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Influence of pathogenic bacterial determinants on genome stability of exposed intestinal cells and of distal liver and spleen cells

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INFLUENCE OF PATHOGENIC BACTERIAL DETERMINANTS

ON GENOME STABILITY OF EXPOSED INTESTINAL CELLS

AND OF DISTAL LIVER AND SPLEEN CELLS

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INFLUENCE OF PATHOGENIC BACTERIAL DETERMINANTS ON GENOME STABILITY OF EXPOSED INTESTINAL CELLS AND OF DISTAL LIVER AND SPLEEN CELLS

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ABSTRACT

Most bacterial infections can be correlated to contamination of consumables such as food and water. Upon contamination, boil water advisories have been ordered to ensure water is safe to consume, despite the evidence that heat-killed bacteria can induce genomic instability of exposed (intestine) and distal cells (liver and spleen). We hypothesize that exposure to components of heat-killed *Escherichia coli* O157:H7 will induce genomic instability within animal cells directly and indirectly exposed to these determinants. Mice were exposed to various components of dead bacteria such as DNA, RNA, protein or LPS as well as to whole heat-killed bacteria via drinking water. Here, we report that exposure to whole heat-killed bacteria and LPS resulted in significant alterations in the steady state RNA levels and in the levels of proteins involved in proliferation, DNA repair and DNA methylation. Exposure to whole heat-killed bacteria and their LPS components also leads to increased levels of DNA damage.

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"Wisdom is not a product of schooling but of the life-long attempt to acquire it". – Albert Einstein

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Alas1 – aminolevulinic acid synthase 1

ATP – adenosine triphosphate

BAK – BCL-2-homologous antagonist/killer

BAX – BCL-2-associated X protein

BER – base excision repair

CDC – Centre for Disease Control and Prevention

CDK – cyclin dependent kinase

CNF – cytotoxic necrotizing factor

CpG – cytosine-guanine dinucleotide

COX2 – cyclooxygenase subunit 2

Cyp7a1 – cytochrome p450 family 7, subfamily a, polypeptide 1

DAPI - 4',6-diamidino-2-phenylindole

DD – death domain

DNA – deoxyribonucleic acid

DNMT – DNA methyltransferase (1, 3a and 3b)

Dusp1 – dual specificity protein phosphatase 1

E. coli – Escherichia coli

ERK – extracellular signal-regulated kinase

Esm1 – endothelial cell-specific molecule 1

FADD – Fas-associated death domain protein

FAK – focal adhesion kinase

Gadd45g – growth arrest and DNA-damage-inducible protein

Gast1 – glutathione S-transferase alpha 1

H. felis – Helicobacter felis

H. pylori – Helicobacter pylori

HBV – hepatitis B virus

HDAC – histone deacetylases

HIV – human immunodeficiency virus

HPC – hepatitis C

HPV – Human Papilloma Virus

HTLV-1 – human t-cell lymphotrophic virus type 1

HUS – Hemolytic-uremic syndrome

IARC – International Agency for Research of Cancer

IgA – immunoglobulin A

IHC – immunohistochemistry

IL – interleukin (1-12)

IFNγ – interferon γ

JNK – c Jun N-terminal kinase

KDO – 2-Kept-3-deoxyoctulosonic acid

LPB – LPS binding protein

LPS – lipopolysaccharide

MALT – mucosa-associated lymphoid tissue

MAPK – mitogen-activated protein kinase

MecP2 – methyl CpG binding protein

MIP2 – macrophage inflammatory protein 2

Mmd2 – monocyte to macrophage differentiation associated 2

mRNA – messenger ribonucleic acid

MTK1 – MAP kinase kinase kinase (or MEKK4)

m5c – 5-methylcytosine

NHEJ – non-homologous end joining

NFAT – nuclear factor activated T cell

PagP – palmitoyltransferase

PAMPS – pathogenic associated molecular pattern

PCNA – proliferating Cell Nuclear Antigen

PGE2 – prostaglandin –E2

PHD – pleckstrin *homology domain*

PMLRAR – promyelocytic leukemia protein-retinoic acid receptor fusion protein

pRB – retinoblastoma protein

PRR – post replication repair

RBC – red blood cell

Rho – ras homolog gene family

RNOS – reactive nitrogen oxide species

RNA – ribonucleic acid

ROS – reactive oxygen species

S. typhi – *Salmonella enteric enterica serovara Typhinmurium*

STX – shiga toxin

Tff3 – trefoil factor 3

TNF-R – tumour necrosis factor receptor

TLR – toll-like receptor

TNF-α – tumour necrosis factor α

TRAFs – TNF receptor associated factor

TRAD – TNF receptor associated death domain

T3SS – type 3 secretion system

VEGF – vascular endothelial growth factor

YopH – *yersinia* protein tyrosine phosphatase

CHAPTER 1: GENERAL INTRODUCTION

Evidence indicates a relationship between an infection originating from a bacterial source and instability of the genome (Lax and Thomas 2002). However, a substantial lack of knowledge about possible mechanisms involved in the development of genomic instability has interrupted the progression of research in this area. Many questions have remained unanswered; for example: why are there only a small number of cancer cases reported as being caused by bacterial infection? Do the bacteria have to be alive to induce instability in the genome? What is the mechanism responsible for induction of genome instability?

I hypothesize that exposure to heat-killed bacteria determinants may trigger genome instability of exposed and distal cells. Thus, the focus of this thesis is on the analysis of influence of heat-killed bacteria or their determinants on present new findings supporting the hypothesis of the bystander-like effect, and the molecular mechanisms thought to be involved in inducing genome instability.

1.1. *Escherichia coli* O157:H7

The serotype of *E. coli* identified as O157:H7 was first isolated in 1982, when a number of people in Michigan and Oregon developed clinically distinctive gastrointestinal illness when they consumed contaminated hamburger meat (Riley *et al,*. 1983). Analysis of stool samples from infected victims identified serotype *E. coli* O157:H7 contamination. Since the initial report of an outbreak, sporadic cases (Pai *et al.,* 1988) and full outbreaks (Duncan, *et al.* 1986), resulting from acute and chronic exposure

to these bacteria have been on an increase within Canada, United States and the United Kingdom.

E. coli is a facultative anaerobic chemoorganotrophs, capable of both respiratory and fermentative metabolism. Certain strains of *E. coli* serve a useful function in the human body by suppressing the growth of harmful bacterial species and by synthesizing appreciable amounts of vitamins (Riley *et al.,* 1983). *E. coli* colonizes the lower intestinal track of animals and survives when released into the natural environment, allowing widespread dissemination to new hosts. *E. coli* is responsible for infections of the enteric, urinary, pulmonary and nervous systems. *E. coli* O157:H7 is an enterohemorrhagic strain, producing large quantities of one or more related potent toxins that cause severe damage to the lining of the intestine (Riley *et al.,* 1983). The acute disease caused by the bacterium is called hemorrhagic colitis and is characterized by severe cramping and diarrhea.

Patients with infections from pathogenic *E. coli* present a wide spectrum of clinical manifestations of symptoms, including but not limited to severe abdominal cramps, watery diarrhea (which can later develop into bloody diarrhea), and little or no fever (Griffin *et al.,* 1988). Infections can also be asymptomatic occurring without bloody diarrhea (Belongia *et al.,* 1991). The intensity of infection of the intestinal tissue has been associated with Hemolytic-uremic syndrome (HUS) and thrombotic thrombocytopenic purpura (Control, 1999) and has the potential to become fatal (Pavia *et al.,* 1990).

E. coli O157:H7 are also known as *Enterohaemorrhagic E. coli* (EHEC) based on their capability of producing Shiga toxin (Stx) (O'Brien *et al.,* 1983). Stx are believed to be the major precipitants of the vascular lesions responsible for the formation of histopathological basis of HUS (Richardson *et al.,* 1988). After ingestion of the EHEC, Stx are released within the intestinal portion of the digestive system and are subsequently absorbed across the epithelium into the circulation (Hurley *et al.,* 2001). When the Stx reaches a target cell, the Stx binds to the glycolipid receptor globotraiosylceramide (Gb3), which is expressed in the vascular tissue of organs such as the intestinal tract (Proulx *et al.,* 2001). Proulx *et al.* (2001) identified that the internalization by receptor-mediated endocytosis is followed by the interaction of Stx with subcellular components that results in protein synthesis inactivation, DNA damage or apoptosis. Also, the Stx produced by *E. coli* O157:H7 could induce mRNA expression, such as endothelium-1, which could additionally lead to cell damage/death (Bitzan *et al.,* 1998).

1.2 Sources of Bacterial Contamination

Humans are exposed to foreign bacteria daily through contact with many sources such as computer keyboards, phones and door handles. Most exposures to bacteria are not harmful to humans; however, exposures have been identified as major causes in medical pandemics in countries around the globe.

1.2.1. Sources of Exposure

Food- and water-borne transmission of bacteria is the most important means of spreading bacterial infection (Riley *et al.,* 1983). In Europe, approximately 14,000 cases of human bacterial infection between 2000-2005 were identified through the consumption

of contaminated vegetables. Of these 14,000 cases 62% were analysed and serogroup *E. coli* O157 was identified as the cause (Fisher and Meakins, 2006).

Another method of exposure and more crucial to this thesis, is the exposure of an organism through contaminated drinking water (CDC, 1999). It is estimated in the United States, during a single outbreak in Milwaukee in 1993, 403,000 individuals were infected by a pathogen within the water supply (MacKenzie *et al.,* 1994). Another case of waterborne bacterial infection occurred in North Battleford, Saskatchewan, where approximately 6,000 people developed gastroenteritis in April 2001 (Stirling *et al.,* 2001).

Waterborne outbreaks of enteric disease occur during at least two possible events (Barwick *et al.,* 2000). First, drinking water supplies are not sufficiently monitored and treated for contamination from surface water such as fecal matter (Barwick *et al.,* 2000; Kondro *et al.,* 2000). This may be caused by lack of monitoring equipment, improper scheduled testing of the water or lack of training of individuals responsible for the maintenance of water quality. The second possible cause occurs when surface water, which is contaminated by pathogens (e.g., *E. coli* O157:H7), is used for recreational purposes. Lakes and rivers in arid areas are preferred locations for larger communities as well as intensive livestock operations. An infamous and widely publicized case in recent Canadian history was in a small community named Walkerton, Ontario. In May 2000, a contamination of *E. coli* O157:H7 and *Campylobacter* spp. was identified at Pump Station 5 of the municipal water supply. In total, a community of 4,800 citizens reported 2,300 cases of gastroenteritis, 65 were hospitalized, 27 developed HUS and several individuals died (Hrudey *et al.,* 2003). A formal inquiry was held by the Government of

Canada which identified the cause of the outbreak to be from *E. coli* O157:H7 and *Campylobacter* spp. Further analysis identified the source of the bacteria to be surface run off of fecal matter from nearby livestock farms combined with human error in the processing of the water.

Bacterial contamination of a water source was also observed in 1996 in multiple schools within the City of Sakai, Japan. Japan Health Services reported that ten patients were identified as being ill due to bacterial contamination. Bacteria most likely were transmitted through the ingestion of food or water contamination between 1991 and 1995. However, in 1996, there were 9,451 cases and 12 deaths from *E. coli* O157:H7, representing patients from multiple outbreak including the Sakai City, Japan incident (Michino *et al.,* 1999). Health officials were notified when an alarming number of young children were hospitalized for symptoms associated with bacterial infection of the gastro-intestinal tract. Indicators of the infection were abdominal pain, diarrhea, bloody diarrhea, fever, vomiting and/or nausea, suggesting an *E. coli* O157:H7 infection. Scientists also identified that the children being diagnosed with these symptoms all attended a small school in the City of Sakia, Japan.

Teachers and school board representatives in Japan had encouraged students to eat food prepared and provided by the school cafeteria. All of the food in question had been consumed, and therefore it was difficult to identify the specific cause of the outbreak. Further interviews by food inspectors and public health nurses, using a questionnaire to determine the day of onset, identified contamination from a nearby white radish sprout farm. The outbreak was identified as *E. coli* O157:H7 in the water supply to the farm producing the consumed vegetables (Michino *et al.,* 1999).

1.2.2. Sources of Contamination

Most common source of contamination of water is at the point where run-off from fields enters the water cycle and finally into rivers and lakes. Reviews conducted of waterborne bacterial outbreaks over the past 50 years within North America, determined that over 68% of waterborne bacterial outbreaks were preceded by high intensity precipitation events (Curriero *et al.,* 2001). In most of the cases, excess of heavy rains or abnormally high rates of water from snowmelt caused intense runoffs from nearby farms. The excess water accumulates within nearby rivers and lakes that are major sources of drinking water for communities.

Farm run-off has been identified as a source of water contamination for the surrounding geographical areas (Girones *et al.,* 2010). Livestock manure is a major source for *E. coli* O157:H7 and is an identified source for surface-water contamination (Kondro, 2000; Johnson *et al.,* 2003). Concerns about the risk of contamination from agricultural practices were raised at numerous public hearings during the development of the pump station in Walkerton during 1976. Poor geographical location and lack of monitoring allowed contaminated water from the surrounding area to enter shallow wells and eventually resulted in contamination (Hrudey *et al.,* 2003).

Research on *E. coli* O157:H7 has indicated that the duration of the bacteria to incubate within the host is approximately two to eight days (Boyce *et al.,* 1995; Nataro and Kaper, 1998). The duration of symptomatic *E. coli* O157:H7 infection was estimated to be after a $14 - 16$ days of incubation, using data from outbreaks including daycare facilities in Minnesota from 1988 (Armstrong *et al.,* 1996). The possibility of secondary transmission of *E. coli* O157:H7 (person to person) accounts for as little as four percent

of reported infections (Seto and Hoskins 2007), indicating that the major source of infection is from consumption of a contaminated food/water source. Due to this concern and other health related illnesses associated with waterborne bacteria, the Government of Canada developed a multi-barrier approach for water quality protection. This multi-barrier approach is an integrated system of processes, procedures and tools that communally prevent or reduce the risk of drinking water being contaminated in a "source to tap" model (Figure 1.1) (Lim et al., 2002).

In Canada, from 1986 to 1993, approximately 150 suspected bacterial outbreaks from contaminated drinking water sources were reported to Health Canada (Bryan*, 1996). In recent studies, there were 20,000 infections identified and 100 deaths associated with contamination in the USA annually. These recent outbreaks, combined with medical records, suggest that drinking water may be a substantial source of bacteria causing endemic (non-outbreak related) gastroenteritis (Daniel *et al.,* 2002). Within a three-year span in the early 1990s, of the 205 children under the age of 15 years from Alberta diagnosed with HUS, 77% were likely infected by *E. coli* O157:H7 (Rowe et al., 1998). Lethbridge and regions of southern Alberta have been identified as a high livestock density area and has one of the highest levels of gastroenteritis in Canada stemming from *E. coli* O157:H7 (Khakhria and Johnson, 1996).

1.2.3. Preventative Methods

"Source to tap" refers to the continuous monitoring of drinking water from wells, lakes, rivers, etc., through the treatment process and testing at numerous residential locations within the community. Under this approach (Figure 1.1), all potential controls and limitations are identified. Limitations could include risks of pathogens or

contaminants passing through the barrier. Independently, the barriers may be inadequate to kill pathogens, but together an integrated network of procedures, offers greater assurance that the water will be free of pathogens (Lim *et al.,* 2002). This method is also identified to help with the sustainability of water supply systems.

A multi-barrier approach to water monitoring, treatment, distribution and early detection is the most contemporary method used to ensure the quality of the water provided to civilians, however, this approach is not 100% guaranteed to prevent such outbreaks. Failure of the multi-barrier (five point water treatment monitoring system) was the reason for such a large portion of the population in Walkerton becoming ill (O'Connor, 2002). Drinking water regulations in the United States and Canada are based on the occurrence of fecal indicator bacteria within the water.

1.2.4. Thermal Treatment

When fecal contamination is detected, the suggested actions promoted by Health Canada (2009) and the U.S. Centers for Disease Control and Prevention (CDC) is to order the citizens of the area to either drink only bottled water or issue a "Boil Water Advisory/Boil Water Order". Boil water advisories and boil water orders are notices to the public that they are to boil their tap water before using it in their homes for drinking, washing and cooking. These measures are identified as preventative measures to protect the public from waterborne infectious agents that could be found in the water.

Boil water advisories/orders can be ordered if an unacceptable amount of bacteria, parasites or viruses are detected in a water system. Quality monitoring occurs at all points along the "source to tap" monitoring system and can be issued at any time during the five

point water treating method. If the water has an unacceptable level of turbidity at the source, the government may also order the water to be boiled. These conditions may stem from inadequate filtration, insufficient or ineffective disinfection during treatment or the possibility of re-contamination during distribution. Water is to be brought to a roiling boil at $100^{o}C$, for a minimum of one minute, to ensure the safety of the water to consume according to Health Canada's Boil Water Advisory Page (2008).

1.3 Bystander Effect

It is generally accepted that any damaging effect (stressor) from an external source (e.g., radiation) will induce damaging effects on cells directly exposed to the stressor (Rzeszowska-Wolny et al., 2009). It was thought that no effects would be experienced in cells that had no direct exposure to the stressor. This traditional view has been challenged by reports of neighbouring and distal cells (that are not exposed to the stressor) expressing damage maintenance behaviour. Observed changes included: sister chromatid exchange (Nagasawa and Little, 1992), chromosome aberrations (Prise et al., 1998) and cell transformation (Sokolov et al., 2007). The phenomenon is known as the bystander effect. For irradiated cells, the bystander effect is defined as the recognition of the occurrence of biological effects in non-irradiated cells as a result of exposures of different or distal cells (Mothersill and Seymour, 2001; Little et al., 2002).

Nagasauce and Little (1999) initially identified the bystander effect by analyzing the effects of cells exposed to α-particles. However, with the use of gene expression analysis as an end point, it was shown that the effects of the stress are transmittable from irradiated cells to non-irradiated cells (Azzam and Little, 2004). Upregulation of the p53

stress response pathway in response to potential DNA damage was supported by the observation that p53 was phosphorylated on serine 15 and micronuclei formation was stimulated in bystander cells. This response was not restricted to cells irradiated with α-particles, but the effect was observed in non-irradiated cells as well.

Further studies on the mechanisms underlying the induced bystander effect by radiation have identified mediating factors. Intracellular and extracellular oxidants, such as reactive oxygen species (ROS) contribute to the effects on distal naïve cells. An increase in the presence of ROS correlated directly with the enhanced secretion of cytokines such as interleukins 1, 8, growth factor-β and tumour necrosis factors (Morgan, 2003).

Similar to chronic infections, the production of ROS has been identified to assist in the promotion of the bystander effect phenomena to distal cells. ROS are produced during infection (Ohshima *et al.,* 1994), and correlate with the effectiveness of the bystander response. However, since the bystander effect initiated from exposure to an ionizing radiation source, we can propose that chronic infection can stimulate the appearance of a bystander response using similar mediatory factors for communicating to distal cells. The bystander-like effect in response to bacterial infection may destabilize the genomes of neighboring/distal cells not exposed to infection.

1.4. Carcinogenesis Induced by Bacterial Infection

1.4.1. History

After the onset of cancers, based on the consumption of tobacco, infections as a group *in toto* may be the most important cause of cancers in humans that is preventable

(Kuper *et al.*, 2000). Early observations identified bacteria present at sites of carcinomas (Lax and Thomas, 2002). This concept created an uneasy and controversial history of carcinogenesis which has evolved our understanding and treatment of tumourogenic and infectious processes (Lax and Thomas, 2002). The theory that microorganisms may be a causative agent contributing to the appearance of cancer had many fallacious leads. Initially, exploration of the origins of cancer hinted that yeast were the cause of cancer or that a cause must be smaller than a cell (Kuper *et al.,* 2000).

Carcinogenesis is a prolonged and complex process that can take years to transform a regular cell to a cancerous cell. Initially, mutations allow a cell to break free of its cell cycle control (normal growth-control mechanisms). This cell must proceed to proliferate while avoiding destruction by the immune system. Once a small tumour has formed, angiogenesis (formation of blood vessels to provide essential oxygen and nutrients to the tumour) must occur before it is allowed to grow further (Carmeliet and Jain, 2000). With the formation of blood vessels, the cancerous cells attain a channel to transport to other parts of the body and settle to form tumours elsewhere. Early studies identified bacteria at sites of carcinogenesis; however, researchers did not identify the long time lag between infection and the development of carcinogenesis. Therefore, the presence of bacteria did not mean causation in the case of the development of cancer.

Despite original interest in the field, research into infectious agents causing cancer failed to have any major significant advances until the 1960s. Within the past half century, research has expanded in the fields of epidemiology and infectious disease biology, epidemiological and serological methods and the biological knowledge base for

understanding infectious agents (Kuper *et al.,* 2000). A major cause for this interest is the theory that significant portions of cancers being treated today are preventable.

In 1772, bronchogenic lung carcinomas frequently appeared in areas of the lung with pulmonary scars from tuberculosis, thus implicating *Mycobacterium tuberculosis* as possible cause of lung cancer (Onuigbo, 1975). In the middle of the 19th century, Rudolf Virchow made one of the first epidemiological observations on the relationship between a chronic inflammation and the origins of cancers (Balkwill and Mantovani, 2001). It was also Peyton Rous, in 1939, who demonstrated the oncogenic potential of the cottontail rabbit papillomavirus Kuper *et al.,*2000). Rous was the first scientist to demonstrate cancer may have an infectious agent with his research demonstrating an RNA virus causing carcinogenesis. In the late 1960s, the response to the field of infectious cancer causing viruses sparked the US Government to create the US Virus Cancer Program. With the creation of such a program, molecular biologists gathered to develop new hypotheses and identify viruses had causative agents of cancer. Unfortunately, focus on the research analysing the bacteria involved was left until decades later (Kuper *et al.,* 2000).

In the 1990s, it was estimated that levels as high as 15% of the cancers worldwide have originated from an infectious agent, exceeding global totals over a million cases per year (Pisani *et al.,* 1997). Within the developed world, this number is estimated to be lower, due to highly monitored and efficient screening of food and water supplies.

1.4.2. Viral Infections and Carcinogenesis

Carcinogenesis occurring from viral infection such as hepatitis B virus (HBV) and human papillomavirus (HPV) is widely accepted primarily due to the mechanistic effects of cellular transformation (Kuper *et al.,* 2000). There are three main mechanisms identified to cause cancer with viral infections. These mechanisms appear to involve: chronic inflammation; direct transformation of cells; and immune-suppression of the host.

Sustained exposure to a virus could induce chronic inflammation, commonly accompanied by formation of ROS and reactive nitrogen species (RNOS) by phagocytes at the site of inflammation (Ohshima *et al.,* 1994). ROS and RNOS have the ability to alter enzyme activity, gene expression and to damage DNA. These events occur along with chronic inflammation resulting in repeated cycles of cell damage, compensated by cell proliferation, favouring carcinogenesis (Cohen *et al.,* 1991). This process increases the rate of cell division and simultaneously promotes an increase in DNA damage and tumourigenesis.

Infectious agents (viruses) may directly transform cells, through the process of inserting active oncogenes into the host genome. Directly, this has the potential to inhibit the ability of the cell to activate tumour suppressor genes/mechanism. Another potential effect of viral infection is oncogene activation in the host cell, causing an increase in proliferation, promoting tumourigenesis. Finally, viral infectious agents could induce immuno-suppression, usually originating from infections (Kuper *et al.* 2000).

It is important to note that infectious agents (particularly viruses) that have been hypothesized as being oncogenic, are generally highly prevalent within the host population (Zur Hausen, 1999). However, virus-associated malignancies are rare among infected individuals and usually occur after a chronic infection.

