube. Next, DI water (7.9 ml) was added to each tube and mixed by vortexing. The Folin-Ciocalteu reagent (0.5 ml) was added to each tube and a timer was started. After 30 seconds, but before 8 minutes, 20% sodium carbonate (1.5 ml) was added to each test tube and mixed by vortexing. All test tubes were covered in foil and incubated in the dark for 2 hours at room temperature. After 2 hours the absorbance of each tube was read at 765 nm using a Spectronic® Genesys™ 2 spectrophotometer (Spectronic Instruments, Inc.; Rochester, NY).

### **C.5 Preparation of Media**

#### ***C.5.1 MRS Media***

MRS Lactobacilli Broth (55 g) was added to DI H<sub>2</sub>O (1 L) and mixed until blended using stir plate. Media was aliquoted and autocaved before use.

### ***C.5.2 Semi-Defined Medium with 1% Glucose***

Semi-Defined Medium (SDM) was prepared as previously described (Kimmel and Roberts 1998). Glucose (10 g), tween 80 (1 g), ammonium citrate (2 g), sodium acetate (5 g), magnesium sulfate (0.1 g), magnesium sulfate\*H<sub>2</sub>O (0.05 g), dipotassium phosphate (2 g), yeast nitrogen base (5 g) and casitone (10 g) were added to 1 L purified water and mixed until dissolved. The SDM was aliquoted and autoclaved before use.

## **C.6 Microtiter Plate Experiment**

### ***C.6.1 Preparation of 1, 2, 4, 10, 20, 30 and 40% GT and EGCG Treatments***

Frozen cultures of *Lactobacillus acidophilus* NCFM and *Lactobacillus gasseri* ATCC 33323 were propagated in MRS broth and incubated overnight at 37°C before use. For experiments, a 1% inoculation (0.1 ml) using the bacteria grown overnight was made into SDM (amounts varied depending on GT and EGCG concentration). Then, the corresponding amount of GT or EGCG was added to make the 1, 2, 4, 10, 20, 30 and 40% GT and EGCG treatments.

### ***C.6.2 Microtiter Plate Protocol***

Cell growth was monitored in 96 well plates at OD<sub>600nm</sub> using a BMB LabTech FLUOstar Optima plate reader. The cultures were grown in 200 µL of media for 22 hours at 37°C.

## **C.7 Growth Rate Curve Experiments**

### ***C.7.1 Preparation of 1, 10, and 30% GT and EGCG Treatments***

Frozen initial starter cultures of *Lactobacillus acidophilus* NCFM and *Lactobacillus gasseri* ATCC 33323 were inoculated into 10 ml of MRS broth and incubated overnight at 37°C before use. For experiments, a 1% inoculation (0.1 ml) using the bacteria grown overnight was made into SDM (amounts varied depending on GT and EGCG concentration) in a test tube. The corresponding amount of GT or EGCG was then added to the test tube to make the 1, 10 and 30% GT and EGCG treatments (i.e. for a 10% GT treatment, 1 ml of GT was added to 8.9 ml of SDM). A final volume of 10 ml was reached in each test tube. All conditions were run in triplicate.

### ***C.7.2 Growth Rate Curve Experiment Protocol***

After test tubes were inoculated, the broth cultures were incubated at 37°C in between readings. Readings were taken at OD<sub>600 nm</sub> using a Spectronic® Genesys™ 20 spectrophotometer (Spectronic Instruments, Inc.; Rochester, NY) at varying time points (typically over a 12 hour period).

## **C.8 Plating Experiments**

### ***C.8.1 Making Agar Plates***

Agar (1.5%) was added to MRS broth and autoclaved. The agar solution was tempered in a 55°C water bath for approximately one hour. The agar solution (20 ml per plate) was poured into petri dishes and gently spread to remove any bubbles. The agar

solution solidified at room temperature before the plates were inverted and stored in the refrigerator.

### ***C.8.2 Preparation of 1, 10, and 30% GT and EGCG Treatments***

Frozen initial starter cultures of *Lactobacillus acidophilus* NCFM and *Lactobacillus gasseri* ATCC 33323 were inoculated into 10 ml of MRS and incubated overnight at 37°C before use. For experiments, a 1% inoculation (0.1 ml) using the bacteria grown overnight was made into SDM (amounts varied depending on GT and EGCG concentration) in a test tube. Then, the corresponding amount of GT or EGCG was added to the test tube to make the 1, 10 and 30% GT and EGCG treatments (i.e. for a 10% GT treatment, 1 ml of GT was added to 8.9 ml of SDM). A final volume of 10 ml was reached in each test tube. All conditions were run in duplicate.