1.4.3. Bacterial Infection and Carcinogenesis

The induction of cancer, based on a previous infection is a highly debated subject. The role of viruses, such as Hepatitis B virus (HBV) in the process of carcinogenesis, is more widely accepted because of the direct mechanistic effects of gene expression alterations resulting from cell transformation (Kuper *et al.,* 2000). Induction of carcinogenesis by bacterial infection remains controversial due to the lack of evidence/research dedicated to supporting the correlation between infection and cancer development. This controversy is based on the argument of the unknown molecular mechanism(s) that could promote the development of carcinogenesis.

This field of carcinogenesis was revolutionized when *Helicobacter pylori* was identified as a source of chronic infections in the digestive tract of its host. This infection has been identified to cause stomach ulcers, followed by a onset of gastric carcinomas (Parsonnet *et al.,* 1991) and mucosa-associated lymphoid tissue (MALT) lymphomas (Wotherspoon *et al.,* 1991). The data to identify the precise mechanism of infectious agents inducing carcinogenesis is currently unavailable; however, an image is emerging that several bacteria have the ability to contribute to different stages of tumourigenesis.

Today, the International Agency for Research on Cancer (IARC) has identified that there is insufficient evidence to recognize some pathogens as carcinogenic to

humans. The pathogens are human papilloma viruses (HPV) and hepatitis C viruses, human immunodeficiency virus type 1 (HIV-1) and human T-cell lymphotrophic virus type 1 (HTLV-I). *Helicobacter pylori, Opisthorchis viverrini* and *Schistosoma haematobium* have recently been added to this list. This list is relatively new and other pathogens such as *E. coli* O157:H7 bacteria can potentially be added as causes of carcinogenesis (Herrera *et al.,* 2005).

1.4.3.1. Epidemiology Study

The relationship between bacteria and the onset of carcinogenesis is not accepted given that much of the evidence is based on epidemiological studies, which are open to a range of interpretations (Falk *et al.,* 2000). Animal models provide a tool for testing evidence in support of the theory that bacteria could induce cancer. Efforts have been dedicated to identify similarities between animal models and real world epidemics. However, bacterial effects within animals are often different from those observed in humans. A common method is the use of *H. pylori* infection of Mongolian gerbils for the induction of gastric adenocarcinoma similar to that of human infection (Ikeno *et al.,* 1999). Several other experiments have used mice as a model organism to identify the effect of infection of multiple bacterial species on the genome. In general, major similarities were identified between the studies such as increases in proliferation, increases in avoiding apoptosis and alterations in gene expression (Nedrud *et al.,* 1999).

Most of the past research has been focused on *H. pylori,* which is linked to gastric carcinoma with MALT lymphomas. *H. pylori* infect the gastric mucosa of approximately half of the world's population. Although infection is asymptomatic in approximately 85% of patients, *H. pylori* is an important pathogen because in the remaining 15%, the

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infection is associated with development of peptic ulcer disease or gastric cancer which is the second leading cause of cancer death world-wide (Peek and Blaser, 2002). Most of the research to support bacteria causing carcinogenesis is based on *H. pylori*; however, other bacterial infections have been known to have a link with cancer and infection. A strong case for the epidemiological support is *Salmonella enterica* serovar *Typhimurium* (*S. typhi)*, which could lead to chronic bacterial carriage in the gallbladder (case control study comparing an outbreak case in New York in 1922 with test animals) (Lax and Thomas, 2002). Another outbreak with supporting data indicating that bacteria were able to induce carcinogenesis occurred in Aberdeen in 1964, where carriers of the Gram-negative bacteria *Salmonella enterica* were more likely to contract hepatobiliary carcinoma than controls (Caygill *et al.,* 1994) . Analysis of the data also suggested a strong association between chronic carriers, incidence of and hepatobiliary carcinoma and the development of tumourigenesis in infected individuals (Mager, 2006).

1.4.3.2. Immunological Mechanisms

Although evidence based on epidemiological studies is persuasive, the data identifying the complete molecular mechanisms of genome instability caused by infection remains unclear. A theory has emerged indicating that chronic inflammation can lead to formation of reactive oxygen species and reactive nitrogen oxide intermediates that cause DNA damage (Rouse and Jackson 2002). The cellular response was further supported by the analysis of *Helicobacter felis* (*H. felis*) infected mice (Kocazeybek *et al.,* 2003). Chronic infections also upregulate signaling molecules supporting the idea that a bystander-like effect could be the cause of other cells emitting a stress response within the host. The idea that distant naive cells may also be affected by receiving a signal from

exposed tissues is similar to a phenomenon described for genotoxic stress exposure and gene therapy termed the bystander effect (Mothersill and Seymour, 2001; Little, *et al.,* 2002). The activation of an ERK (extracellular signal-regulated kinases) pathway may result in up regulation of transcription factors that support angiogenesis, proliferation, and avoidance of apoptosis (Lara-Tejero *et al.,* 2000). It is important to note that not all infections lead to inflammation. *H. pylori* may infect millions globally, but in most cases the infection does not cause any health concerns or inflammatory responses from the tissue, resulting in about 10% of infected individuals contracting carcinomas or ulcers (Rolig *et al.,* 2011).

1.4.3.3. Angiogenesis

Angiogenesis is a physiological process that involves induction and growth of blood vessels throughout the human body (Penn, 2008). All cells require some form of transport to deliver the essential nutrients needed for the growth of cells. Angiogenesis, while being fundamentally important during development and tissue repair, is also important in the process of transitioning tumour cells from a dormant state to one that is malignant. Tissue development for the blood vessels is induced by a chemical signal, vascular endothelial growth factor (VEGF) (Ria *et al.,* 2003).

VEGF is part of the system that restores the oxygen supply to tissues when blood circulation is inadequate. Normally, it is activated during embryonic development, however if tissue damage is detected, or if a blood vessel is blocked and a bypass is required, then an increase in VEGF signalling must take place (Holmes *et al.,* 2007). When overexpressed, VEGF can contribute to alteration of a tumour cluster from benign

(non-spreading) to malignant, resulting in the release of cells or chemical signals through the blood stream of the individual.

Bacterial infection affects the expression of the VEGF signal by activating the Rho pathway in infected cells. This pathway is a major target of bacterial toxins. Rho proteins are pivotal in the cellular control and the consequences of the deregulation has yet to be fully investigated (Lax and Thomas, 2002). Researchers are not sure what mechanism bacteria use to alter the Rho pathway, but they have identified the potential that toxins such as Cytotoxic necrotizing factor (CNF) produced by *E.* coli that is directly affected by the Rho protein and effecting DNA synthesis, inhibiting cytokinesis and modulating apoptosis factors (Lax and Thomas, 2002).

Focal adhesion kinase-1 (FAK), part of the Rho protein family, is recognized as a molecular switch, and plays a crucial role in control of cell proliferation, apoptosis, gene expression and multiple other common cell functions (Lax and Thomas, 2002). Researchers have hypothesized that the bacterial toxins (potentially the Shiga toxins) interact with Rho protein families, such as FAK, and are the most effective method of developing inflamed tissue into tumourous cells (Lax, 2005). The interaction between bacterial toxins and the Rho proteins occurs rapidly as the bacteria attach to the host cells, and in most cases, the activation is irreversible (Lax, 2005).

1.4.4. *Promotion of Proliferation*

The process of growing or multiplying by rapidly producing new cells is a highly conserved process in eukaryotic cells (Celli and Finlay, 2002). It is also known that deregulated proliferation can promote chromosomal instability by facilitating the

acquisition of alterations in genes encoding such as loss of tumour suppressor genes (Coschi and Dick, 2012). Proliferation of cells is controlled by many factors that help monitor cell growth, alter cell division or halt division when applicable. Neoplasia, the abnormal proliferation of benign or malignant cells, is caused by many factors, one being the disruption of cyclin dependent kinase (CDK) pathways (Pasz-Walczak *et al*., 2001).

Cyclins are a family of proteins that control the progression of cells through the cell cycle by activating cyclin dependent kinase (CDK) pathways (Kuper *et al.,* 2000). Bacteria have evolved to utilize this pathway to induce alteration in Cyclin D1, a regulatory subunit of a quaternary protein containing CDK kinase that assists in regulation of cell progression through the cell cycle (Lax, 2005). The expression of genes like *Cag E,* promotes the activation of the cell cycle regulatory molecule Cyclin D1. Cyclin D1 can interact with other sub-units of regulatory factors (CDK4 and CDK6), impeding the cell-cycle inhibitory function of the retinoblastoma protein (pRB).

Although direct mechanisms have not been identified, bacterial toxins are known to modulate intracellular signalling pathways directly related to the development of tumours and some toxins have been shown to activate extracellular signal-regulated kinases (Lax, 2005). Activation of such signals stimulates the cell to synthesize DNA and to promote proliferation. It has been identified in *Pasteurella multocida*, that promotion of these two signals is correlated with promotion of RhoA-mediated signal transduction that results in FAK activation (Thomas *et al.,* 2001) This activation allows newly formed cells to bond to existing tissue surrounding the infected area to continue proliferation and avoid apoptosis.

1.4.5. Suppression of Apoptosis

Apoptosis is programmed cell death in which transformed cells are prevented from proliferating and developing into tumours (Navarre and Zychlinsky, 2000). Bacteria, such as *H. pylori*, also have the capability to release tumour necrosis factor α (TNF-α) from CD8$^+$ T cells (Lax and Thomas, 2002) . The TNF-α binds with a membrane receptor on the infected cell (TNF-R), which contains a death domain (DD) (Lax and Thomas, 2002). This domain is essential for interaction with TNF-receptor associated factors (TRAFs). This domain also interacts with (NF)-κB (nuclear factor) and inhibits apoptosis of the cell.

The DD also interacts with TNF-receptor-associated death domain protein (TRAD) and Fas-associated death domain (FADD). This interaction results in the manipulation of the Caspase cascade (alternatively known as the "executioner proteins") that results in inhibition of BAX and BAK. BAX (BCL-2-Associated X protein) is a pro-apoptotic protein that interacts with organelle membranes (believed to interact with mitochondria) and initiate a cascade-signalling pathway to induce apoptosis (Thomas *et al.,* 2001). BAK (Bcl-2 homologous antagonist/killer) is another pro-apoptotic protein that interacts with mitochondria to induce apoptosis (Figure 1.1).

Although the exact mechanism of avoidance of apoptosis is still debated among scholars, a common idea is that the introduction of the TNF-α factor has a direct effect on the cell's ability to self-regulate its life cycle (Yu *et al.,* 2000). Test results indicate that CNF (which also suppresses apoptosis) toxin found in many *E. coli*, induces an increase in COX2 (cyclooxygenase subunit II) levels (Thomas *et al.,* 2001). Overexpression of COX2 has been identified to cause the development of tumours through the

overexpression of Bcl-2 (B-cell lymphoma 2) and the suppression of apoptosis (Lax and Thomas, 2002). However, in colorectal cancers, cells treated with *H. pylori* have been identified to produce COX2 and cytokines (Romano *et al*., 1998) and have been linked to tumour invasiveness (Gupta and DuBois, 2001). The exact mechanism is yet to be proven, however research on colorectal cancer is being conducted.

1.4.6. Evasion of the Immune System

The human immune system is in a constant battle with bacteria. The biological "arms race" has also been a focus for the study of bacterial infection and their ability to evade the immune system to survive (Finlay and Mcfadden*.,* 2006). A wide range of bacteria has evolved and incorporated methods of monitoring and evading phagocytes.

1.4.6.1. Modulation of the Pathogen Surface

E. coli have evolved techniques to evade the immune system by altering their appearance (Yu *et al.,* 2012) . The immune system acts on surface signal recognition of foreign invaders to identify and neutralize the bacteria. Some bacteria have developed a carbohydrate capsule to mask their surface (identity), as well as a process to accumulate proteins from the host organism and incorporate them into the capsule (Finlay and Mcfadden*.,* 2006). This mimicry and camouflage is used by many pathogenic and non-pathogenic bacterial species to prevent phagocyte clearance. The bacterial capsule is not a solid shell, having pores which allow filamentous adhesion (by fimbriae and pili) to host cells (Finlay and Mcfadden*.,* 2006).

Bacterial capsules are an effective method of hiding many bacterial surfaces and avoiding opsonisation. Even with the capsule, the host immune system could potentially

identify predominant structures on the bacterial surface as key signatures (Christie *et al.,* 2005). Bacteria have developed methods of altering these molecules so that they are less recognizable within the host. The most common modulation of bacterial surfaces, for Gram-negative bacteria, is alteration of the lipid A structure found in the LPS outer cell wall. Bacteria have sensor molecules and enzymes (e.g., *Salmonella* has a two-component sensor PhoP/PhoQ) that assesses host environment and modifies the structure of Lipid A using a Lipid A palmitoyltransferase (PagP) (Kawasaki *et al*., 2004). This modification makes Lipid A 100-fold less active for the immune responses to identify and eliminate.

1.4.6.2. Bacterial Subversion of Phagocytes

The actual size of bacteria, makes them ideal phagocyte targets and therefore bacteria have developed methods of avoiding phagocytosis (Finlay and Mcfadden, 2006). Many bacteria secrete several type 3 secretion system (T3SS) effectors that neutralize phagocyte activity. An example is secretion of YopH, a tyrosine phosphatase that dephosphorylates key actin cytoskeleton proteins such as paxilin and p130cas, that disrupts the structure of actin, causing an alteration in cell activity (Finlay and Mcfadden, 2006).

Many bacteria, which are internalized, generally try to avoid destruction in a phagosome. Most infectious bacteria try to avoid phagocytes by inhibiting inflammation of host cells, but some pathogens activate inflammatory pathways. T3SS secretions of effectors bind to caspase pathways and activate downstream pro-inflammatory pathways creating a niche (Finlay and Mcfadden, 2006). These niches for pathogens can cause serious inflammatory diseases and provide host cells with ideal proliferating conditions

that will further promote the infection/inflammation process (Finlay and Mcfadden, 2006).

1.4.6.3. Blockade of Acquired Immunity

Most pathogens avoid an acquired immune response by avoiding any interaction with the immune system, and only a very few pathogens have direct interference with acquired immunity. *H. pylori* produce a vacuolating toxin (VacA), which blocks T cell proliferation. T cells are lymphocytes, which are a group of white blood cells that are essential for cell-mediated immunity (without antibodies). VacA inhibits the receptor/IL-2 signalling pathway, resulting in a decrease of nuclear factor activated T cells (NFAT), which is a global regulator of immune response genes (Gebert *et al.,* 2003).

Another strategy is to secrete enzymes such as IgA (Immunoglobulin A) proteases that degrade immunoglobulin (antibodies). IgA is an antibody that is found on mucosal surfaces, indicating that it is important for humoral defence (Cerutti and Rescigno, 2008). The IgA protease is an ideal tool for bacteria because of its ability as an auto-transporter mechanism and self-cleavage reaction to secrete itself out of the bacterium mimic the host cell's ability to neutralizing threats before they reach the cells (Kaetzel *et al., 1992)*. These mechanisms produce proteins possessing an amino-terminal signal sequence (with features required for passage through the *sec* translocon), a passenger domain and a carboxy-terminal β-domain (Henderson *et al.,* 1998). Proteins mediate their own translocation (hence the term autotransporter) across the outer membrane by virtue of their b-domains; the b-domain forms a pore in the outer membrane through which the passenger domain of the molecule is translocated to the cell surface (Henderson *et al.,* 1998).

1.4.7. Chronic Inflammation

Chronic inflammation is a common feature of infections, which is associated with induction of pro- and anti-inflammatory cytokines (Kawai *et al.,* 2004). Inflamed cells generate mediators such as free radicals, prostaglandins and cytokines that interact in different phases of the inflammation process. Chronic exposure to these mediators leads to an increase in cell proliferation, mutagenesis, oncogene activation and angiogenesis (Shacter *et al.,* 2002). Free radicals (ROS or RNOS) serve as a protection mechanism by killing invading pathogens; however, they also have the capability to induce a state of cell destruction or proliferation and provide a selective advantage for clones of cells with DNA damage. ROS and RNOS can oxidize and damage DNA either directly or through interactions with other free radicals or cellular components (Burcham *et al.,* 1998).

Chronic inflammation could also spawn carcinogenesis through nitric oxide and its derivatives produced by phagocytes. Nitric oxide can oxidize to nitrogen dioxide, which can directly induce DNA damage. The nitric oxide also has the ability to react with a superoxide anion to form peroxynitrite, which may be cytotoxic itself or can decompose into hydroxyl radicals and nitrogen dioxide. This also induces lipid peroxidation, which itself could interact directly with DNA to cause mutations, e.g., 4-hydroxynonenal (Burcham *et al.,* 1998). Once DNA damage has occurred, the cell with the DNA damage has the potential to give rise to a clone of altered cells, an event that is enhanced by the proliferative response of the host cells to compensate for tissue damage caused by the initial inflammation.

Prostaglandins are a group of fatty acid derivatives generated by COX, and are involved in numerous body functions such as the contraction and relaxation of smooth

muscle, the dilation and constriction of blood vessels, control of blood pressure, and

modulation of PGE-2 (prostaglandin-E2) (Balzary & Cocks, 2006). COX is released by

blood vessel walls in response to inflammation, which acts on the brain to induce fever,

as well as other organs directly involved in the stress response system (spleen) and the

filtration of the blood (liver) (Byrne *et al.,* 2003). Theories have been developed that

recognize the potential for tissues with a large vascular flow to be affected by a distant

infection by the interaction between distant cells and prostaglandins released by infected

cells, providing support for distal tissues being affected by phenomena like the bystander

effect (Byrne *et al.,* 2003).

1.5 Epigenetic Alteration and Mechanisms of Genome Instability

Epigenetic modification refers to changes in gene expression that do not involve

alteration of the original DNA sequence of a cell and that is reversible and transmitted

during the mitosis and meiosis (Portela and Esteller, 2010). Epigenetic modification can

include processes that contribute to the regulation of chromatin structure, genome

integrity/instability, alterations in the expression of tissue specific genes, embryonic

development, replication timing, genomic imprinting and chromosome inactivation (most

common is X-chromosome inactivation in female embryogenesis) (Portela and Esteller,

2010). Epigenetic changes include reversible DNA methylation, histone modifications

and small non-coding RNA-induced silencing. Histone modifications are universal and

evolutionally conserved and a required component of the epigenetic mechanism of

transcriptional regulation in eukaryotic cells, DNA methylation can occur in both

prokaryotic and eukaryotic cells. Epigenetic modification involving mammalian genomic

DNA involves methylation at the 5' position of the cytosine (C) residue within the

cytosine-guanine dinucleotide (CpG), resulting in the formation of 5-methylcytosine

(m5C) (Portela and Esteller, 2010) as well as hydroxymethylation. Hypermethylation of

gene promoters or hypomethylation of various parts of the genome, may contribute to

autoimmune disease development or potential carcinogenesis/tumourigenesis (Portela

and Esteller, 2010).

Epigenetic alterations have been correlated with carcinogenesis in human patients,

but evidence from test models lack the specific epigenetic changes capable of inducing

carcinogenesis (Koturbash *et al.,* 2009). Although genetic and epigenetic alterations have

been characterized in diverse cancers, the crucial steps that result in cancer remain

unknown. More important, it is unknown how damage is transmitted from direct

exposure (inflamed tissues infected by bacteria and/or its components) to the distal

organs. Early detection of such events has been shown using whole bacteria and filtered

water contaminated with bacteria, indicating that such exposures can trigger substantial

molecular responses directly in exposed and distal cells (Koturbash *et al.,* 2009).

1.5.1. γH2AX

H2AX in one of the many genes coding for the H2A histone, a major

component of the nucleosome, and therefore essential for the structure of chromatin. In

eukaryotes, DNA wraps around the nucleosome, consisting of core histones H2A, H2B,

H3 and H4. H2AX has been shown to be phosphorylated at serine 139, altering it to

become γH2AX. H2AX is involved in DNA repair and becomes phosphorylated soon

after the appearance of double strand breaks (DSBs) and has been implicated both in

homologous recombination and non-homologous end joining DNA repair pathways

(Burma *et al.,* 2001). H2AX is thought to have a function in recruitment of DNA repair

factors and DNA-damage signalling proteins. Hyper phosphorylation of H2AX may be linked to chromatin fragmentation prior to apoptosis (Park *et al.,* 2003).

1.5.2. DNMT1

DNA methyltransferases (DNMTs) are the enzymes involved in cytosine methylation. Mammalian DNMTs are composed of three main structural regions. The first region is the N-terminal regulatory domain, responsible for the localization of DNMTs in the nucleus. The N-terminal domain plays a regulatory role, and contains a proliferating cell nuclear antigen-binding domain (PBD), a nuclear localization signal (NLS), a cysteine-rich zinc finger DNA-binding motif (ATRX), a polybromo homology domain (PHD), and a PWWP tetrapeptide chromatin-binding domain (Tang *et al.,* 1994).The second region is the C-terminal catalytic domain, which consists of several α-helical and β-sheet structures and is characterized by the presence of six conserved amino acid motifs, namely I, IV, VI, IX and X (Hermann *et al.,* 2004). Motif I and X fit together to form a large part of the binding site for the methyl donor (S-adenosyl-L-methionine, SAM). Motif IV contains a prolyl-cysteinyl dipeptide that provides a thiolate to the active site. Motif VI contains a glutamyl residue that protonates the 3 position of cytosine. Finally, the third region is the central linker, which consists of repeated GK dipeptides (Tang *et al.,* 1994).

DNA methyltransferase 1 (DNMT1) activity is required for maintenance of DNA methylation, which is needed for proper organization of chromatin domains. DNMT1 recruits chromatin-modifying enzymes including HDAC1, HDAC2 and histone methyltransferase Suv39h1 (Geiman *et al.,* 2004). This suggests that DNMT1 may play an important role in transcriptional regulation and chromatin remodelling in mammalian

cells (Geiman *et al.,* 2004). Aberrant DNA methylation may be involved in carcinogenesis as a result of; 1) increased frequency of point mutations in a gene because of deamination of 5-methylcytosine to thymine, 2) possible association of aberrant DNA methylation with the loss of alleles, and 3) repression of gene transcription through methylation of CpG islands in regulatory regions of specific genes, including tumour-suppressor genes. Over expression of DNMT1 has been detected in several human cancers(Etoh *et al.,* 2004). In reference to gastric cancer, it has been reported that DNMT1 mRNA expression levels were significantly higher in cancer tissues than in normal gastric mucosae (Etoh *et al.,* 2004).

In past studies, it was found that increased DNMT1 mRNA expression correlated significantly with the CpG island methylator phenotype (defined as frequent DNA hypermethylation of C-type CpG islands, that are methylated in a cancer-specific but not an age-dependent manner) in gastric and colorectal cancers (Etoh *et al.,* 2004). Current research has indicated that DNMT1 expression was also increased in gastric cancers, suggesting that DNMT1 over expression has some significance during gastric carcinogenesis. DNMT1 protein over expression showed no significant correlations with either the cellular type (gastric type *versus* intestinal type) or the presence, absence, or degree of intestinal metaplasia (a precancerous lesion for adenocarcinomas with an intestinal phenotype), in corresponding noncancerous mucosae, suggesting that DNMT1 protein over expression is associated with gastric carcinogenesis regardless of the cellular origin or phenotype (Etoh *et al.,* 2004).

1.5.3. DNMT3A and DNMT3B

DNMT3A and DNMT3B are similar in their primary structure; however these enzymes are coded by different genes located on different chromosomes and regions of chromosomes 2p23 and 20q11.2, respectively (Xie *et al.,* 1998). This methyltransferase, methylates CpG dinucleotides, without preference for hemimethylated DNA, and is responsible for the *de novo* methylation of DNA. DNMT3A and DNMT3B activity is usually reduced significantly within adult organisms and is primarily used during the development of the embryo to silence genes no longer required, or detrimental to survival of the embryo. The expression of *DNMT3A* is ubiquitous, while *DNMT3B* is expressed at very low levels in most tissues except testis, thyroid and bone marrow (Xie *et al.,* 1998). Past research has identified an increase in DNMT3B in tumour cell lines indicating that DNMT3B may play a significant role in the development of tumourigenesis (Robertson *et al.,* 1999).

The architecture of the DNMT3A and DNMT3B enzymes is consistent with the general structure of the DNMTs. The DNMT3A and DNMT3B domains are hypothesized to interact with the chromatin, and the regulatory regions of these enzymes have the capability to bind with various transcriptional repressors (Kim *et al.,* 1998). DNMT3A can bind co-repressor RP58, oncogenic factor PML-RAR or HP1b protein, whereas DNMT3B can be associated with Sin3a SUMO-1/Ubc9 and ATP-dependent chromatin remodelling enzyme (hSNF2H).

DNMT3A and DNMT3B may also interact with DNMT1 and activate HDAC1, which deacetylates histones and represses gene transcription. This indicates that DNMT3A and DNMT3B may be involved in chromatin remodelling associated with

modulation of gene transcription. DNMT3A exhibits a lower level of enzymatic

methyltransferase activity as compared to DNMT1. This may indicate that DNMT3A

requires a small protein or co-factors for optimal activity. Furthermore, DNMT3A

exhibits a preference for methylation sites that are flanked by pyrimidines. Although

DNMT3A is highly specific for CpG methylation, this enzyme is also able to methylate

cytosine at the CpA and CpT dinucleotides; however, function of this DNA modification

is still unknown (Ramsahoye *et al.,* 2000).

1.5.4. MeCP2

Another important enzyme, which plays a major role in mammalian development

and correlates with chromatin-associated gene silencing, due to methylation maintenance,

is labelled as MeCP2. MeCP2 (Methyl CpG binding protein2) plays a significant role in

methylation-mediated gene silencing and chromatin remodelling of hemi-methylated

regions of a chromosome (Fan and Hutnick, 2005). MeCP2 specifically recognizes

methylated regions of DNA and represses gene transcription, either directly affecting

DNA or interacting with known co-repressor proteins, which include members of histone

deacetylase protein families. MeCP2-associated methylation is identified as specific to

lysine 9 of histone H3. These proteins are able to form complexes with HDAC, co-

repressor (Sin3a) and ATP-dependent chromatin remodelling proteins, which are

involved in the stabilization of heterochromatin (Fuks *et al.,* 2003). MeCP2 is also known

to set up a relapsing cycle of repression within mammalian cells, which may be required

for maintenance of repressed genes initially, but could also promote additional rounds of

methylation following histone modification. DNMT1 and MeCP2 associate together in

order to perform maintenance methylation of the genome (Fuks *et al.,* 2003).

1.6 Possible Role of Bacterial Infection in Inducing Genomic Instability

Modern agricultural practices (mostly livestock) have become one of the largest contributors of water contamination (Kondro, 2000). Research conducted within recent years has identified that heat- killed bacteria (whether pathogenic *E. coli* O157:H7 or non-pathogenic DH5α) induce genome instability (Koturbash *et al.,* 2009). It was identified that water containing only heat-killed bacteria continued to have the capacity to induce genome instability in the host. The effect remained even after water contaminated with heat-killed bacteria was filtered through a 0.45 µm filter. This indicates that whole bacteria are not required to induce genetic and possibly epigenetic changes, but rather only a single component of the bacteria.

Bacterial components could be present within a solution, separate from the breakdown of bacterial cells. It has been hypothesized that these components may induce cell proliferation in mucosal cells (Olaya *et al.,* 1999). LPS has been proposed as a potential component of bacteria capable of inducing genome instability in the host. This assumption is supported by tests conducted *in vitro* with an assortment of bacterial components provided within water extracts (Olaya *et al.,* 1999). Some Gram-negative bacteria posses a glycolipid component in their outer wall, called LPS. LPS are known to interact in cell proliferation and cell signalling mechanisms of infected cells (Luderitz *et al.,* 1982). LPS possess the capability of surviving heat-shock exposure without major alterations to structure or function (Rietschel *et al.,* 1993).

Genome instability is a fundamental event in carcinogenesis, permitting the initiated cell to alter and evolve into transformed cells resulting in tumourigenesis

(Coleman and Tsongalis, 1999). It has been hypothesized (Luderitz *et al.,* 1982) that LPS can induce genome instability by primarily two mechanisms:

1) Repeated exposure of cells to LPS causes an inflammatory response resulting in production of free oxygen radicals, inducing DNA damage. It has been demonstrated (Yamada *et al.,* 2006) that exposure to relatively low doses of LPS significantly increases the concentration of H_2O_2 (hydrogen peroxide, an active oxygen species).

2) Repeated LPS treatment accelerated urothelial proliferation (Kawai *et al.,* 2004). Cell proliferation can be caused by cytokines, induced by an immune response, caused by the signalling effects of LPS. Epidermal cells, capable of accelerated proliferation, consist of several kinds of cytokines including IL-6, TNF, and IL-8 and are known to be involved in the process of pathogenesis and proliferation of cells exposed to environmental stressors (Kawai *et al.,* 2004). This indicates that the cytokine network, induced by LPS, may play a significant role in cell proliferation in inflammation-induced hyperplasia. There may also be a possibility that cytokines such as TNF (Kudo *et al.,* 2009) and IL-8, which have chemotactic, and angiogenesis activity, may be involved in the development of the tumours.

LPS were isolated in the 1930s (Bolvin *et al.,* 1933) and their structure had been determined to consist of three distinct regions: (i) a hydrophilic lipid A moiety, responsible for the endotoxic properties of LPS (Du *et al.,* 1999), (ii) core nucleotides (five known structures identifies as R1, R2, R3, R4 and K12) made up of three 3-deoxy-D-manno-2-octulosonic acid (KDO) and three heptose residues situated along variable

sugars attaching the lipid A structure (Netea *et al.,* 2002) to the third structure, (iii) the O-polysaccharide antigen (accessible to the host immune system inducing response) (Netea *et al.,* 2002) (Figure 1.2). Lipid A is a unique lipid found in bacteria and consists of a phosphorylated β-1,1-linked glucosamine disaccharide, on which long fatty acid chains are attached (Rietschel, 1982; Rietschel *et al.,* 1987). Lipid A is hypothesized to be responsible for induction of the expression of the cytokines by the LPS structure, although KDO and the polysaccharide component could also cause this activity (Rietschel *et al.,* 1987). Finally, the O-polysaccharide antigen component of LPS has been identified to posses over 170 serotypes, in *E. coli* yet only a few have been implicated in diseases in humans (Gibbs *et al.,* 2004).

Studies on a range of Gram-negative bacteria have produced information supporting the idea that LPS from certain bacterial strains such as *E. coli* and *Salmonella spp.*, are more potent than that of other strains (e.g. *Bordertella pertussis*). Even more interesting, LPS from *E. coli* can stimulate macrophages, inducing expression of tumour necrosis factor α (THF-α), various interleukins, macrophage inflammatory protein 2 (MIP-2), interferon γ (IFN-γ) and macrophage chemotactic protein 5 (Hirschfeld *et al.,* 2001). Expression of these genes can be associated with variation in the structure of the LPS molecule. Lipid A of *E. coli*, consists of a biphosphorylated β-1,6-linked glucosamine disaccharide substituted with a 3-hydroxyl myristoyl (C14:0) group at the 2,2`, and 3` positions of where the 2` and 3` fatty acid chains are esterified (Rietschel *et al.,* 1987). Lipid A isolated from other species might differ in terms of the presence of 2,3-diamino-2,3-dideoxy-D-glucose instead of D-glucosamine, and number of acyl groups, chain lengths, symmetrical distribution or substitution of phosphate groups (Figure 1.3) (Seydel

et al., 2000). Research by the Ulrich Seydel group has identified that the conical shape of

LPS, such as the one seen in the LPS structure of *E. coli*, gives LPS high activity with

cell wall proteins as compared to the LPS possessing a cylindrical conformation

identified in *Porphyromonas gingivalis* (Schromm *et al.,* 2000).

Based on published data (Netea *et al,.* 2004), researchers have proposed a

hypothesis of why the conical orientation of *E.* coli LPS can induce cellular homeostatic

instability (Figure1.4). When the Lipid A in the LPS molecule assumes a conical

structure, Toll-like receptors (TLRs) can be engaged, which are a major component of

LPS-mediated signalling (Du *et al.,* 1999). CD14 on the cell membrane binds to the LPS,

then transfer lipopolysaccharide to the receptor TLR4, which is the signalling chain of the

receptor complex. CD14 is a glycolipid-anchored membrane glycoprotein expressed on

cells of the myelomonocyte lineage including monocytes, macrophages, and some

granulocytes. They function as receptors for the complex of LPS and LPS-binding

protein (LPB). These cell surface domains trigger activation of intracellular signalling

complexes, composed of interleukin receptors and adaptor proteins. This can lead to

nuclear translocation of nuclear factor kappa-light-chain-enhancer of activated B cells

(NF-$_K$B) and transcription of cytokine-encoding genes (Golenbock and Fenton , 2001).

Overall, several suggestions can be drawn from the existing literature:

- Ingestion of contaminated food and water is a source of genomic instability of
  exposed and distal cells identified as the bystander-like effect
- Induction of genome instability is potentially caused by an epigenetic
  modification

- Events involved in the regulation of cellular functions are essential
  components of genome stability

It is still unclear whether infection with pathogenic *E. coli* is associated with carcinogenesis. Knowledge of molecular pathways involved in induction of genomic instability in response to infection is still missing. Analysis of chromatin structure in cells exposed to pathogens, identification of the component of the bacteria capable of induction of the genomic instability, and identification of the molecular pathways leading to it need to be further explored.

1.7 Hypothesis

The current study aims to identify the component of the heat-killed bacteria (*E. coli* O157:H7) that induces genomic instability of cells directly exposed to the bacteria and the naive cells of distal tissues affected by the bystander effect in *in vivo* exposed mice.

Exposure to heat-killed bacteria will result in alteration of genomic stability of the exposed cells. Therefore it is hypothesized that components of the aforementioned bacteria (DNA, RNA, proteins or LPS) will result in alteration of genomic stability of the exposed cells. Additionally, it is hypothesized that distant naive cells may also be affected by receiving a signal from exposed cells, by a bystander-like phenomena. Finally, it is hypothesized that Lipopolysaccharides (LPS) from *Escherichia coli* O157:H7 Gram-negative bacteria is a triggering agent to induce genomic instability
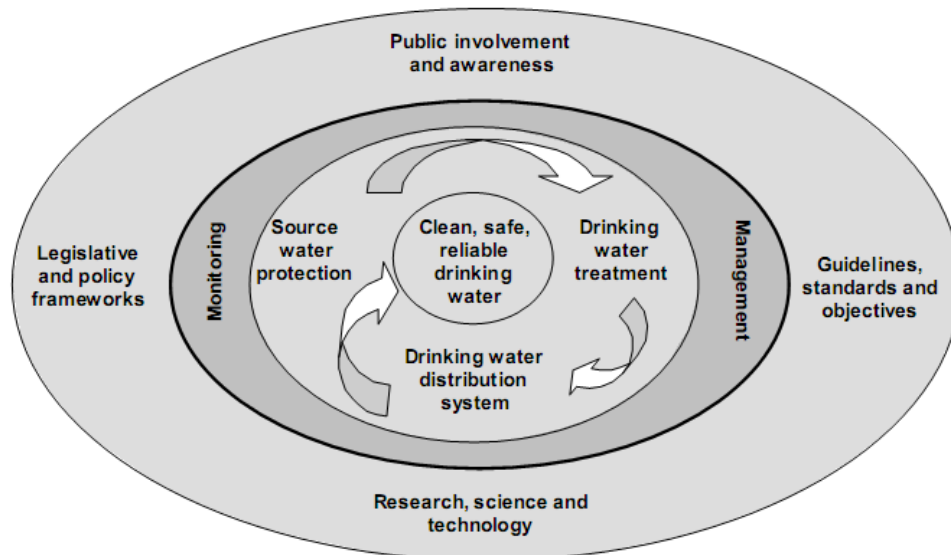
Figure 1.0. The Multi-Barrier Approach to ensure the ingested water meets safety standards. Federal-Provincial-Territorial Committee of Drinking Water of the Federal-Provincial-Territorial Committee on Environmental Occupational Health and the Water Quality Task Group of the Canadian Council of Ministers of the Environment (Lim *et al.,* 2002).
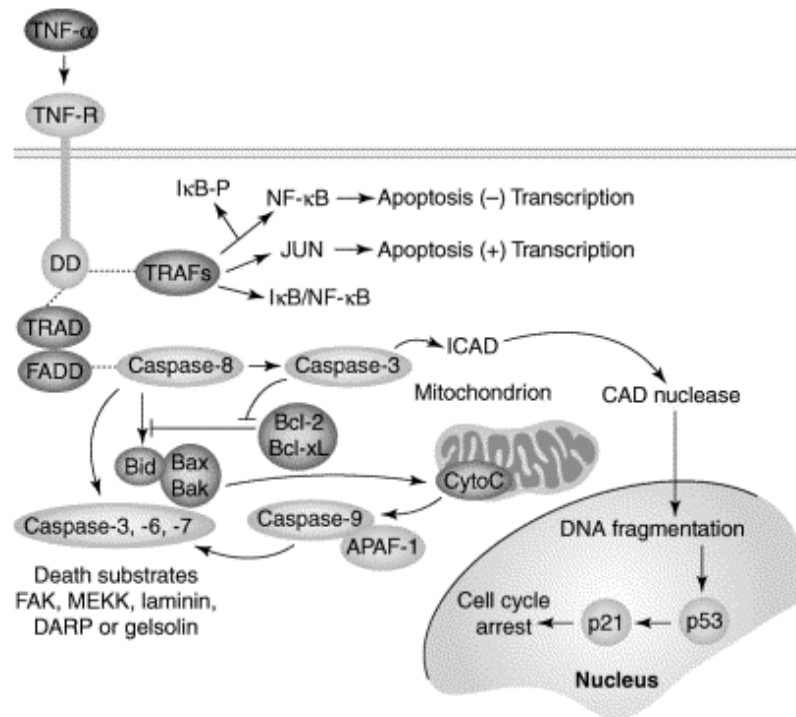
Figure 1.1. Apoptosis. Tumour necrosis factor α (TNF-α) acts through a membrane receptor (TNF-R) whose death domain (DD) interacts with TRAFs, that regulate the nuclear factor (NF)-κB, suppressing apoptosis. TNF-α also interacts with caspase-8 through TRAD and FADD to activate the caspase cascade affecting the Bax and Bak pathways. (Lax and Thomas, 2002).
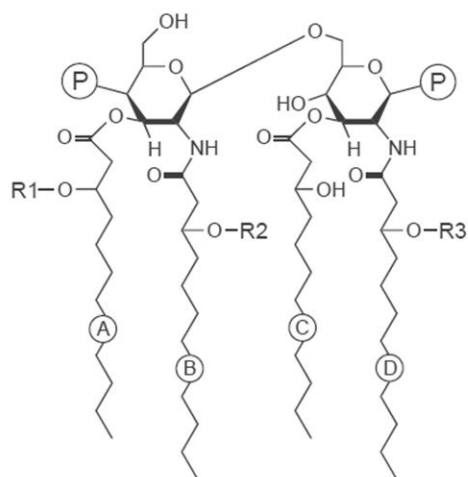
Species	A	B	C	D	R1	R2	R3
E. coli	14	14	14	14	14:0	12:0	H
Salmonella spp.	14	14	14	14	14	12:0	14
P. gingivalis[a]	15	17	16	17	H	16:0	H
N. meningitidis	12	14	12	14	H	12:0	12:0
R. sphaeroides	10	12	10	12	H	cis$^{\Delta 5}$12:1	H

Figure 1.2. Structural components of LPS consisting of a lipid component (lipid A) and polysaccharide chains. Various bacterial species are represented. β-1,6-linked glucosamine disaccharide substituted with two negatively charged phosphate and saturated fatty acids carrying radicals; R1, R2 and R3.   *P. gingivalis*[a], superscript a indicating an anaerobic organism. (Netea *et al.,* 2002).
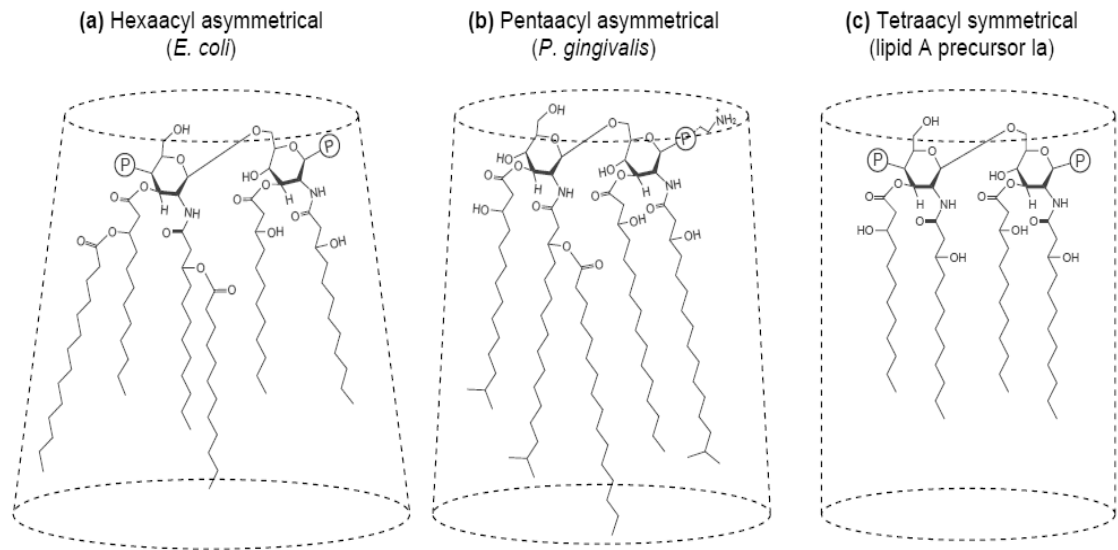
Figure 1.3. Structural alterations in LPS structures. A) Hexaacyl asymmetrical lipid A from *E. coli* creates a conical conformation. B) Pentaacyl asymmetrical structure of lipid A from *Porphyromonas gingivalis*. C) Tetraacyl symmetrical lipid A precursor adopts a strictly cylindrical shape. (Seydel *et al.,* 2000).

Chapter 2: Genomic instability in liver cells caused by an LPS-induced bystander-like effect

Abstract

Bacterial infection has been linked to carcinogenesis; however, there is lack of knowledge of molecular mechanisms that associate infection with the development of cancer. Research indicates that the pathogenic ability of bacteria is not a significant factor in induction of genome instability of exposed in mice or distal non-exposed tissue (Koturbash *et al.,* 2009). We analyzed possible effects of the consumption of heat-killed *E. coli* O157:H7 cells and cellular components on genome instability of naïve liver cells. Four week old mice were provided water supplemented with whole heat-killed bacteria or bacterial components (DNA, RNA, Protein or LPS) for a two week period. Additional groups of mice were provided a two week sample of water with supplements of whole heat-killed bacteria or its components, followed immediately by an additional two weeks of receiving uncontaminated tap water before sacrificing. Liver samples were collected, post mortem, for analysis of the response of naïve tissues to the bacteria or their components. Liver cells responded to exposure of whole heat-killed bacteria and LPS with alteration in levels of proteins involved in proliferation, DNA methylation (*de novo* or maintenance) (MecP2, Dnmt1, Dnmt3a and 3b) or DNA repair (Ape1 and Ku70) as well as with changes in the expression of genes involved in stress response, cell cycle control and bile acid biosynthesis. Other bacterial components analysed in this study did not lead to any significant changes in the tested molecular parameters. This study suggests that lipopolysaccharides are a major component of Gram-negative bacteria that induce genomic instability within naïve cells of the host.

2.1. Introduction

There is clear evidence linking environmental exposures to the onset of carcinomas (Parkin *et al.,* 2005). Viral infections such as HIV, HCV and HBV have a prominent effect on the development of carcinomas during and after infection. The influence of some bacteria on the effects of genome stability is significant but not widely accepted. *Helicobacter pylori* and its association with the development of gastric cancer is one of the best examples (Suerbaum, & Michetti, 2002). Presence of a common intestinal bacteria such as *E. coli* may facilitate the development of various malignancies (Horie *et al.,* 1999). *E. coli* infections can also be associated with hematological and non-hematological malignancies (Brook *et al.,* 1998).

Bacteria can promote carcinogenesis by induction of chronic infection, leading to disruption of the cell cycle and alterations in cell growth and DNA damage (Ferrero *et al*., 2000). Even though a link between cancer induction and bacterial infection exists, it is unclear if living or heat-killed cells, or even remnants of the bacteria can trigger genome instability and cancer. Yamamoto *et al.* (1992) conducted tests which exposed urinary bladders to heat killed *E. coli,* which resulted in a 40x enhancement of tumourigenesis in pre-initiated tumour sites. The most common organs in which infection-associated malignancies take place are within the liver, colon and stomach (Yamamoto *et al.,* 1992).

The liver is continuously exposed to a variety of antigens and toxins derived from the gut, where ingested substances are absorbed into the blood stream (Jirillo *et al.,* 2002). The liver is exposed to bacterial determinants and/or toxins through its

physiological role of detoxification of the blood; specifically, the hepatocytes are involved in clearance of endotoxins (Jirillo *et al.,* 2002).

Exposure to bacterial pathogens and/or their components most frequently occurs through consumption of contaminated food or water. Contamination is more frequently identified in rural communities with a high frequency of large livestock farms (Gannon *et al.,* 2005). Upon identification of water contaminated by *E. coli* O157:H7, Health Canada imposes a boil water advisory. Boiling of contaminated water is intended to kill the bacteria and prevent infections, but bacterial remnants such as proteins and LPS remain intact and have the capability to interact with cells of the gastro-intestinal tract. Even though, epidemiological evidence identifies links between bacterial infection and cancer induction, it is still unclear, which/if any, component of the heat-killed bacteria could produce a genomic instability response in naïve cells of the host. Based on the literature, it can be hypothesized that exposure to heat-killed bacteria or their components, causes genomic instability in cells that does not require direct contact with a bacterial cell or its constituents. This response may represent a bystander-like effect.

The purpose of this study was to analyze of genome stability of an indirect target organ (liver) in mice following digestive tract exposure to DNA, RNA, protein or LPS extracted from heat-killed bacteria. Another objective was to analyse the ability of the mouse organs to react to initial exposure to bacteria/bacterial components and return to a physiological level comparable to the control.

2.2. Methods

2.2.1. Animal Model

Four-week-old C57BL/6 male mice and all subjects were handled and cared for according to the requirements set by the Canadian Council for Animal Care and Use. The mice were housed in cages with a 12 hr light/dark cycle and provided water (with or without treatment) and food pellets *ad libitum*. Water consumption, food intake and body weight were monitored for any significant changes. Mice were housed in groups according to the determinant from the bacteria they were exposed to (e.g., all mice in one compartment would receive the LPS-rich solution). The two and four week groups were housed within the same component with half of the mice removed for each temporal experimental endpoint.