### ***C.8.3 Spiral Plater Protocol***

A Whitley Automatic Spiral Plater was used for plating experiments. First, the vacuum was turned on. Two small white containers were autoclaved with DI water prior to using the spiral plater. The container in the back of the machine was filled approximately 1/3 of the way with ethanol (70%). Then, the spiral plater was turned on. The settings were set for 50 µL, autovalve, F<sub>2</sub> and log. The machine was washed 3 times. Next, a test run using the P-ink (blue dye) was done to confirm that the machine was plating properly. Finally, the samples were plated by pushing the button with a spiral symbol on it. The machine was washed 3 times in between each sample and 10 times after use.

#### ***C.8.4 Plate Count Experiment***

The 1, 10 and 30% GT and EGCG treatments in SDM with *L. acidophilus* and *L. gasseri* were prepared as described above. The test tubes containing the cultures were stored at 37°C in between plate count data collection using the spiral plater. Plate count data was collected over a 30-40 hour period. After all the treatments were plated, the MRS plates were stored inverted in an anaerobic chamber for 24 hours. The colonies on each plate were counted using a ProtoCOL colony counter.

**APPENDIX D: HPLC Analysis of Green Tea Aliquots**



## **D.1 MATERIALS AND METHODS**

GT and EGCG were prepared as previously described in this thesis. The EGCG sample (828  $\mu\text{g/ml}$ ) was used as a standard for determining the amount of EGCG in the GT sample.

High performance liquid chromatography (HPLC) analysis was performed by Lisa Oehrl Dean using a Dionex Summit Analytical HPLC with a Phenomenex Hyperclone ODS (C18) column (120  $\text{\AA}$ , 250 x 4.00 mm). The mobile phase A was 0.1% formic acid in water and mobile phase B was 0.1% formic acid in acetonitrile. A flow rate of 1.5 ml/min (gradient) was used with UV detection at 250 nm.

## **D.2 RESULTS**

Using the EGCG (828  $\mu\text{g/ml}$ ) as a standard, the EGCG content of the GT sample was determined to be 173  $\mu\text{g/ml}$  (Figure D.1).

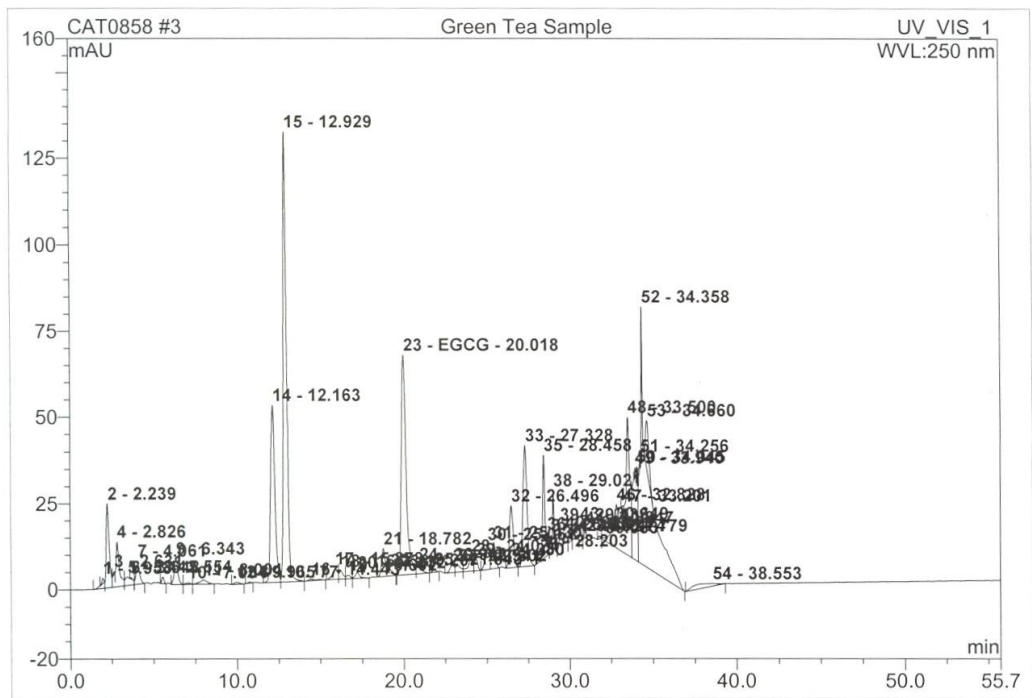


Figure D.1: HPLC analysis results for GT sample