The bacteria were grown to OD_{600} 0.2, and then 1.25 ml of bacterial suspension was heat-killed and added to one litre of water to get approximately $6x10^6$ bacteria/litre. For this study, six treatment groups were created: Group 1 (control, tap water); Group 2 (heat-killed bacteria); Group 3 (DNA prepared from group #2); Group 4 (RNA from group #2); Group 5 (protein from group #2); Group 6 (LPS from group #2). Animals (eight per experimental group) were sacrificed either immediately after treatment (4 animals in each group) or two weeks later (4 animals in each group). Concentrations provided to the animals were; DNA at 430 µg/L, RNA at 72.7 µg/L, protein at 9.6 µg/L and LPS was 50 µg/L. The concentration of DNA, RNA, protein or LPS used in the experiment was approximately is ~15,000-fold higher than what was expected to be produced from amount of bacteria stated earlier: DNA extracted from a single *E. coli* cell weighs ~ $5x\ 10^{-9}$ µg, thus $6x10^6$ bacteria would weigh $30x\ 10^{-3}$ µg, which ~15,000-fold less DNA than was used in the experiment. Also, typical bacterial cell contains 0.1 pg of RNA; thus $6x10^6$ bacteria would yield 0.6 µg of RNA, which 200-fold less than used in our

experiment. Average bacteria contain approximately $200 \times 10^{-9} \mu$g of protein (Zubkov et al., 1999); thus 6×10^6 bacteria would yield 1.2 µg of protein, or 8 times less than used in our experiment. The use of increased concentrations of DNA, RNA and protein was intentional to ensure a large concentration of bacterial components was present in the water to induce a response to the contaminant. Proportionally higher concentration of bacteria in water was not possible to achieve without causing the water to be turbid. During the experiment, the treated animals did not consume more water than the non-treated animals. Animals in the four week test group received normal water for two weeks following initial treatment (Figure 2.0). Animals were sacrificed either 2 or 4 weeks (dependant on the test group) after the start of the treatment. Liver and muscle tissue samples were harvested and processed for molecular testing or fixed in paraformaldehyde for immunohistochemical analysis.

2.2.2. DNA extraction

DNA was extracted from the *E. coli* using a Qiagen DNAeasy kit (Qiagen) in accordance with the manufacturer's specifications. Please refer to Qiagen DNAeasy kit DNA Extraction from Cell Culture section for protocol. Heat-killed *E. coli* O157:H7 were provided by Dr. James Thomas (University of Lethbridge).

2.2.3. RNA Extraction

RNA was extracted from the *E. coli* using TRIzol® Reagent following the manufactures protocols.

2.2.4. Bacterial Protein Extraction

One ml of bacterial suspension was centrifuged at 5,000 x g for 20 minutes at 4^0C. Next, 500 µl of Lysis buffer (1% Sodium Dodecyl Sulphate) was added and each sample

was sonicated for 30 seconds. Cell debris was removed by centrifugation at 10,000 x g for 30 minutes. Supernatants containing the proteins were transferred to new tubes.

2.2.5. Lipopolysaccharide Extraction

The cells were harvested by centrifugation (Eppendorf ® 5415R Centrifuge) at the speed of 1,000 rpm for 15 minutes. Approximately 500 mg of dried bacterial cells were re-spun in 15 ml of 10 mM Tris-HCl, pH8.0, 2 mM $MgCl_2$, DNase (100 µg/ml) and RNase (25 µg/ml). The cell suspension was compressed twice at 15,000 psi, followed by two 30 second bursts of sonication (Braunsonic® 1510). Once again, 100 µg/ml of DNase and 25 µg/ml of RNase were added into solution.

The cell suspension was then incubated for two hours at 37^0C. Five ml of 0.5 EDTA (tetra sodium salt)/10 mM Tris (pH 8.0), 2.5 ml of 20% SDS/10 mM Tris (pH 8.0) and 2.5 ml of 10 mM Tris-HCl (pH8.0) were added to a final volume of 25 ml. The sample was then vortexed and centrifuged at 50,000 g for 30 minutes at 20^0C to remove peptidoglycan. The supernatant was collected into separate 1.5 ml tubes. Pronase was added to the final concentration of 200 µg/ml. The samples then were incubated overnight at 37^0C with constant agitation. Two volumes of 0.375 mM $MgCl_2$/95% EtOH was added, mixed and cooled to 0^0C. After it had cooled, the sample was centrifuged at 12,000 g for 15 minutes at 0-4^0C. The pellet was re-spun in 25 ml of 0.1 M EDT, 2% SDS, 10 mM Tris-HCl (pH 8.0). The sample was once again sonicated at ¾ intensity for a 30 seconds burst and set to incubate at 85^0C for 30 minutes. Upon removal of the sample, it was allowed to cool to room temperature and the pH was brought to 9.5 with addition of 4N NaOH. Next, pronase was added to a final concentration of 25 µg/ml followed by incubation at 37^0C overnight with constant agitation.

Samples were taken out of the incubator and 0.375 mM $MgCl_2$/95% EtOH was added and mixed /cooled to 0^0C in a -20^0C refrigerator. After it had cooled, the sample was centrifuged at 12,000 g for 15 minutes at $0-4^0C$. The pellet was re-spun in 25 ml of 0.1 M EDTA, 2% SDS, 10 mM Tris-HCl (pH 8.0). The sample was once again sonicated at ¾ intensity for a 30 second burst, then centrifuged at 1,000 rpm for five minutes to remove insoluble Mg/EDTA complexes. The pellet was washed in a small volume of water, re-centrifuged and the previously saved supernatant was added again. $MgCl_2$ was added to a final concentration of 25 mM and centrifuged at 200,000 g for two hours. Finally, the pellets were spun in distilled water. The final product was a total extract of LPS and possible other components of the bacterial cell wall (impure LPS rich solution).

The above-mentioned procedure for purification of LPS does not completely exclude addition of portions of the bacterial cell wall (Darveau & Hancock, 1983). Other components such as proteins may also be included in the LPS rich solution, therefore the extract is identified as a crude LPS-rich solution.

2.2.6. mRNA expression analysis and semi-quantitative RT-PCR

Total RNA was extracted from 100 mg of the mouse liver tissue using 1 ml TRIzol® Reagent (Invitrogen, Burlington, ON) according to the manufacturer's instructions. After homogenization, using a plastic pestle, RNA extraction was completed following the TRIzol® protocol. Tissue from the four animals exposed for two weeks to LPS or to whole heat-killed bacteria, as well as control animals were used for the gene expression analysis. The mRNA expression analysis was performed by Genome Quebec (Montreal, QC) with an Illumina MouseWG-6 v2.0 Expression BeadChip. Data produced from the Chip assay, was analysed using an Ingenuity IPA Network Analyser and

significance was calculated with the use of ANOVA and Significance analysis of microarrays (SAM) test.

RT-PCR was carried out on a Bio-Rad Laboratory's CFX96 Real-Time PCR Detection System (Mississauga, Ontario), using *Taq* DNA polymerase (Fermentas, Burlington, Ontario). Each reaction contained 2 µl of cDNA, prepared with RevertAid[TM] H Minus First Strand cDNA Synthesis Kit (Fermentas, Burlington, Ontario), 10 pM of forward and reverse primers, 2 mM MgCl2, *Taq* buffer with KCl, and 0.625 units of *Taq* DNA polymerase. Specific primers were designed by myself using integrated DNA Technology primer design software (Oligo Perfect™ Designer) (Table 2.0).

Primers for the analysis were designed to amplify exon sections, with the use of the online exon library provided by Invitrogen®. Table 2.0contains the sequence for the forward and reverse primers, size of cDNA fragment produced and the ideal annealing temperature identified using temperature gradient analysis. A heat-map showing ANOVA analysis of the mRNA expression, was produced with the assistance of IPA Network® program.

2.2.7. Immunohistochemical Analysis

Paraffin embedding and sectioning of the tissue was conducted at Pantomics (Richmond, CA) with a predetermined random pattern of tissue placement on the slide created by a random third party. Tissue sample labels were recorded and replaced with a random numbered system to ensure no predetermined knowledge was given to either Pantomics or the individual quantifying the data visualized by the fluorescent probes. Staining of the slide consisted of a DAPI staining and fluorescent antibody probe for

PCNA and γH2AX proteins (both probes were provided by Santa Cruz Biotechnology, Santa Cruz, CA).

Slides were baked at 60^0C to fix the tissue to the slide and immediately placed into 100% xylene to start the deparafinization and rehydration process. The slides were transferred to baths of 100% ethanol followed by baths with decreasing concentrations of ethanol (95%, 80% and 70%, respectively) and then placed in 1x PBS for 15 minute intervals in each bath. Slides were placed in 10 mM sodium citrate buffer (6.0 pH) and microwaved to a boil for 10 minutes on high power followed by a 20 minute interval of cooling at room temperature. The slides containing tissue were fixed in 300 μl of 4% paraformaldehyde in 1x PBS for 20 minutes at room temperature and then washed in 1x PBS (three times for five minute intervals).

The slides were treated with goat serum/1x PBS (1:200) for 1.5 hours at room temperature followed by a wash consisting of three intervals of five minutes each in unused 1x PBS. The primary antibody (either PCNA or γH2AX at 1:500 and 1:350, respectively) was added to goat serum/1x PBS (1:200) overnight at 4^0C. The slides were washed (similarly to previous cycles) to remove excess antibodies and goat anti-mouse serum (1:200) for PCNA antibody and anti-rabbit 594 serum (1:200) for γH2AX antibodies was added. These solutions also contained fluorescent probes (secondary antibodies containing a fluorophore) that bind to the specific antibodies (either PCNA or γH2AX) and emit fluorescent light indicating location of the specified proteins. Slides were washed again in 1x PBS for four intervals of five minutes each and then in 300 μl of DAPI staining (5% in 1x PBS) for one minute. Slides were then dehydrated by being submerged in a bath for 1x PBS followed by baths with increasing concentration of

ethanol (70%, 80% and 95%, respectively) and then placed in a 100% ethanol bath for 15 minutes intervals in each bath. Cover slides were then mounted over the samples with Entellan® Rapid Mounting Media containing xylene.

Samples were examined with a Zeiss confocal microscope and quantified without prior knowledge of the predetermined pattern created by an independent third party. Each tissue sample was digitally sectioned into several equal portions and cells expressing PCNA or γH2AX were recorded by counting.

2.2.8. Western Blot Analysis

Tissue samples for protein analysis were snap-frozen in liquid nitrogen immediately after extraction from the mice. Tissues were sectioned (~25 mg), washed thoroughly, sonicated in 1% SDS and small aliquots of extracts were isolated for protein analysis using Bradford dye reagents from BioRad (Hercules, CA). Tissue samples were placed in 300 μl of 1% SDS within a 1.5 ml microcentrifuge tubes. Tissues were lysed with the use of a sonicator.

Protein quantification was completed with the use of a spectrophotometer. Each sample was diluted to 1/10 in distilled H_2O and 25 μl of the diluted sample was placed in a new cuvette. To each sample, 1.25 μl of Bradford dye (diluted in 1:5 concentration in double distilled H_2O) was added and mixed thoroughly. Samples were loaded into the spectrophotometer (595 nm) and data was transferred to Microsoft Excel® to identify aliquots to be used in Western Blot analysis. Each sample aliquot was standardized to be 2 mg/ml.

Equal amounts of protein (~20 µg) were used for SDS polyacrylamide gel electrophoresis at 150V for one hour (Laemmli, 1970). Smaller predicted proteins such as PCNA (36 kDa) and MeCP2 (53 kDa) were identified using a 12% polyacrylamide gel; Ku70 (70 kDa), DNMT3a (85 kDa) and DNMT3b (96 kDa) were identified using a 10% polyacrylamide gel and DNMT1 (138 kDa) with an 8% gel. Each protein extract was analyzed three times to ensure significance of the results.

Proteins were transferred to PVDF membranes (GE Healthcare Biosciences, Piscataway, NJ). A piece of PVDF membrane, equal in size to the gel and activated in a 100% methanol bath for one minute, was placed in direct contact with the gel. The gel and membrane were placed in a BioRad® blotting apparatus applying pressure and an electrical current to ensure the proteins within the gel were transferred to the membrane. The apparatus was placed in a tank filled with 1X blotting buffer consisting of an electric charge of 100V for 60 – 90 minutes to ensure complete transfer.

Membranes were removed from the blotting buffer and stained with Ponceau S Stain to ensure the transfer was complete and then distained in distilled water. The membrane containing the proteins were activated in a bath of 100% methanol for one minute and submerged in a blocking solution consisting of 5% milk (2% fat) in 1X PBS-Tween for one hour. The blocking solution was removed and replaced with a primary antibody solution (0.5% milk [2% fat] in PBS-Tween [5%]) to incubate overnight on a shaker at 4^0C. The primary antibody solution was removed and the membrane washed 5x in 1x PBS, for five minutes each wash. Then, a secondary antibody was diluted, in 0.5% milk in PBS-Tween, and then was added and the membranes left on the shaker for three

hours. A second wash cycle to ensure removal of excessive antibodies was conducted (five times for five minutes each interval).

After an additional wash in 1X PBS for 10 minutes, the antibody binding was visualized by an ECL PLUS immunoblotting detection system (GE Healthcare Biosciences). The reaction produced a chemiluminescence signal that was captured by GE ECL Hypefilm (GE healthcare Biosciences). The membrane was then rinsed in distilled water, to remove the ECL and incubated with an Actin antibody (1:10000 dilution) in a 5% milk (2% fat) medium (repeat of the secondary antibody and detection method), which was used as a loading control.

2.2.9. Statistical analysis

To identify significant alterations, statistical analysis of the data was conducted for every experiment with a significance confidence level of a minimum of 95% ($p \leq .0.05$). A comparison between different treatments was performed, using ANOVA for continuous responses and statistical tests for contingency tables such as Fisher's exact test. The analysis of data was performed using the software packages Stat View and Analyze It for Excel and checked using the statistical analysis program SPSS 15.

2.2.10. Image J

Image J is a computational analysis tool used to identify alterations in expression of Western blot analysis. Image J is a Java-based image processing program allowing for the comparison of exposure of fluorescent probes to a pre-determined value (control). Image J can calculate area and pixel value statistics of user-defined selections and

intensity threshold objects. The exposure is calculated to a value based on the threshold and transferred to Microsoft Excel for statistical analysis.

2.3. Results

2.3.1. Experimental set-up and tissue selection for experiment

To analyze whether or not exposure to heat-killed bacteria and their components (DNA, RNA, protein or LPS) triggers any changes in genome instability and cell proliferation in exposed animals, drinking water was supplemented with heat-killed *E. coli* O157:H7 in the amount of 6×10^6 bacteria per litre of water, or 440 µg/L of DNA, 73 µg/L of RNA, 100 µg/L of complete protein extracts or 50 µg/L of crude LPS for two week duration of exposure. Based on the supplement received, the test subjects (C57BL/6 male mice) were divided into six test groups; Control, DNA exposure, RNA exposure, protein exposure, LPS-rich solution exposure, and exposure to whole heat-killed bacteria.

The liver was chosen as an indirect target organ, because of its capacity to detoxify the host blood from possible toxins and pathogens (Jirillo *et al.,* 2002). Muscle cells were used as a control that should be neutral to bacterial exposure.

2.3.2. Exposure to LPS from heat-killed bacteria leads to increased expression of PCNA in liver cells

Protein analysis identified a significant increase in the expression of PCNA in animals exposed to heat-killed whole bacteria (*E. coli* O157:H7) (1.41 fold increase) or LPS (1.26 fold increase) when compared to the control group for a 2 week exposure duration (Figure 2.1). Analysis of samples from the four week group showed that PCNA levels in these two groups were still increased, as compared to the control, albeit to a

lower extent. Exposure to whole heat-killed bacteria led to a 1.2 fold increase and LPS exposure has an increase by 1.19 fold (Table 2.0). Other treatment groups did not show any significant alteration in the expression of PCNA.

To support the data obtained by Western Blot analysis, an immunofluorescence analysis of PCNA protein was performed on tissues paraffin embedded on microscope slides. We also found a substantial increase in PCNA in the liver tissue of animals exposed to both the whole heat-killed bacteria and LPS (Figure 2.2.A). Exposure to bacteria triggered a significant increase of 1.46 fold in the two week test group and 1.27 fold in the four week test group as compared to control, respectively. Exposure to LPS resulted in an increase in PCNA expression of 1.38 fold and 1.24 fold in two week and four week test groups, as compared to the control, respectively (Figure 2.2.B & C).

2.3.3. Exposure to LPS from heat-killed bacteria leads to increased levels of γH2AX

Immunofluorescence analysis identified an alteration in the level of γH2AX between test groups (Figure 2.3). Exposure to the whole heat-killed bacteria resulted in an increase in γH2AX detection of 2.7 fold for the two week group and 3.25 fold for the four week group. Exposure to the LPS has resulted in a 1.95 fold increase in detection of γH2AX for the two week group and a 3.3 fold for the four week exposure group. Exposure to other bacterial components did not result in an altered level of γH2AX (Table 2.2).

2.3.4. Exposure to LPS from heat-killed bacteria leads to an increase in the frequency of DNA damage in the liver tissue

To analyse whether any DNA damage was induced by infection within the tissue of the liver, different proteins that could be activated in response to genomic stress-induced DNA damage were profiled. First, we tested the protein level of Ape1, involved in base excision repair pathways. A significant decrease in expression of Ape1 was identified when animals were exposed to LPS (1.19 fold decrease) and whole heat-killed bacteria (1.13 fold decrease) in the two week group (Table 2.3) as compared to the control. All other determinants did not result in any significant difference in expression compared to the control. In the four week group, levels of expression returned to comparable levels to those seen in the control group (Figure 2.4).

Second, we tested the level of Ku70 protein, which is involved in non-homologous end-joining of damaged DNA; NHEJ is the predominant double strand break repair pathway in mammalian cells (Kocazeybek *et al.,* 2003). A 2.5 fold increase was observed upon exposure of the mice to whole heat-killed bacteria and a 1.67 fold increase upon LPS exposure (Figure 2.5). In contrast, levels of Ku70 were not changed in animals exposed to DNA, RNA and protein purified from the heat-killed bacteria. Levels of Ku70 returned to levels comparable to the control group after two additional weeks of recovery; bacterial LPS exposure resulted in a return to levels identified within the control group (Table 2.4).

2.3.5. Exposure to LPS from heat-killed bacteria leads to an increase in expression of maintenance and de novo DNA methylation enzymes

It has been previously identified that genomic instability is accompanied by an alteration in DNA methylation. It can be hypothesized that epigenetic regulation of genome stability, in part occurs as a result of the action of proteins involved in the methylation of the genome (Li *et al.,* 2012). We first analyzed the level of proteins involved in maintenance of DNA methylation, such as MeCP2 and Dnmt1. The level of both of these proteins was increased in whole-bacteria and LPS treatment groups (Figure 2.6).

MeCP2 protein levels were significantly increased after two weeks of exposure to LPS (1.2 fold increase) and remained increased in four week samples (1.28 fold increase). Exposure to the whole heat-killed bacteria resulted in a significant increases in expression by 1.76 fold in the two week sample and by1.37 fold in the four week samples (Table 2.5). All other samples did not have any significant alterations in expression of MeCP2.

Analysis of Dnmt1 upon exposure to LPS showed a significant 1.25 fold increase in the two week sample, and a 1.24 fold increase in the four week sample. Exposure to whole bacteria also resulted in increase in the level of DNMT1 protein: 1.71 fold and 1.67 fold increases were observed in the two and four week samples, respectively (Figure 2.7, Table 2.6).

The protein level of an enzyme involved in *de novo* DNA methylation, DNMT3A, also increased upon exposure to whole bacteria and LPS samples. A two week exposure

resulted in a 1.96 fold increase for whole bacterial exposure and a 1.25 fold for LPS exposure. The analysis of four week samples showed that the levels of DNMT3A dropped and showed no significant alteration of expression levels as compared to the control group; there was a 0.97 fold change in LPS samples, and 1.001 fold change in whole bacteria samples (Figure 2.8).

The level of DNMT3B protein, also involved in *de novo* DNA methylation was increased upon exposure to whole bacteria and LPS samples. A two week exposure resulted in a 1.57 fold increase for whole bacterial exposure and a 1.18 fold for the LPS exposure. Analysis of the four week samples identified a significant increase in DNMT3B protein expression within liver tissues of animals exposed to LPS (124.8 fold increase) but no significant difference for animals exposed to whole heat-killed bacteria compared to the control group (Figure 2.9). Analysis of MeCP2 (Table 2.6), DNMT1 (Table 2.7) and DNMT3A (Table 2.8) in DNA, RNA and protein treatment groups did not show any significant changes.

2.3.6. Exposure to LPS or whole heat-killed bacteria leads to alterations in mRNA expression within liver tissues

Since only exposure to whole bacteria and LPS triggered changes in proliferation and DNA methylation, the microarray analysis only used tissue from animals exposed to whole bacteria or LPS. Results identified alterations in expression of many genes. To decrease the number of genes with altered expression, only genes that have been identified to have an expression of 2 fold higher or lower than the control expression were considered for further analysis. Whole heat-killed bacteria exposure increased the expression of interleukin L1, 6, 4, 17B and Tumour Necrosis Factor, and decreased

expression of Glycine C-Acetyltransferase genes. LPS exposure increased the expression of CCL6, FADS2, PLIN2, PNRC1 and RXRA genes. A number of transcripts were altered in similar manner upon the exposure to the heat-killed bacteria and LPS. *DUSP1*, *GADD45G*, *TFF3*, *ESM1*, *MMD2*, *GSTA1*, *CYP7A1* and *ALAS1* genes changed their transcription levels in response to both whole heat-killed bacteria and LPS (Figure 2.10).

To confirm the changes in expression of the aforementioned genes, real-time quantitative polymerase chain reaction (RTPCR) analysis was performed. RTPCR confirmed upregulation of the *DUSP1* gene, which was found to be upregulated by 2.7 fold in response to LPS and by 2.2 fold in response to whole bacteria. Results also confirmed an increased expression of the gene *ALAS1* in the LPS group (a 5.21 fold increase was indicated in the microarray) but not in the whole bacteria group (Figure 2.10.B i and ii).

Microarray analysis identified a significant decrease in transcription levels of the *GADD45G*, *TFF3*, *ESM1*, *MMD2*, *GSTA1* and *CYP7A1* genes. RTPCR was used to confirm microarray analysis and quantify expressions of these genes. *TFF3* expression was found to decrease 12 fold and 8 fold for the test groups exposed to LPS and bacteria, respectively (Figure 2.10.B iii). Expression of the *ESM1* gene was identified to decrease 9.5 fold for the test group exposed to LPS and 7.9 fold for the group exposed to whole bacteria (Figure 2.10.B iv). Expression of *MMD2* gene was decreased by 8 fold and 3.8 fold for test groups exposed to LPS and whole bacteria, respectively (Figure 2.10.B v). Exposure to LPS decreased the expression of *GSTA1* by a 3.58 fold, whereas exposure to whole bacteria did not change GSTA1 expression, as measured by RTPCR (Figure 2.10.B.vi). *CYP7A1* gene expression was decreased by 2.5 fold for LPS groups and 1.7

fold for the group exposed to whole bacteria (Figure 2.10.B vii). *GADD45G* gene

expression decreased by 5.1 fold in response to LPS and by 5.0 fold in response to whole

bacteria (Figure 2.10.B viii).

2.4 Discussion

Previously, it was shown that exposure to heat-killed bacteria resulted in an

increase in cell proliferation and genome instability of non-exposed liver cells (Koturbash

et al., 2009). This research attempted to identify which component of bacteria triggers

this response. Exposure to LPS and not to DNA, RNA or proteins resulted in an increase

in the level of PCNA, the level of Ku70 protein and the levels of proteins coding for

DNA methylation enzymes. Furthermore, it was identified that a set of 8 genes (DUSP1,

GADD45G, TFF3, ESM1, MMD2, GSTA1, CYP7A1 and ALAS1) were upregulated

upon exposure to whole bacteria and LPS in liver. Below we discuss these findings in

detail.

2.4.1. PCNA, γH2AX and Ku70 levels increase in liver cells of animals exposed to whole

bacteria and LPS

PCNA amounts increased in liver tissue after two weeks of exposure to whole

bacteria and LPS. No such increase was observed upon exposure to DNA, RNA or

protein extracts prepared from heat-killed bacteria. It is important to note that PCNA

levels remained significantly higher even after two weeks of recovery (four week

sample). High levels of PCNA are associated either with an increase in cell proliferation

or with an increase in DNA damage. It is interesting to note that such a response may not

necessarily be triggered by direct exposure of liver cells to LPS. In fact, in healthy mice,

most of the bacteria and LPS molecules probably do not penetrate the mucous layer of the intestine. It cannot be excluded, however, that a small amount of LPS is absorbed into the portal blood and passed through the liver cells. Thus, it is possible that the increase in PCNA levels is in part due to direct contact of liver cells with LPS. Is the increase in PCNA due to cell proliferation (cell division) or due to damage to DNA? Unfortunately this study did not distinguish between these two possibilities.

Exposure to LPS can result in direct or indirect damage to DNA via ROS or RNOS pathways (Sanlioglu *et al.,* 2001).   Thus, the increase in PCNA levels could reflect an increase of DNA repair activity. Also, without continuous exposure to the pathogenic bacteria and/or its components, the levels of DNA damage and thus DNA repair will potentially decrease, requiring fewer proteins such as PCNA. This could explain the lower levels of expression detected from the four week samples compared to the two week test, although these levels were still significantly higher in four week samples than in the control. Exposure to DNA, RNA and protein did not induce any significant alterations in PCNA expression.

γH2AX immunofluorescence analysis identified that exposure to LPS and heat-killed bacteria caused an increase in the phosphorylation of the H2AX protein. This increase is correlated with an increase in the level of Ku70 protein as shown with Western Blot analysis. The increase in the γH2AX phosphorylation identifies DNA alteration, mainly DNA strand breaks. Changes to DNA structure lead to multiple epigenetic modifications including phosphorylation of serine 139 on histone H2A. Recruitment of γH2AX activates homologous recombination and non-homologous end joining DNA repair pathways (Burma *et al.,* 2001). These results are supported by

previous research conducted by Koturbash *et al.,* (2009), where it was identified that heat-killed bacteria (pathogenic or non-pathogenic) induced higher levels of γH2AX within the liver tissue, indicating the presence of DNA double strand breaks and instability within the genome.

Exposure to whole bacteria or LPS induced an alteration in expression of DNA repair proteins within the liver. Ku70, a NHEJ protein, was significantly increased in whole bacteria and the LPS exposure groups of the two week samples, indicating a large increase in the level of DSBs. However, for the same group, the BER protein Ape1 was shown to have lower expression. Ku70 is a key component in the NHEJ process, that is known to be the predominant method of DSB repair (Kuper *et al.,* 2000). LPS and other endotoxins increase expression of NF-κB that in turn correlates with expression of COX-2 (Wadleigh *et al.,* 2000). NF-κB is an inducible transcription factor that regulates a wide variety of genes that have been identified to respond to inflammatory signals (Baeuerle and Baltimore, 1996). Um *et al.*, (2001) has shown that Ku70 expression correlates with the expression of NF-κB and COX-2, potentially affecting cell proliferation. Cells with inhibited COX-2 and/or NF-κB genes were identified to have limited ability to repair their DSBs and proliferate (Lim *et al.,* 2001).

The amount of proteins associated with methylation of the genome, whether due to *de novo* synthesis or maintenance, Dnmt3A and Dnmt3B significantly increased with LPS and bacterial exposure but not in response to other molecules. The constitutive expression of MeCP2 is caused by its ability to perpetuate its own expression. This cycling of expression results in continual expression of the MeCP2 protein and potential to repress genes and manipulate chromatin structure (Chahrour *et al.,* 2008). In this

experiment, the expression of DNMT1, 3A and 3B returned to normal levels in animals that were allowed to recover by consuming uncontaminated water for the additional two week period after the initial exposure. This may indicate reversibility of potential changes in DNA methylation. This may also suggest that constant presence of a causative agent, such as LPS, is required for triggering changes in DNA methylation.

The results identify that the naïve cells, distant from the exposed tissue, can be affected by exposure to LPS and whole heat killed bacteria. This indicated that within the two week exposure to multiple components of the bacteria, LPS was identified to be a key bacterial component inducing a response in distal cells and responsible for potential genomic instability. Altered levels of PCNA and γH2AX, increased expression of Ku70 and proteins involved in DNA methylation, in response to bacteria and LPS supported the hypothesis that a bystander-like effect induced genomic instability.

2.4.2. Exposure to LPS and whole bacteria result in changes in the expression of eight different genes

Microarray analysis of liver cells in animals exposed to LPS or whole heat-killed bacteria showed differential expression of eight genes that were verified with RTPCR. Altered expression of the eight genes (*DUSP1*, *GADD45G*, *TFF3*, *ESM1*, *MMD2*, *GSTA1*, *CYP7A1* and *ALAS1*) could have detrimental effects on the host. Dual specificity phosphatase 1 (Dusp1) expression was found to be altered in fibroblasts exposed to oxidative/heat stress and upon stimulation with growth factors (Abraham and Clark, 2007). In this study, Abraham and Clark identified Dusp1 as having a potential role in the cellular response to environmental stress as well as in the negative regulation of proliferation and an inflammatory response. An increase in expression of *Dusp1* may

have occurred to assist in the cells' ability to survive the shock of the LPS-induced reaction (Hammer *et al.,* 2006).

An increased expression level of the gene coding for aminolevulinic acid synthase 1 (Alas1) protein was also identified. This nuclear encoded mitochondrial enzyme is the first and rate-limiting enzyme in the heme biosynthetic pathway (Red Blood Cell (RBC) production). The production of RBCs could accelerate with influx of this specific protein and any other in an eight step process (Abraham and Clark, 2006). Tumour cells may increase the expression of enzymes in this process, to assist in the production of RBCs, to oxygenate new tumour cells throughout the body.

Growth arrest and DNA-damage-inducible 45 gamma (Gadd45g) is a protein, identified as a stress sensor, that modulates the response of mammalian cells to genotoxic/physiological stress and modulates tumour formation. The transcription of the aforementioned gene has been quantified at alternative levels in response to stressors inducing growth arrest (Abell *et al.,* 2007). Gadd45g protein also responds to environmental stresses by mediating the activation of p38/JNK pathway via MTK1/MEKK4 kinase (Abell *et al.,* 2007). A decrease in transcription level of *Gadd45g* gene inhibits the production and dimerization of MEKK4, allowing cellular proliferation, differentiation, inflammation and tumourigenesis (Ip and Davis, 1998).

Another mRNA with decreased gene transcription level was identified as *Tff3* (Trefoil factor 3). The function of the encoded protein is not well defined; however it is predicted to stabilize the mucus layer and affect healing of the cells themselves. Recently, Tff3 has been identified to be involved in the immune response (Paulsen *et al.,* 2008).

This research has identified very low levels of the Tff3 protein during liver and gastrointestinal tissue damage, and high levels of Tff3 gene transcription briefly after the tissue was repaired. Our analysis showed that the transcription level of *Tff3* was increased within the two week of exposure to whole heat-killed bacteria of LPS. It remains to be shown whether similar changes would be found within the four week sample group.

The expression of the gene coding for endothelial cell-specific molecule 1 (Esm1) protein was found to be lower in liver cells from the LPS and whole bacteria group. Esm1 is regulated by cytokines, identifying potential involvement in pathogenic infections. *Esm1* expression has been shown to be increased in the presence of pro-angiogenic growth factors, such as VEGF (vascular endothelial growth factor) or FGF-2 (fibroblast growth factor 2). A significant decrease in transcription of *Esm1* gene, correlates with previously reported decrease in transcription level of pro-angiogenic growth factors such as VEGF or FGF2 genes (Béchard *et al.,* 2001).

Macrophage differentiation associated 2 (*Mmd2*) was reduced in expression for LPS and whole heat-killed bacteria test groups. Mmd2 is involved in the immune response and in differentiation of monocytes to macrophages. Since the response to bacteria/LPS may trigger an immediate immune response upon which monocytes differentiate into macrophages, it can be suggested that the expression of Mmd2 is no longer required at two weeks post exposure. It is possible that *Mmd2* expression was increased in the first two days of exposure and then decreased at two weeks post exposure. It remains to be shown whether *Mmd2* levels would return to normal levels after a two weeks recovery period.

Glutathione S-transferase alpha 1 (*Gsta1*) mRNA was downregulated in our experiments. Gsta1 has enzymatic functions associated with the detoxification of electrophilic compounds such as carcinogens, environmental toxins and products of oxidative stress. These highly polymorphic enzymes alter the susceptibility of the organism to carcinogens, toxins and alter the effectiveness of some pharmaceutical drugs. The decrease in expression identified in our experiment implicated that the liver tissue was highly susceptible to damage caused by ROS.

Finally, analysis showed a decrease in the steady state RNA levels of the *cytochrome p450 family 7, subfamily a, polypeptide 1* (*CYP7A1*) gene. Cyp7a1 is involved in drug metabolism and synthesis of bile acid and steroids from cholesterol within liver tissue. Conversion of cholesterol into bile acid is controlled by this protein and is the main process of removing cholesterol from the body (Holt *et al.,* 2003). Removal of cholesterol from the body is important to the overall homeostatic state of organism. Even though there seems to be no connection with an immune response for this particular protein, altered levels affect the entire organism through inhibition of elimination of cholesterol.

This work is the first to show that a heat-killed bacterial component known as LPS can lead to distinct molecular changes in the liver. It is important to note, that many changes in liver cells after a two week exposure to LPS returned to levels similar to the control group, indicating the recovery period for such as alteration is short. However, it is also important to note, that changes in the levels of protein and expression of mRNAs in liver samples after exposure to whole heat-killed bacteria were more pronounced than after the exposure to LPS. This indicates that LPS may contribute to genome instability

caused by bacterial contaminants in the intestine or blood, but it is not the only component. Toxins released by the bacteria upon death may also have some negative effect. Since filtering the water would not remove released toxins (such as Shx), it is possible that exposure to these toxins may had an additive effect to changes in the stability of cells in direct contact or distal naïve.

PCNA is used to identify the proliferation of cells; however, it has also been identified to be associated with the DNA damage repair process (Shivji *et al.,* 1992). The results from this experiment indicate a positive correlation between PCNA and γH2AX, which could be interpreted as a possible increase in proliferation and/or an increase in DNA damage within the liver tissue. Another possible product of the bacterial exposure is circulating inflammatory and anti-inflammatory cytokines produced by affected cells of the host (Murata *et al.,* 1998). Further research with the expansion into the inflammatory or anti-inflammatory field is required to identify every component of the bacteria that could induce the effects of carcinogenesis on liver tissue. Another remaining question is whether the bystander effect can induce genomic instability in other organs and tissues throughout the body. Thus our study may serve as a roadmap for further analysis of toxic and genotoxic effects of water contamination.
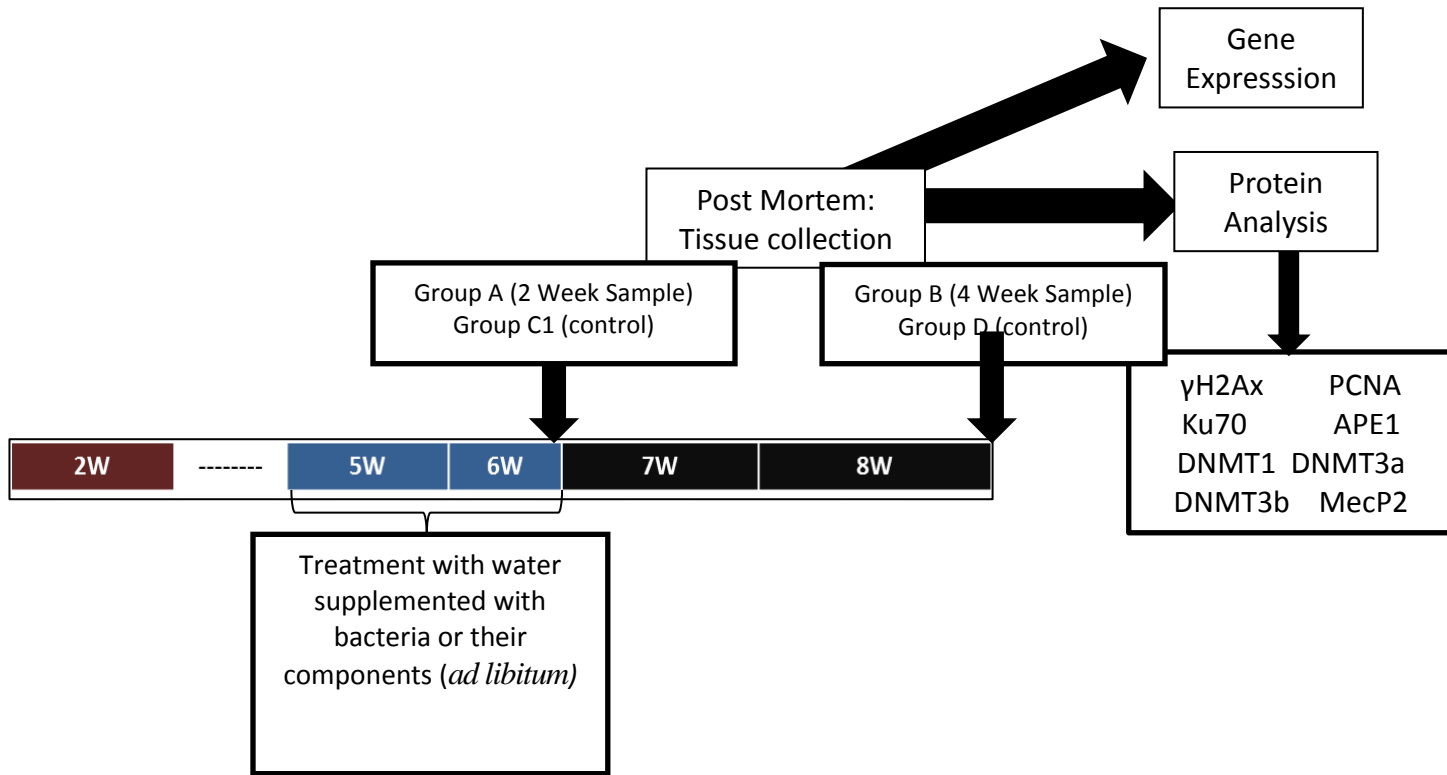
Figure 2.0. Experimental design to analyze potential genomic alterations induced in the liver cells of mice. Four-week-old animals received treatment water for two weeks. First set of four animals (per group) was sacrificed immediately after this treatment, whereas the second set of four animals was sacrificed in two weeks, after receiving normal tap water.
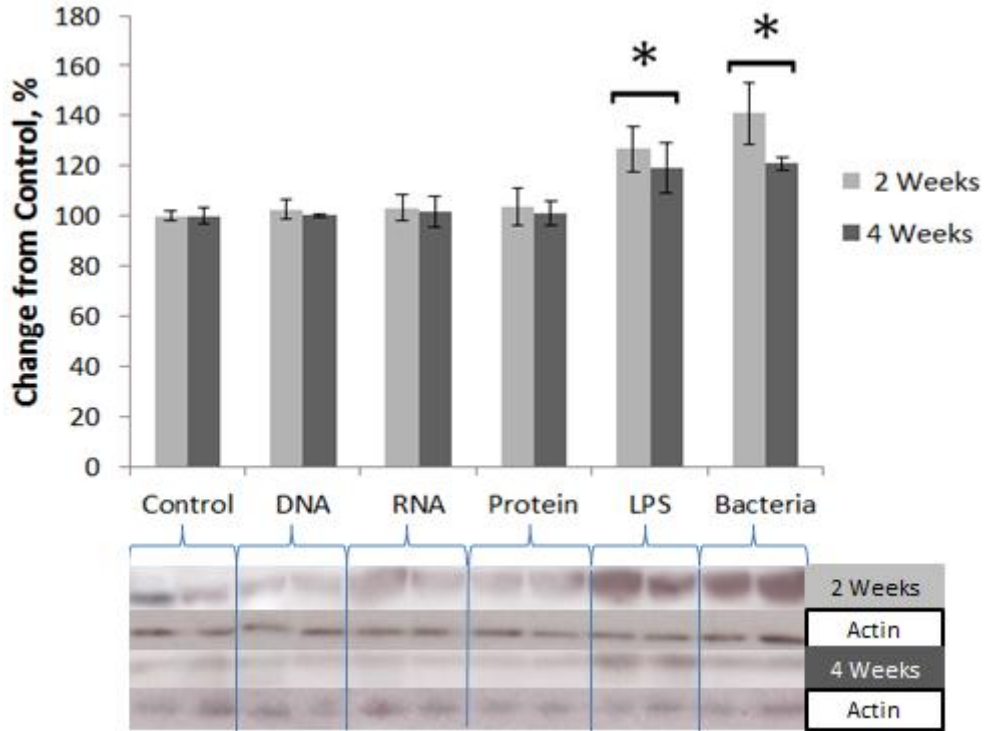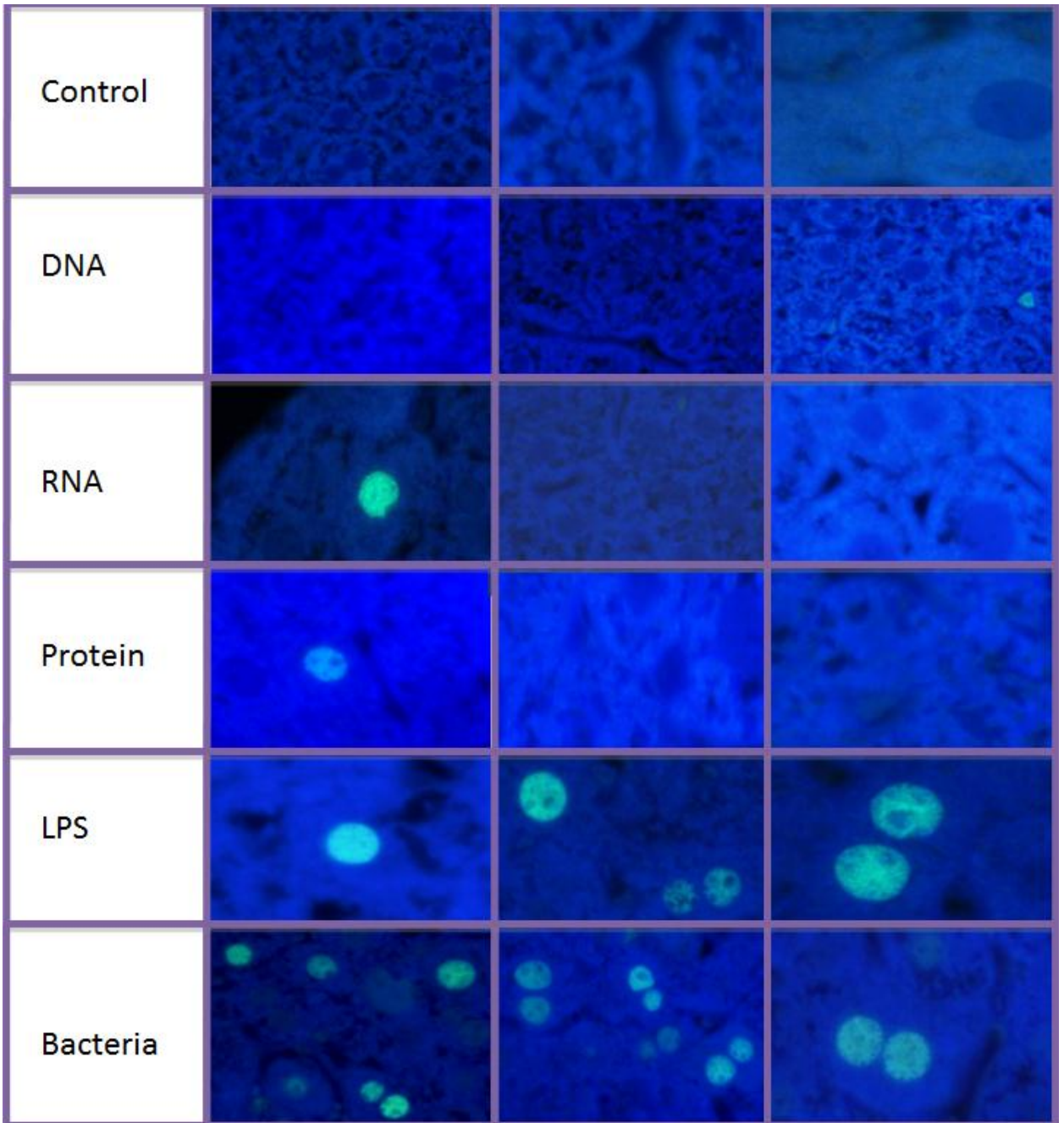
Figure 2.1. Western blot analysis of PCNA protein levels in liver tissue of mice exposed to whole heat-killed *E. coli* O157:H7 bacteria and DNA, RNA, protein, and LPS extracted from heat-killed bacteria. Bars show the average protein levels (with SD) as compared to the control set at 100%. Asterisks and bars show significant increases from non-exposed controls through the analysis of data using one way ANOVA test.

Table 2.1. Western blot analysis of PCNA quantified with Image J. program. Average±SD represents the average protein expression±standard deviation, compared to the control test. Asterisks identify significant increases from non-exposed controls.

Test	2 Week Average ± SD	4 Week Average ± SD
Control	100±1.9	100±3.1
DNA	102.6±3.6	100.1±0.4
RNA	103.2±5.1	101.5±6.15
Protein	103.7±7.4	101.3±4.9
LPS	*126.8±9.0	*119.3±10.0
Bacteria	*141.0±12.3	*120.9±2.5

A

Control			
DNA			
RNA			
Protein			
LPS			
Bacteria			

B

Control			
DNA			
RNA			
Protein			
LPS			
Bacteria			
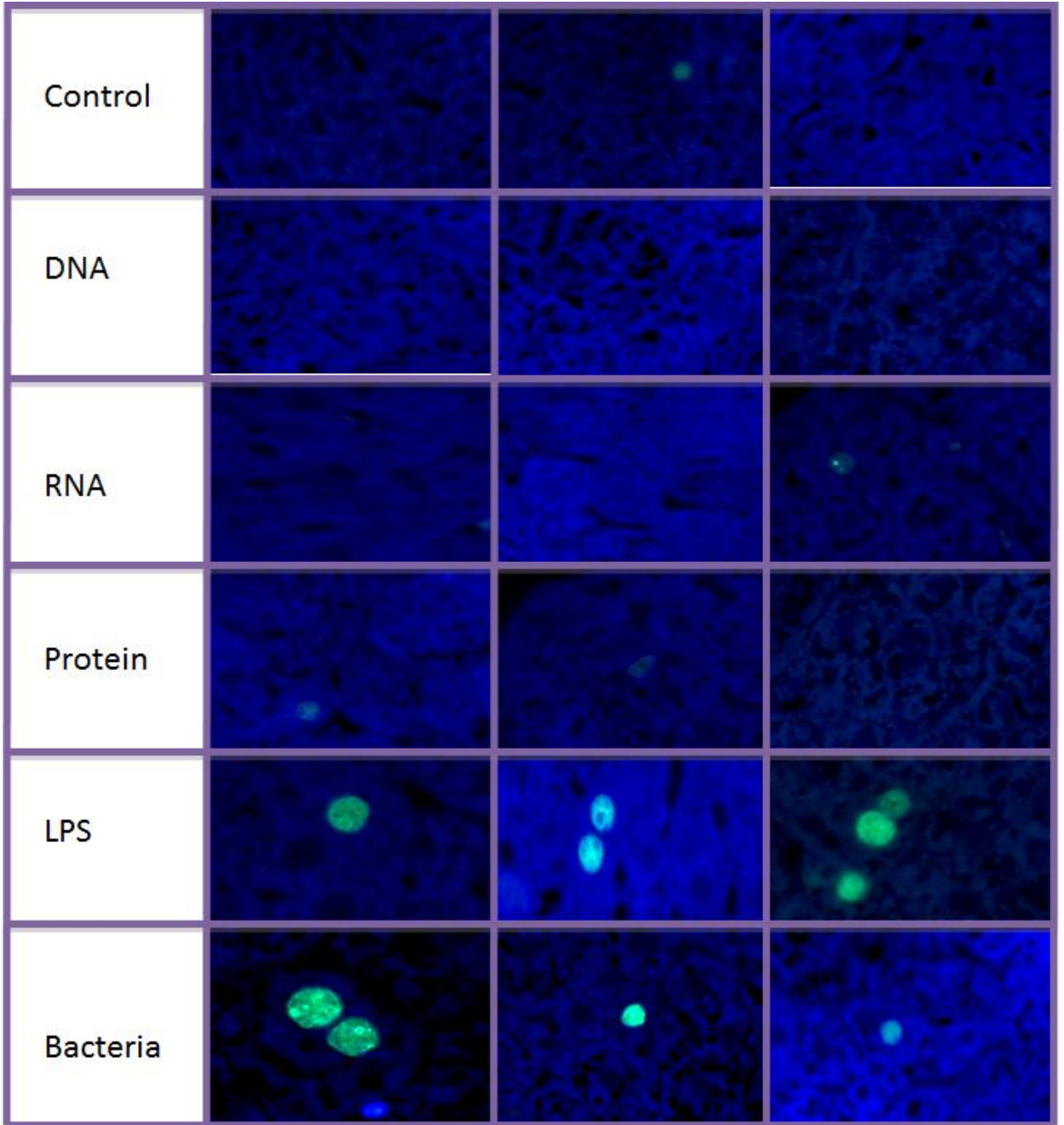
C.
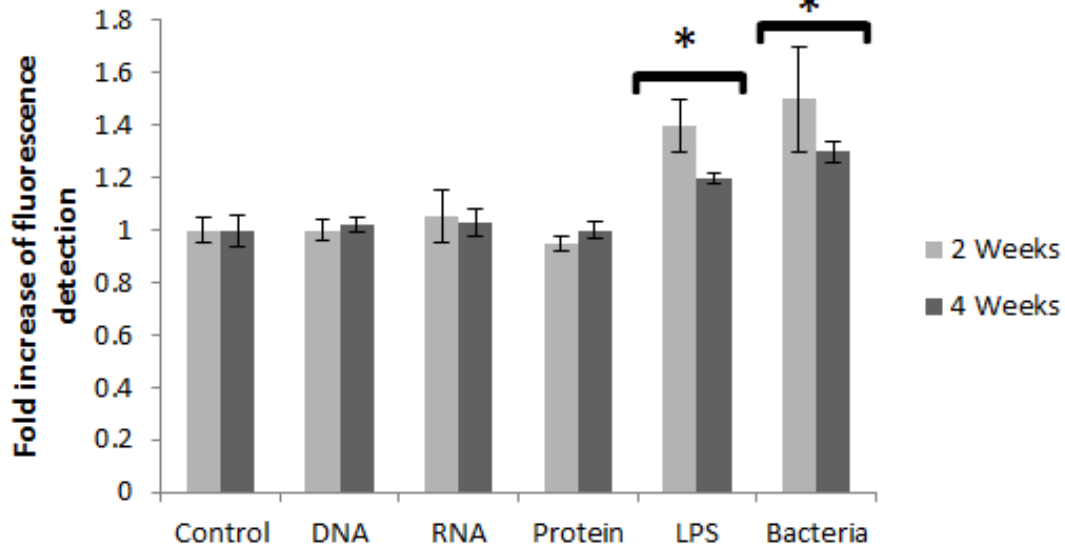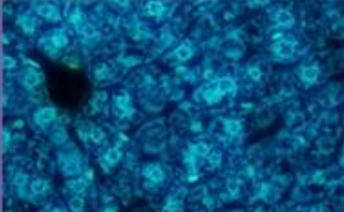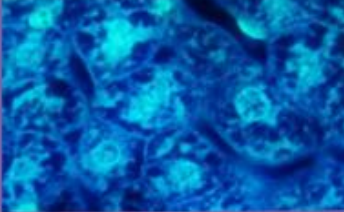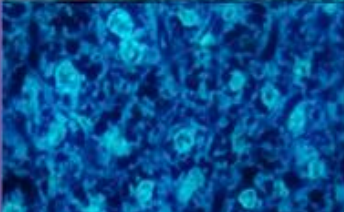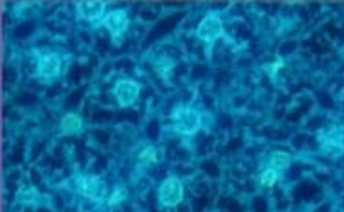
Figure 2.2. Immunohistochemical analysis of liver tissue samples stained with DAPI staining and Green Fluorescent antibody for PCNA. A, Images of liver samples taken from individual animals within test Group A (2 Week sample). **B,** Images of liver samples taken from individual animals within test Group B (4 Week sample). C, Quantification of PCNA-positive cells. Bars represent the average (with SD) number of PCNA cells. Asterisks show significant differences from control.

Table 2.2. Imunohistochemical analysis of PCNA protein expression quantified. Average±SD represents the average protein expression±standard deviation, compared to the control test detected via analysis of images. Asterisks identify significant increases from non-exposed controls.

Test	2 Week Average ± SD	4 Week Average ± SD
Control	1.0±0.05	1.0±0.06
DNA	1.0±0.04	1.02±0.03
RNA	1.05±0.1	1.03±0.05
Protein	0.95±0.03	1.0±0.03
LPS	*1.4±0.1	*1.2±0.02
Bacteria	*1.5±0.2	*1.3±0.04

A.

Test	2 Week - Sacrifice		4 Week - Sacrifice
Control			
DNA			
RNA			
Protein			
LPS			
Bacteria			

B.
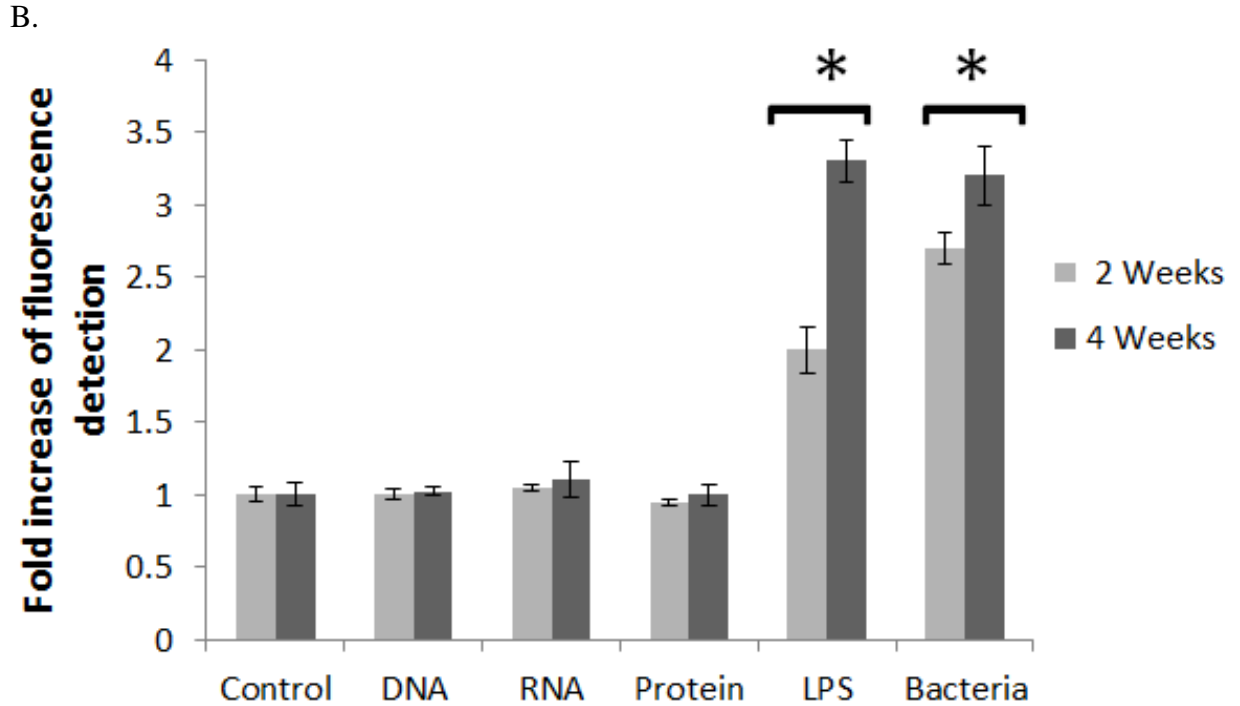
Figure 2.3. Immunohistochemical analysis of liver tissue samples stained with DAPI staining and Green Fluorescent antibody for γH2AX. A. Images taken from animals in the group exposed to tap water (control), DNA, RNA, protein, LPS and whole heat-killed bacteria. B. Bars show the average (with SD) fold difference in number of γH2AX-positive cells between treated and control groups. Asterisks show significant difference from control.

Table 2.3. Imunohistochemical analysis of γH2AX protein expression quantified. Average±SD represents the average protein expression±standard deviation, compared to the control test detected via analysis of images. Asterisks identify significant increases from non-exposed controls.

Test	2 Week Average ± SD	4 Week Average ± SD
Control	1.0±0.05	1.0±0.08
DNA	1.0±0.04	1.0±0.03
RNA	1.0±0.02	1.1±0.1
Protein	1.0±0.02	1.0±0.07
LPS	*2.0±0.16	*3.3±0.14
Bacteria	*2.7±0.11	*3.2±0.20
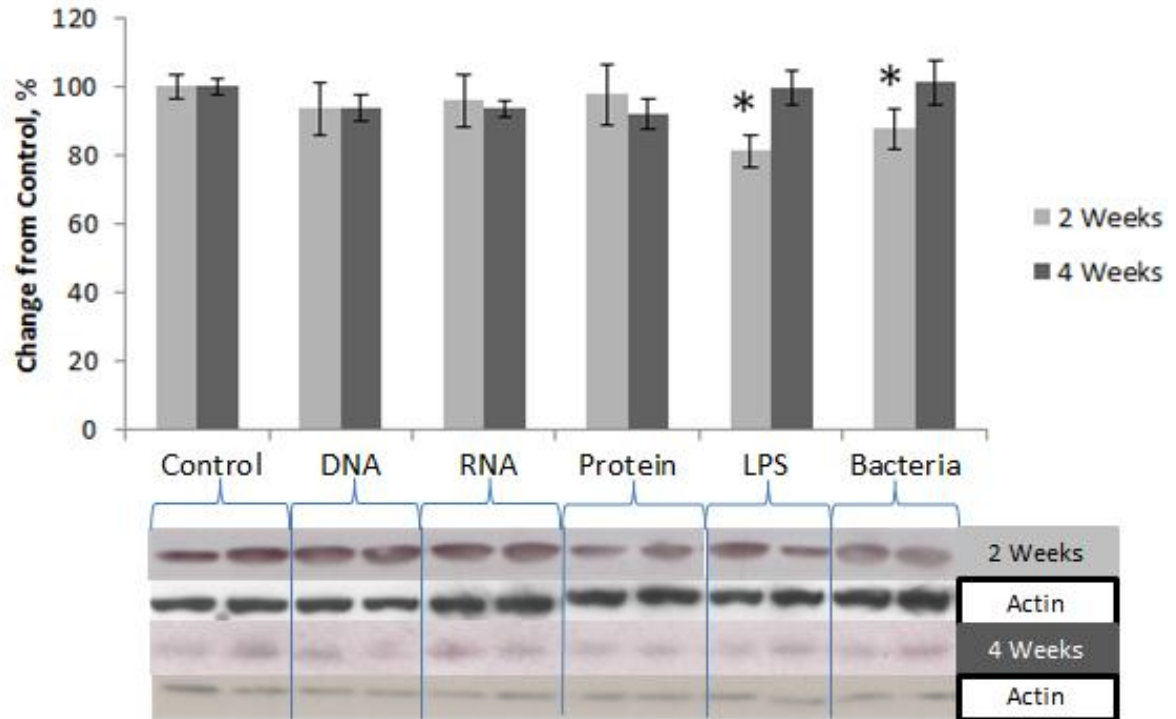
Figure 2.4. Western blot analysis of Ape1 protein levels in liver tissue of mice exposed to whole heat-killed *E. coli* O157:H7 bacteria and DNA, RNA, protein, and LPS extracted from heat-killed bacteria. Bars show the average protein levels (with SD) as compared to the control set at 100%. Asterisks and bars show significant decrease from non-exposed controls through the analysis of data using one way ANOVA test.

Table 2.4. Western blot analysis of Ape1 quantified with Image J. program. Average±SD represents the average protein expression±standard deviation, compared to the control test. Asterisks identify significant decreases from non-exposed controls.

Test	2 Week Average ± SD	4 Week Average ± SD
Control	100.0±3.6	100.0±2.6
DNA	93.8±7.6	93.6±3.8
RNA	95.9±7.7	93.6±2.4
Protein	97.7±8.7	92.0±4.6
LPS	*81.3±7.8	99.5±5.0
Bacteria	*87.7±5.7	101.3±6.4
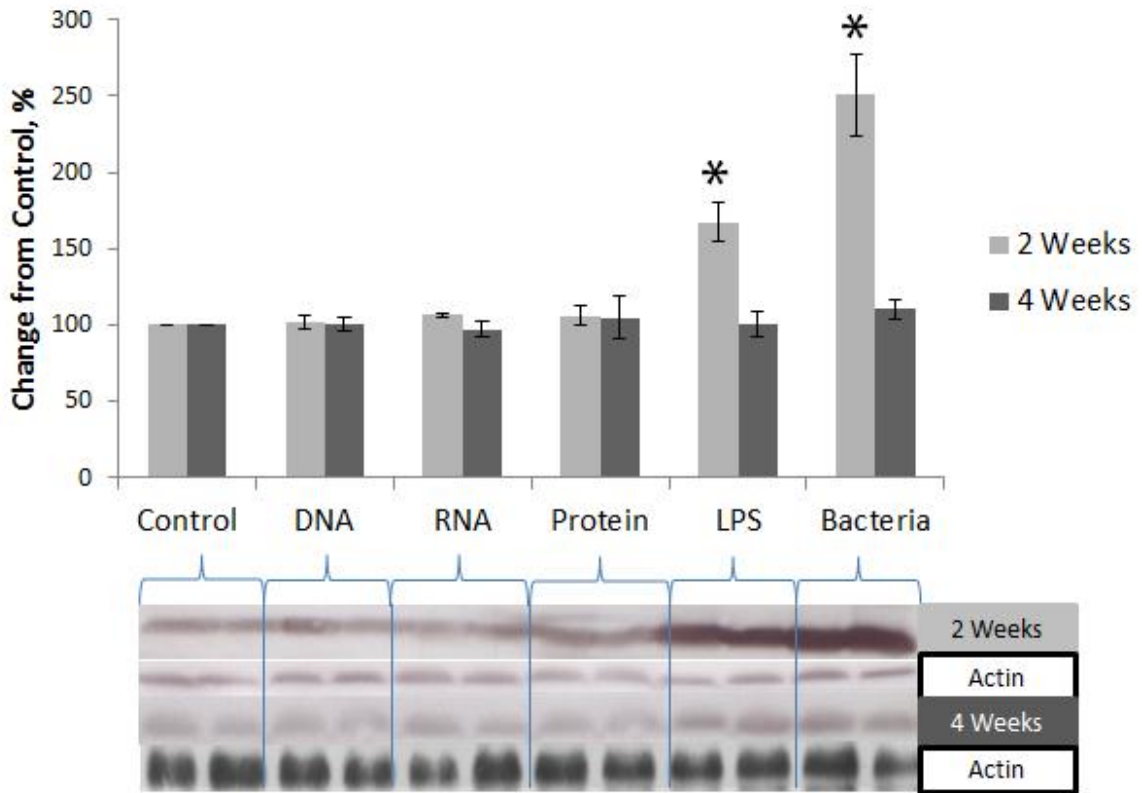
Figure 2.5. Western blot analysis of Ku70 protein levels in liver tissue of mice exposed to whole heat-killed *E. coli* O157:H7 bacteria and DNA, RNA, protein, and LPS extracted from heat-killed bacteria. Bars show the average protein levels (with SD) as compared to the control set at 100%. Asterisks and bars show significant increase from non-exposed controls through the analysis of data using one way ANOVA test.

Table 2.5. Western blot analysis of Ku70 quantified with Image J. program. Average±SD represents the average protein expression±standard deviation, compared to the control test. Asterisks identify significant increases from non-exposed controls.

Test	2 Week Average ± SD	4 Week Average ± SD
Control	100.0±2.3	100.0±1.3
DNA	102.1±4.6	100.3±5.0
RNA	106.6±7.4	96.7±5.1
Protein	105.1±5.8	104.9±14.5
LPS	*167.4±12.4	100.2±8.13
Bacteria	*250.5±27.2	107.1±7.9
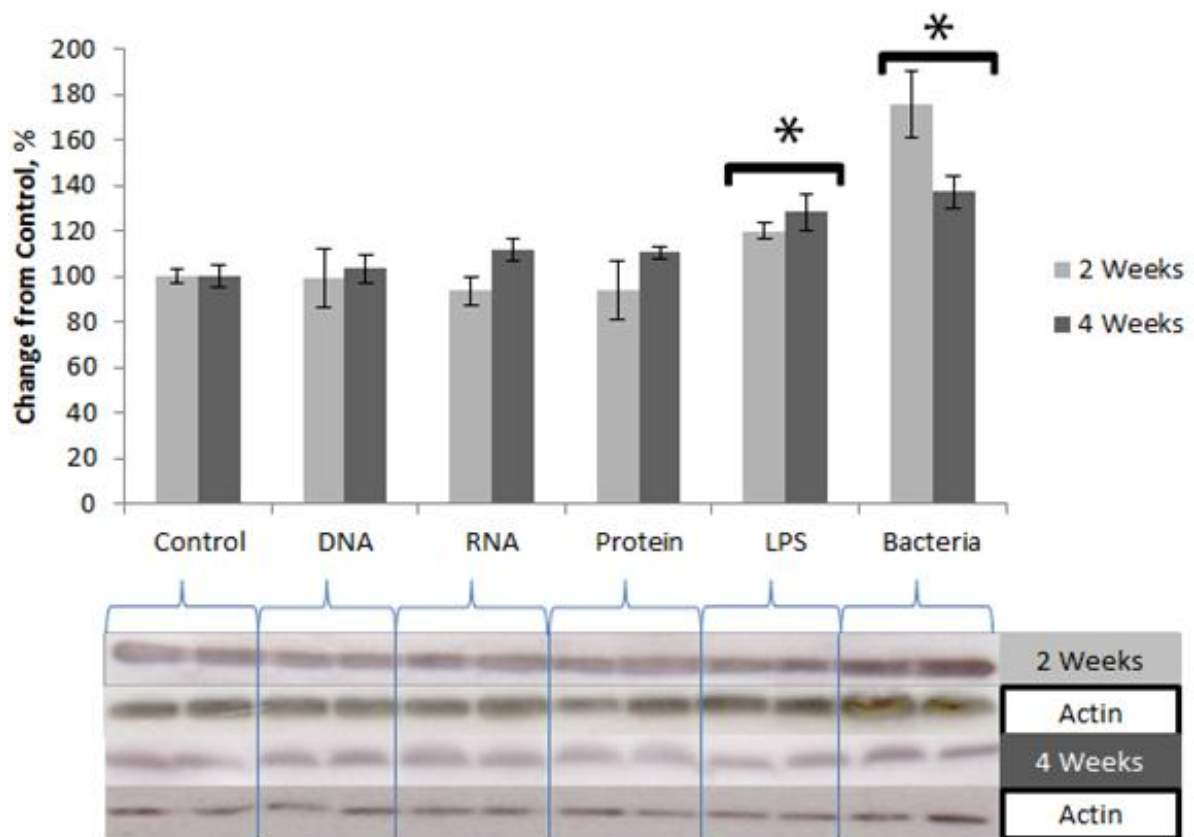
Figure 2.6. Western blot analysis of MeCP2 protein levels in liver tissue of mice exposed to whole heat-killed *E. coli* O157:H7 bacteria and DNA, RNA, protein, and LPS extracted from heat-killed bacteria. Bars show the average protein levels (with SD) as compared to the control set at 100%. Asterisks and bars show significant increase from non-exposed controls through the analysis of data using one way ANOVA test.

Table 2.6. Western blot analysis of MeCP2 quantified with Image J. program. Average±SD represents the average protein expression±standard deviation, compared to the control test. Asterisks identify significant increases from non-exposed controls.

| Test	2 Week Average ± SD	4 Week Average ± SD
Control	100.0±3.1	100.0±4.6
DNA	99.5±12.8	103.2±6.1
RNA	95.4±6.3	107.6±9.0
Protein	94.1±12.7	105.4±6.5
LPS	*120.6±3.9	*128.4±8.1
Bacteria	*175.6±14.4	*137.1±7.1
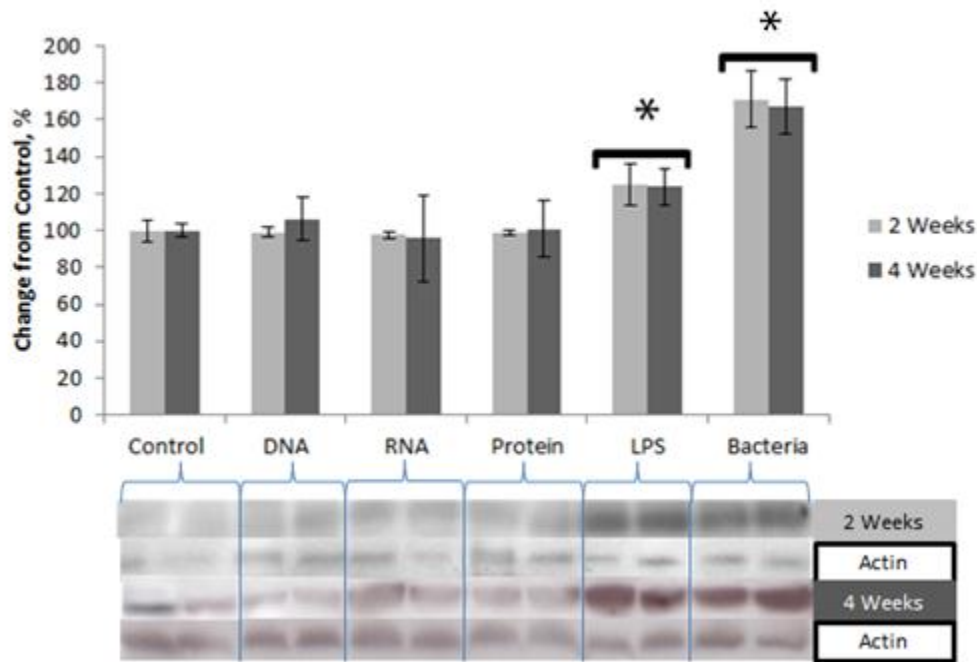
Figure 2.7. Western blot analysis of Dnmt1 protein levels in liver tissue of mice exposed to whole heat-killed *E. coli* O157:H7 bacteria and DNA, RNA, protein, and LPS extracted from heat-killed bacteria. Bars show the average protein levels (with SD) as compared to the control set at 100%. Asterisks and bars show significant increase from non-exposed controls through the analysis of data using one way ANOVA test.

Table 2.7. Western blot analysis of Dnmt1 quantified with Image J. program. Average±SD represents the average protein expression±standard deviation, compared to the control test. Asterisks identify significant increases from non-exposed controls.

| Test	2 Week Average ± SD	4 Week Average ± SD
Control	100.0±5.7	100.0±3.6
DNA	99.1±2.7	106.2±11.8
RNA	97.8±1.9	95.9±23.5
Protein	99.0±1.5	101.0±15.3
LPS	*125.6±11.5	*123.6±9.5
Bacteria	*171.1±15.3	*167.2±14.8
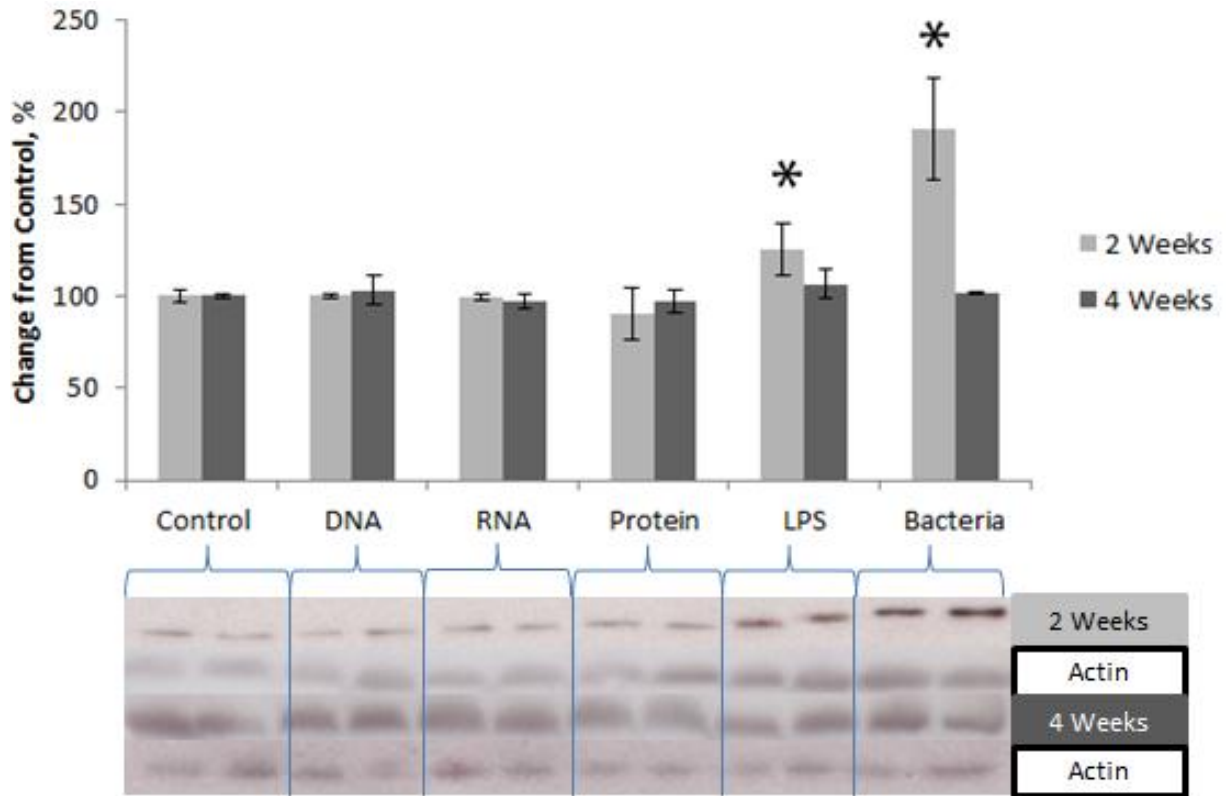
Figure 2.8. Western blot analysis of Dnmt3A protein levels in liver tissue of mice exposed to whole heat-killed *E. coli* O157:H7 bacteria and DNA, RNA, protein, and LPS extracted from heat-killed bacteria. Bars show the average protein levels (with SD) as compared to the control set at 100%. Asterisks and bars show significant increase from non-exposed controls through the analysis of data using one way ANOVA test.

Table 2.8. Western blot analysis of Dnmt3A quantified with Image J. program. Average±SD represents the average protein expression±standard deviation, compared to the control test. Asterisks identify significant increases from non-exposed controls.

| Test	2 Week Average ± SD	4 Week Average ± SD
Control	100.0±0.9	100.0±1.1
DNA	100.3±1.1	103.3±7.4
RNA	99.3±1.8	97.1±3.5
Protein	89.9±14.1	97.1±6.0
LPS	*125.4±13.7	106.3±7.9
Bacteria	*190.9±27.3	101.9±0.3
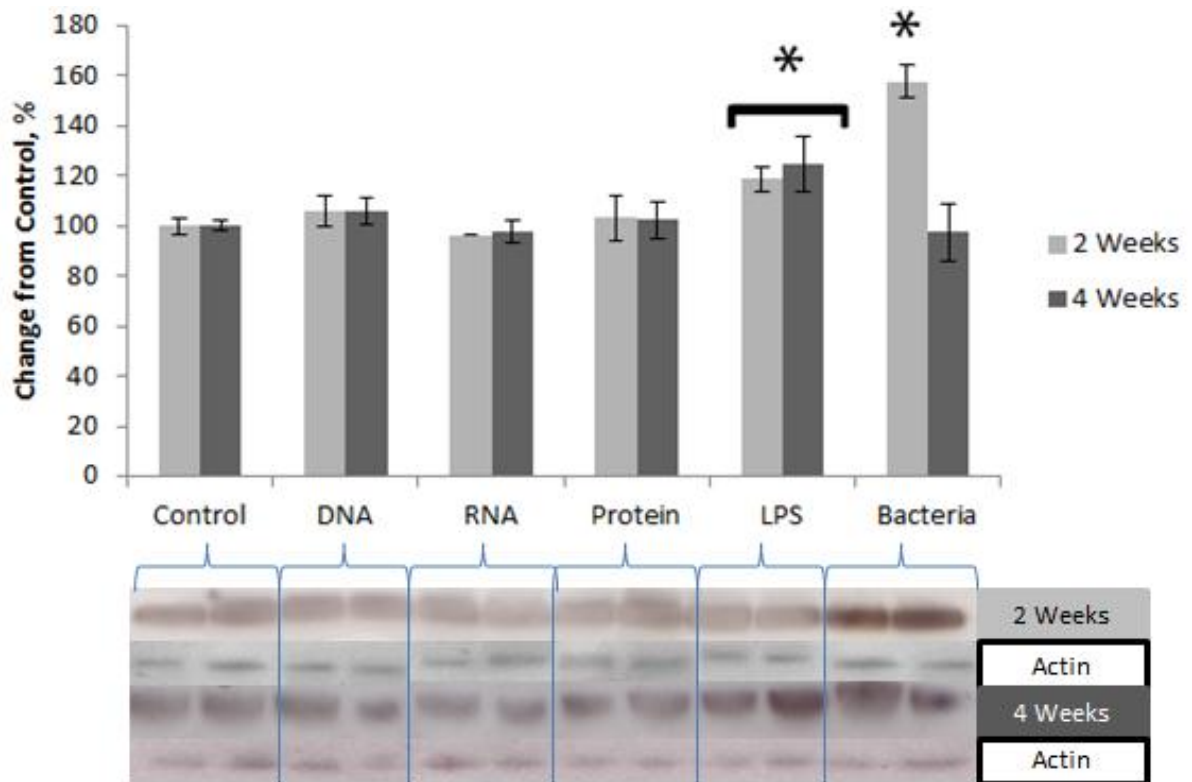
Figure 2.9. Western blot analysis of Dnmt3B protein levels in liver tissue of mice exposed to whole heat-killed *E. coli* O157:H7 bacteria and DNA, RNA, protein, and LPS extracted from heat-killed bacteria. Bars show the average protein levels (with SD) as compared to the control set at 100%. Asterisks show significant increase from non-exposed controls through the analysis of data using one way ANOVA test.

Table 2.9. Western blot analysis of Dnmt3B quantified with Image J. program. Average±SD represents the average protein expression±standard deviation, compared to the control test. Asterisks identify significant increases from non-exposed controls.

Test	2 Week Average ± SD	4 Week Average ± SD
Control	100.0±3.2	100.0±2.1
DNA	106.4±6.2	106.2±7.1
RNA	96.5±0.2	97.8±4.7
Protein	103.2±8.8	102.7±7.4
LPS	*118.8±4.9	*124.8±11.1
Bacteria	*157.8±6.3	97.7±11.5

Table 2.0 Sequence of the forward and reverse primers for RT-PCR analysis from liver samples. All primers created with the DNA Technology primer design software (Oligo Perfect™ Designer).

Protein	Direction	Primers	Ideal Temp.	Size (kbp)
DUSP1	Forward	5'-ACCTTAAAAGCCCCATCACC-'3	57.6°C	1.9
	Reverse	5'-AAATAAGGACCAGCTCCCATG-3'		
GADD45G	Forward	5'-CGGACTCTGGGAATCTTTACC-3'	57.6°C	1.5
	Reverse	5'-CAGAGTCATTGTGCGATCCA-3'		
TFF3	Forward	5'-CATTTTGAAGCTGTCCAGGC-3'	60.0°C	4.3
	Reverse	5'-GACTCCTGGCTCCTTTATTGG-3'		
ESM1	Forward	5'-AGGAAGTGAGATATTGGAAGCTG-3'	54.7°C	7.8
	Reverse	5'-CCAGAGATGAGAAGTGATGGG-3'		
MMD2	Forward	5'-GCACCATTACCTTCTACTCCC-3'	52.4°C	46.4
	Reverse	5'-AATGTATGCCTTGGTCTCCC-3'		
GSTA1	Forward	5'-AGCCAGGACTCTCACTAGAC-3'	57.6°C	0.875
	Reverse	5'-ACTTCTCTTCAAACTCCACCC-3'		
CYP7A1	Forward	5'- TCCAAGAACCACACCATGAG-3'	55.6°C	4.1
	Reverse	5'-CCTTTCTGTAGTCCTGGCTTG-3'		
ALAS1	Forward	5'-GGGCACTGGTCGGTTTAG-3'	52.4°C	7.79
	Reverse	5'-GACTCGGGATAAGAATGGGC-3'		

A
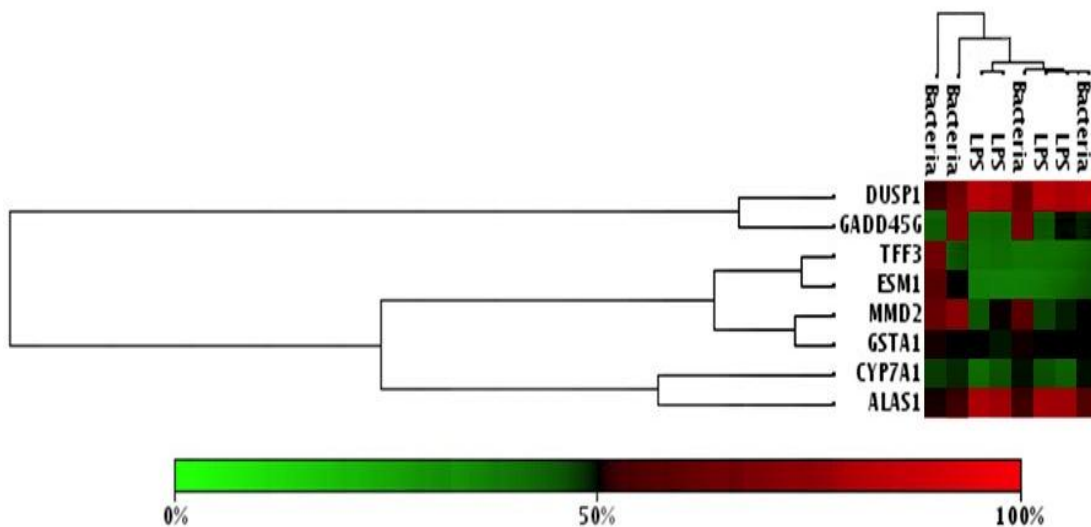
B)i)
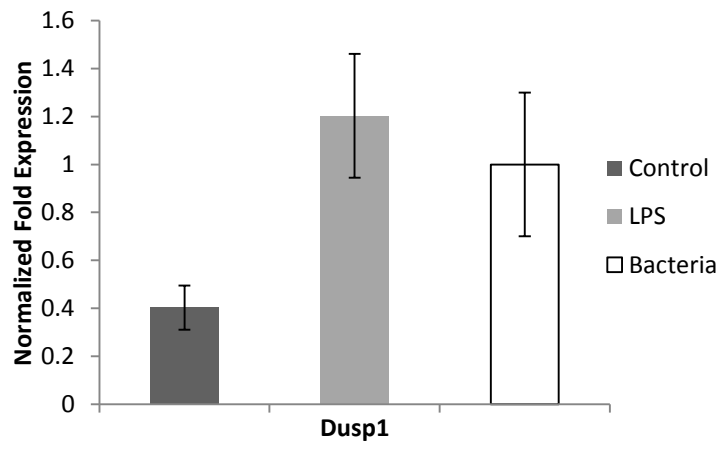
ii)
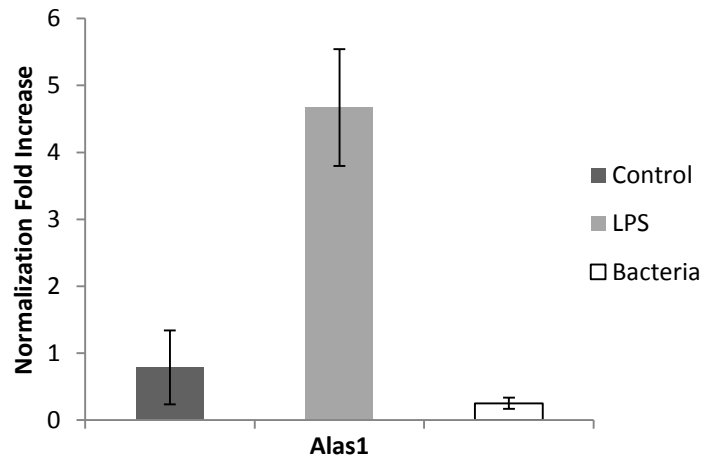
iii)
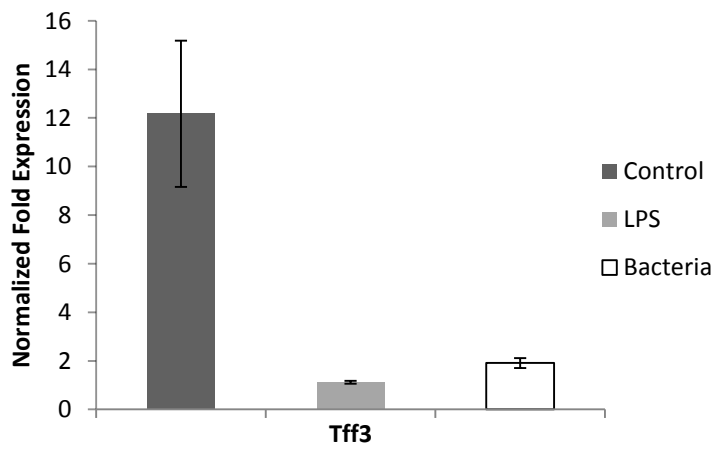
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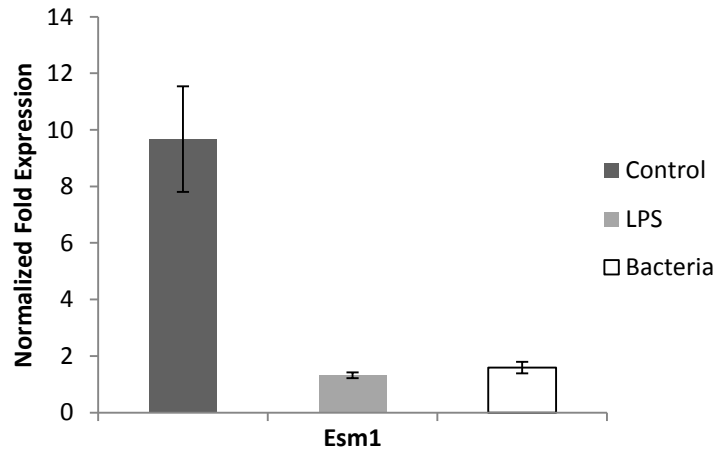
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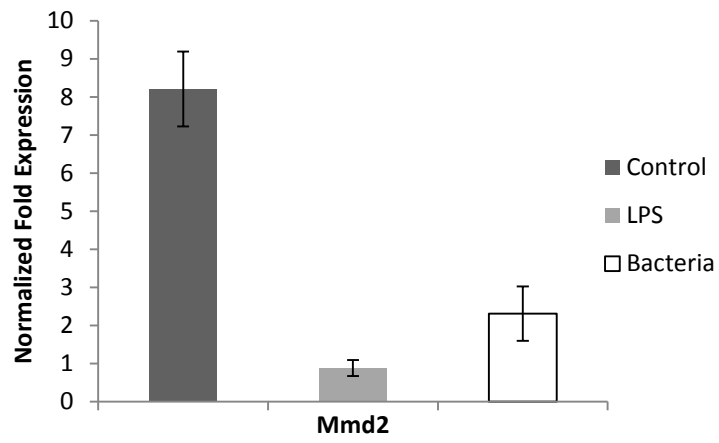
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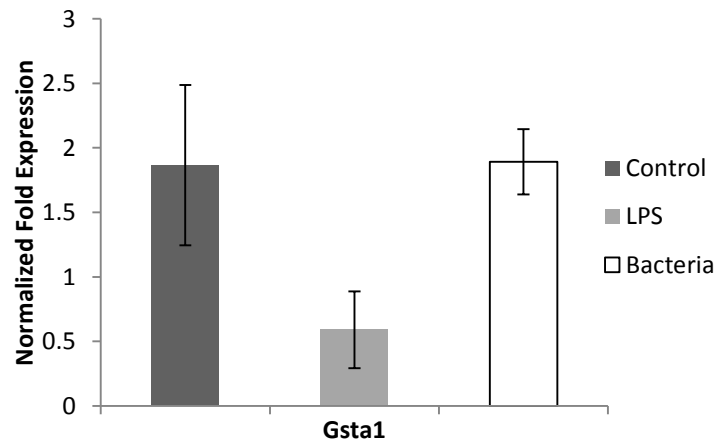
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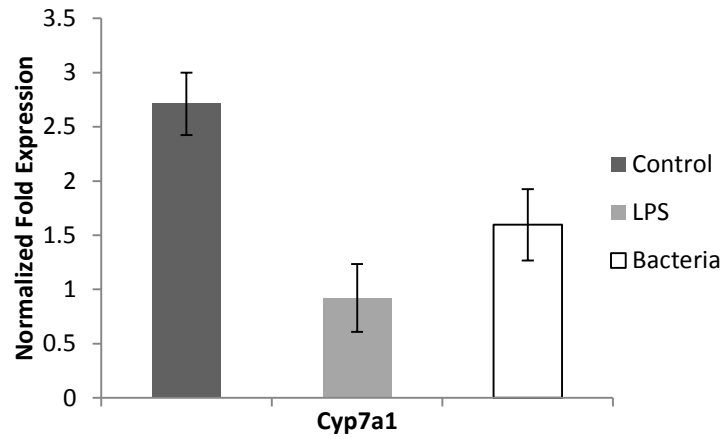
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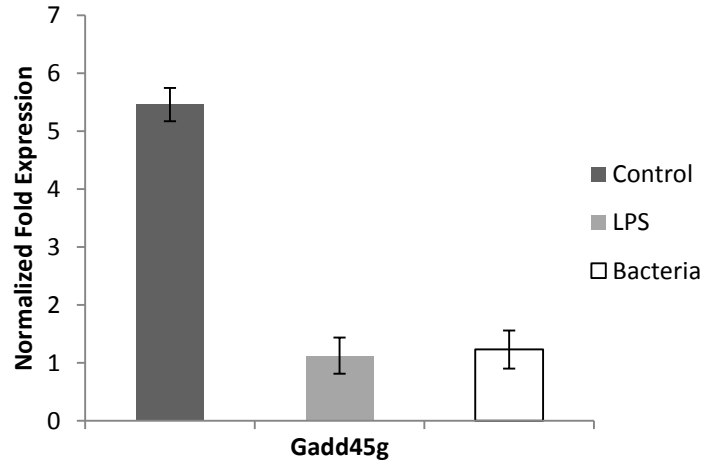
Figure 2.10. Exposure to LPS and Bacteria alters mRNA levels in mouse livers. A. Clustering of differential expression of genes with the use of control as a standard. Red denotes high expression levels, whereas green denotes low expression levels. B. RTPCR gene expression analysis of i) Dusp1, ii) Alas1, iii) Tff3, iv) Esm1, v) Mmd2, vi) Gsta1, vii) Cyp7A1, viii) Gadd45g. Bars show normalized expression levels (with SD) of aforementioned genes in control and two exposed groups, whole heat-killed bacteria and LPS groups. Normalization was conducted with Actin transcription levels.

Chapter 3. Genomic instability in spleen cells is caused by an LPS-induced bystander-like effect

Abstract

The possibility that bacterial components such as LPS could induce genomic instability in distal tissues of the mouse was analysed. Four week old male mice were provided water supplemented with whole heat-killed *E. coli* O157:H7 bacteria or components of the bacteria (DNA, RNA, proteins and LPS). Protein analysis of spleen tissue identified increased PCNA levels and the levels of DNA methyltransferases and DNA repair proteins. Spleen cells responded to exposure of whole heat-killed bacteria and LPS with alteration in the level of PCNA proteins, DNA methylation proteins (MecP2, Dnmt1, Dnmt3a and 3b) and DNA repair proteins (Ape1 and Ku70). Other bacterial components analysed in this study did not lead to any significant alteration in protein expression. The data suggests that lipopolysaccharides are a bacterial component capable of inducing genomic instability in the naïve cells of an exposed host.

3.1. Introduction

Worldwide, it is estimated that between 34 and 76 million people will perish from water-related diseases by 2020 through the ingestion of contaminated water with bacteria such as *E. coli* O157:H7 (Gleick, 2002). Furthermore, epidemiological analysis has identified a correlation between infection and the onset of cancer (Lax and Thomas, 2002). Bacterial based inflammation has been identified to be a preventable cause to many forms of malignancies worldwide, but mechanisms responsible for oncotransfromation have yet to be identified. Researchers have attributed the occurrence of water contamination in rural areas to the presence of a large number of livestock farms (Gannon *et al.,* 2004). Boiling contaminated water kills the bacteria and prevents infections, but the bacterial remnants such as proteins and LPS remain intact.

Even though a link between cancer induction and bacterial infection exists (Suerbaum & Michetti, 2002), it is unclear how heat-killed bacteria can induce genome instability in cells. LPS, one of the most studied and well recognized pathogen associated molecular patterns (PAMPs) and a major component of the outer membrane of Gram-negative bacteria. In this thesis, it is hypothesized to be a component of Gram negative bacteria that can induce genome instability. It is predicted that LPS bind to LPS Binding Protein (LPB), located on the exterior of eukaryotic cell membranes (Doe *et al.,* 1978). The binding of LPS stimulates TLR4 pathways, inducing release of inflammatory cytokines that are required to induce an immune response (Hsu *et al.,* 2011). LPS induced pathways and reactions have been identified, but precisely which cells are directly and indirectly stimulated by LPS *in vivo* is still being perused (Doe *et al.,* 1978).

Upon encounter with macrophages, LPS initiates a cascade of events resulting in release of inflammatory mediators and tissue factors (Wang and Yadav, 2006).

In vivo, LPS injected intravenously or intraperitionealy, have demonstrated a cellular response within spleen tissue and cause the migration of splenic neutrophils (Kesteman *et al.,* 2008). The spleen, the largest lymphoid organ in human body is rich in immune cells, is sensitive to foreign signals, and is involved in the body's response to defend and destroy harmful agents and pathogens (de Porto *et al.,* 2010). Research has reported that when the spleen is not directly exposed to heat-killed bacteria (Koturbash *et al.,* 2009), its cells are still stimulated to response from cells through the phenomena identified as the bystander effect. This research did not identify which component of the Gram-negative bacteria caused the response. It is hypothesized that exposure to heat-killed bacterial components can cause genomic instability in cells and that cells do not require direct contact with components of the pathogen. It is further hypothesized that LPS can be the molecules that lead to a bystander-like response. LPS is thought to trigger a response resulting in activation of various cell types and the production of multiple cytokines in the host (Yadav *et al.,* 2006).

The purpose of this study was to analyze the influence of whole heat-killed bacteria or bacterial components, DNA, RNA, protein or LPS on an indirect target organ (spleen) of mice after a two week exposure to contaminated water.

3.2. Method

Please refer to Chapter 2 for a complete explanation of the methodology. For these experiments, analysis was conducted on spleen tissues from our murine samples.

3.3. Results

To determine whether or not exposure to heat-killed bacteria or its components (DNA, RNA, protein or LPS) can trigger changes in genome instability and cell proliferation in spleen tissue not exposed to the determinants but induced by the bystander-like effect, mice were supplied with drinking water supplemented with heat killed pathogenic *Escherichia coli* (O157:H7) in the amount of 6×10^6 bacteria per litre of water, or 440 µg/L of DNA, 73 µg/L of RNA, 100 µg/L of complete protein extracts or 50 µg/L of LPS rich extracts for a two week duration of exposure. Based on the supplement received, the mice were divided into six main groups. Each experimental group was split into two subgroups: half of all animals were sacrificed two weeks after exposure, whereas the other half was kept an additional two weeks during which they received tap water. This was done to observe if any of the animals would return to a normal homeostatic state after two weeks of consumption of normal water.

Spleen tissue was selected for analysis based on its ability to respond to an immune response to bacterial infection (Kesteman *et al.,* 2008). The spleen is not in direct contact with the pathogenic bacteria and/or its components, but it has been suspected that the spleen function (filtration of the blood) may be induced by a distant signal through signals transferred through the blood stream (Koturbash *et al.,* 2007).

3.3.1 Exposure to LPS from heat-killed bacteria leads to an increased PCNA levels in the spleen tissue.

PCNA is a common protein used to identify the cellular process of proliferation. Primarily, it is a co-factor to DNA polymerase, assisting in the replication of the DNA of a cell, but it is also known as a protein associated with the process of post-replication repair (PRR), in response to DNA damage (Lehmann and Fuchs 2006). Our analysis showed no significant change in PCNA levels two weeks after exposure to water spiked with various bacterial extracts (Figure 3.0). In contrast, analysis of spleen tissue after two weeks of recovery (the four week group) identified a significant increase in the expression of the PCNA protein in the spleen tissue of mice exposed to LPS (1.91 fold increase) and whole heat-killed bacteria (1.85 fold increase) (Table 3.0). Exposure to other components did not result in any significant change (Figure 3.0).

Immunofluorescence analysis of the spleen tissue supported Western Blot data, identifying a significant increase in the LPS and bacterial exposure compared to the control and other exposure groups for the four week test groups (Figure 3.1). Exposure to LPS and heat killed bacteria indicated a significant increase in expression of PCNA protein, compared to the control and the other test groups. No significant differences were observed for the two week groups (Table 3.1).

3.3.2. Exposure to LPS from heat-killed bacteria leads to an increase in the level of Ape1 and Ku70 proteins in spleen tissue.

Protein analysis of specific DNA damage repair proteins, was targeted to identify the effects of determinants on genomic stability of spleen cells. Initial Ape1 analysis identified a significant increase in expression for the exposure groups of LPS and whole

heat-killed bacteria compared to the control group (Figure 3.2). The two week samples yielded significant increases in expression of Ape1 in the exposure group of the heat-killed bacteria (1.09 fold increase) and the test group exposed to LPS (1.11 fold increase). The four week samples exposed to LPS and whole heat killed bacteria resulted in a significant increase in Ape1 expression with values of 1.21 and 1.20 fold, respectively (Table 3.2).

Analysis of Ku70 protein expression was conducted and indicated a significant increase in the amount of Ku70 protein for the test groups exposed to LPS (1.14 fold increase) and whole bacteria (1.11 fold increase) for the two week exposure test. This increase continued during the four week test for LPS (1.28 fold increase) and for the whole heat-killed bacterial test group (1.28 fold increase) (Table 3.3). Other exposure groups did not show any significant alterations to the expression of the Ku70 protein (Figure 3.3).

3.3.3. Exposure to LPS from heat-killed bacteria leads to an increase in protein expression involved in maintenance and de novo methylation in spleen tissues.

To test whether exposure to bacterial components resulted in changes to the level of proteins involved in DNA methylation, protein levels of the DNA methyltransferases Dnmt1, Dnmt3a and Dnmt3b and a protein that binds to methylated DNA, MeCP2 were analysed. Dnmt1 showed significant alterations in the exposure groups for the protein, LPS and whole heat-killed bacteria. Protein exposure from the heat-killed bacteria caused a 1.25 fold increase in Dnmt1 level at two weeks exposure. LPS and bacterial exposure resulted in significant alterations in Dnmt1 levels in the two week exposure group; there was a1.65 fold and 1.64 fold increase upon exposure to LPS and whole heat-killed

bacteria, respectively (Figure 3.4). Exposure to DNA and RNA did not yield significant expression alterations compared to the control. Dnmt1 was not changed in any experimental groups of the four week group (Table 3.4).

MeCP2 levels were increased significantly upon a two week exposure to RNA (1.15 fold increase), proteins (1.41 fold increase), LPS (1.5 fold increase) and to whole heat-killed bacteria (1.54 fold increase). There was a significant increase in expression of MecP2 upon exposure to proteins (1.27 fold increase), LPS (1.28 fold increase) and whole bacteria (1.24 fold increase), but no significant expression was identified for the group exposed to RNA from the heat-killed bacteria for our four week test groups (Figure 3.5). Exposure to DNA did not result in any significant changes to the spleen cells' expression of MeCP2 in either two or four week test groups (Table 3.5).

To analyze whether exposure to bacterial determinants changed the expression of *de novo* methyltransferases, the protein levels of Dnmt3A and Dnmt3B were analyzed. Dnmt3A protein production was identified as significantly increased in the exposure group of the LPS with a 1.29 fold increase and whole heat-killed bacterial exposure group with a 1.34 fold increase (Figure 3.6, Table 3.6). Dnmt3B expression analysis identified an increase in expression during the two week time period for the groups exposed to LPS and whole heat-killed bacteria. LPS exposure increased the expression of the Dnmt3A protein in the spleen tissue by 1.27 fold, while whole heat-killed bacteria exposure resulted in a significant increase of 1.48 fold (Figure 3.7). Exposure to DNA, RNA and protein determinants did not result in altered expression of *de novo* methyltransferase proteins in the two week samples. All levels of expression for Dnmt3A

and B returned to levels comparable to the control group for the four week test groups (Table 3.7).

3.4. Discussion

Alternating protein expression within the spleen, by exposure of distal cells to LPS is further support for the bystander-like phenomena. Previous reports showed that heat-killed bacteria had the ability to cause an effect on distal naive cells to lose genomic stability (Koturbash *et al.,* 2009). This work also identified that the causative agent was apparently smaller than whole heat-killed bacteria since filtered water contaminated with bacteria still resulted in changes in genome stability (Koturbash *et al.,* 2009). The work reported here has identified that a major component of the Gram-negative cell membrane of *E. coli* O157:H7, LPS induced significant alterations within the spleen cells originally distant from the exposure.

Cellular DNA is constantly exposed to a wide spectrum of exogenous and endogenous factors; therefore a variety of DNA repair pathways have evolved, protecting cells from DNA damage (Lodish *et al.,* 2004). Ape1 is abundant in human cells and accounts for nearly all of the basic site DNA repair activity (Wang *et al.,* 2004). The results indicate that LPS is a component of the bacterium that could possibly activate Ape1 expression within distal cells in a manner similar to exposure to whole heat-killed bacteria. Ape1 not only acts as a repair protein, but also as a protein that participates in the response to oxidative stress, regulation of transcription factors, cell cycle control, and apoptosis (Evans *et al.,* 2000). LPS, is a major source for ROS and RNOS that could induce damage of DNA in cells (Sanlioglu *et al.,* 2001). Ape1 was independently

determined to be an oxidation reduction factor and given the alternative name of Ref-1 (Xanthoudakis *et al.,* 1992). Increases in expression of this protein are associated with increased exposure to radical oxygen species and a precursor of cancer cell formation (Xanthoudakis *et al.,* 1992).

Changes in the level of Ku70 protein animals treated with LPS support the hypothesis that LPS is one of the major components from Gram-negative bacteria that induces DNA damage (genomic instability) within distal cells of the spleen. The key element of the NHEJ pathway is the Ku70–Ku80 heterodimer, which binds to DNA double strand damage and recruits DNA protein kinase catalytic subunits, DNA ligase four and XRCC4 intervene in the final ligation step (Valerie *et al*., 2003).This mechanism of DNA repair, however, is non-conservative, because it may lead to erroneous re-joining of broken chromosome ends, causing a loss or amplification of chromosomal material, and even translocation (Khanna and Jackson., 2001).

A ''caretaker'' role has been proposed for the NHEJ pathway, and down regulation of the system was reported in more advanced and metastatic malignancies as compared with benign lesions or less aggressive tumours (Burma *et al.,* 2006). Other studies indicate that NHEJ itself may cause chromosomal rearrangements (Rothkamm *et al.,* 2001). This activation may be a response to failure of other repair mechanisms, such as nucleotide excision repair that plays such an important part in defending the genome from instability and loss of genomic material. On the other hand, the increase in Ku70 protein expression may be related to high proliferation rates that enhance the likelihood of DNA breaks requiring a more reactive DNA repair system. Data indicates that a

substantial up regulation of the key element of the NHEJ pathway is mainly correlated with tumour cell proliferation rate. As the NHEJ system is an error-prone mechanism (Gaymes *et al.,* 2002), this up regulation could increase the genotoxic effect from bacterial infections.

PCNA expression levels in the two week group was comparable to the control, but became significantly higher after the additional two week exposure to uncontaminated tap water. Increased PCNA expression could be associated with an increase in proliferation or an increase in DNA damage. In the murine cultured cells, LPS has been identified to cause an increase in proliferation of exposed spleen cells (Tough *et al.,* 1997). The increase in proliferation may perpetuate genome instability with an increase in the number of cells with altered genome expression or damage, increasing the potential for the development of tumourigenesis.

DNA methylation has been found to influence a variety of processes that affect DNA integrity and function. Genomic expression variations could be altered with epigenetic manipulation from the effects of methylation on the genome. It is possible that hypermethylation of tumour-suppressor genes, leading to gene inactivation, results in a selective growth advantage of the transformed cells (Laird and Jaenisch, 1994). In this experiment, maintenance methylation and *de novo* methylation associated proteins were identified to be overexpressed for the LPS group and bacterial exposure group compared to the control group.

Changes in the level of proteins involved in proliferation, replication, DNA repair and DNA methylation could be a result of the increase of cells producing similar amounts

of proteins, identified by signs of increased proliferation. It can also be suggested that

exposure of the intestine to whole heat-killed bacteria or LPS and probably to some

degree, to protein and RNA may trigger genomic instability in non-exposed spleen cells.

It can be further hypothesized that such changes may contribute to malignancy. Immune

responses have been associated with alterations in methylation by the recognition of CpG

dinucleotide lacking DNA methylation as foreign. Aberrant levels of DNA methylation

have the possibility of activating T-cells auto-reactivity and immunity (Teitell and

Richardson, 2003).
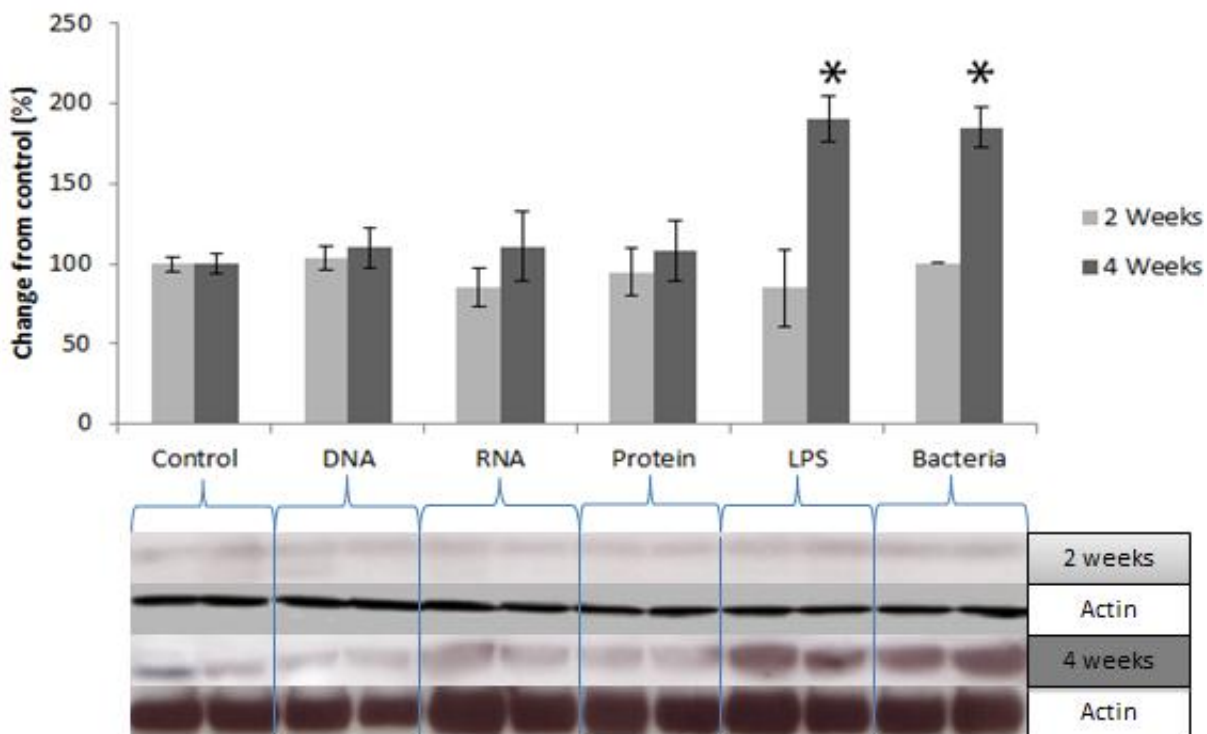
Figure 3.0. Protein expression analysis of PCNA from spleen tissue exposed to DNA, RNA, protein, LPS rich solution or whole heat-killed bacteria. Protein expression variations from mouse models representing each test group by Western Blot analysis Bars represent average expression of the protein (with SD) detected compared to the control set at 100%. Asterisks show significant increase from non-exposed controls through the analysis of data using one way ANOVA test

Table 3.0. Western blot analysis of PCNA quantified with Image J. program. Average±SD represents the average protein expression±standard deviation, compared to the control test. Asterisks identify significant increases from non-exposed controls.

Test	2 Week Average ± SD	4 Week Average ± SD
Control	100.0±4.6	100.0±6.5
DNA	103.8±7.6	110.3±12.5
RNA	85.2±11.9	110.9±21.8
Protein	94.6±14.8	108.2±18.5
LPS	98.0±24.0	*190.9±14.2
Bacteria	100.5±0.2	*185.3±13.1

A.
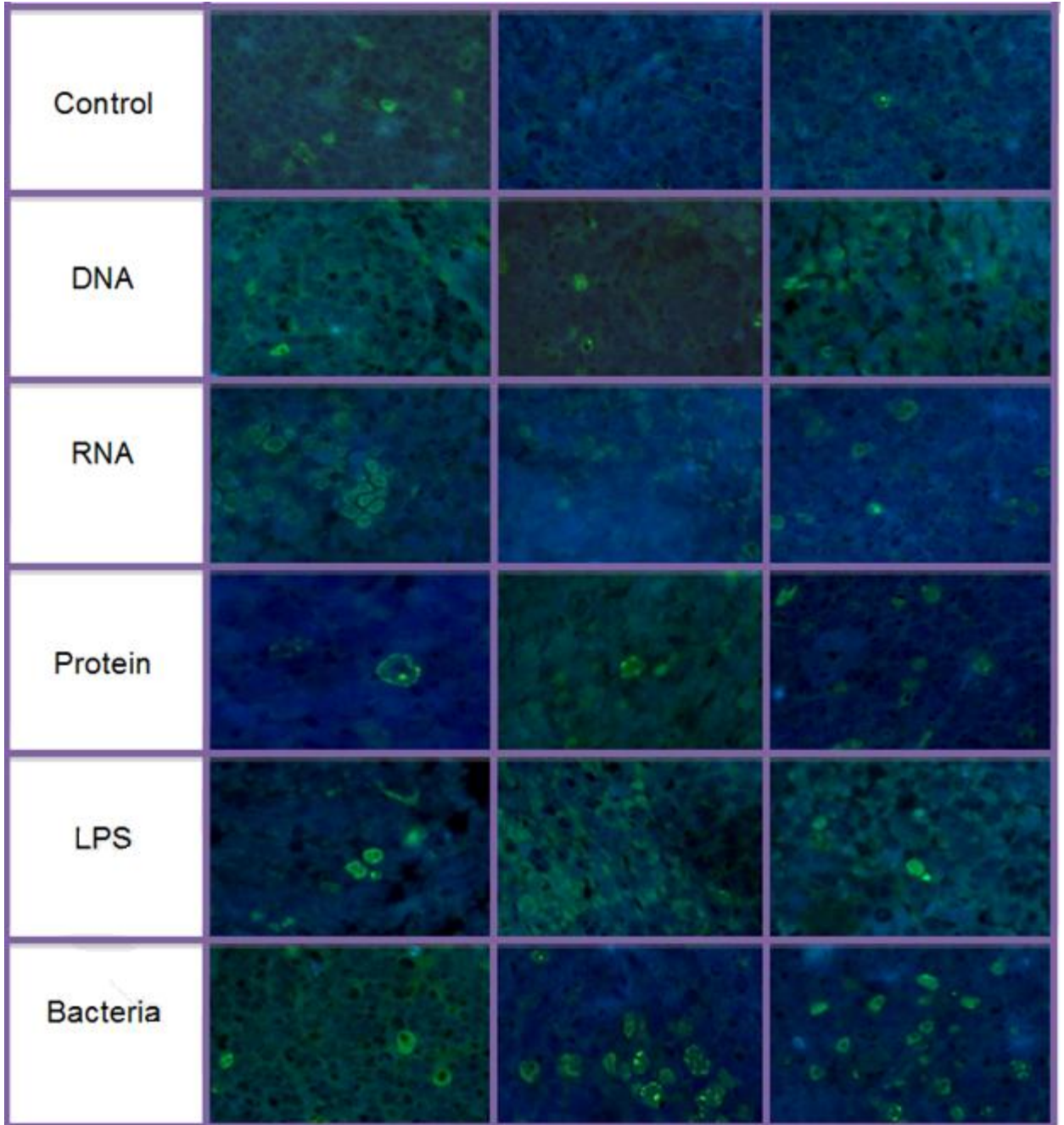
B.

C.
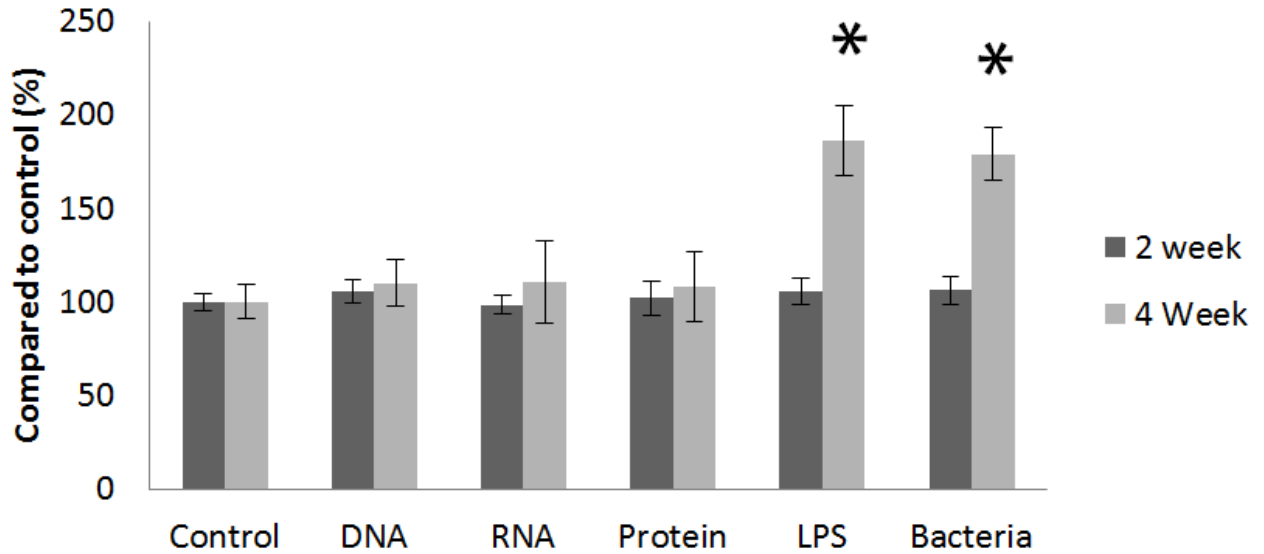
Figure 3.1. Immunohistochemical analysis of spleen tissue samples stained with DAPI and Green Fluorescent antibody for PCNA. A, Spleen samples taken from individuals within test Group A. **B,** Spleen samples taken from individuals within test Group B. is quantified data from images. **C,** Bars represent PCNA expression detected in nucleus of cells. "Y" axis shows the average (with SD) number of PCNA-positive cells. Asterisks show significant difference from control.

Table 3.1. Imunohistochemical analysis of PCNA protein ecpression quantified. Average±SD represents the average protein expression±standard deviation, compared to the control test detected via analysis of images. Asterisks identify significant increases from non-exposed controls.

Test	2 Week Average ± SD	4 Week Average ± SD
Control	100.0±4.8	100.0±9.1
DNA	105.8±6.4	109.2±12.3
RNA	98.6±4.7	114.6±23.6
Protein	102.1±9.3	111.5±19.6
LPS	105.6±6.9	*191.3±18.9
Bacteria	106.2±7.2	*184.0±9.6
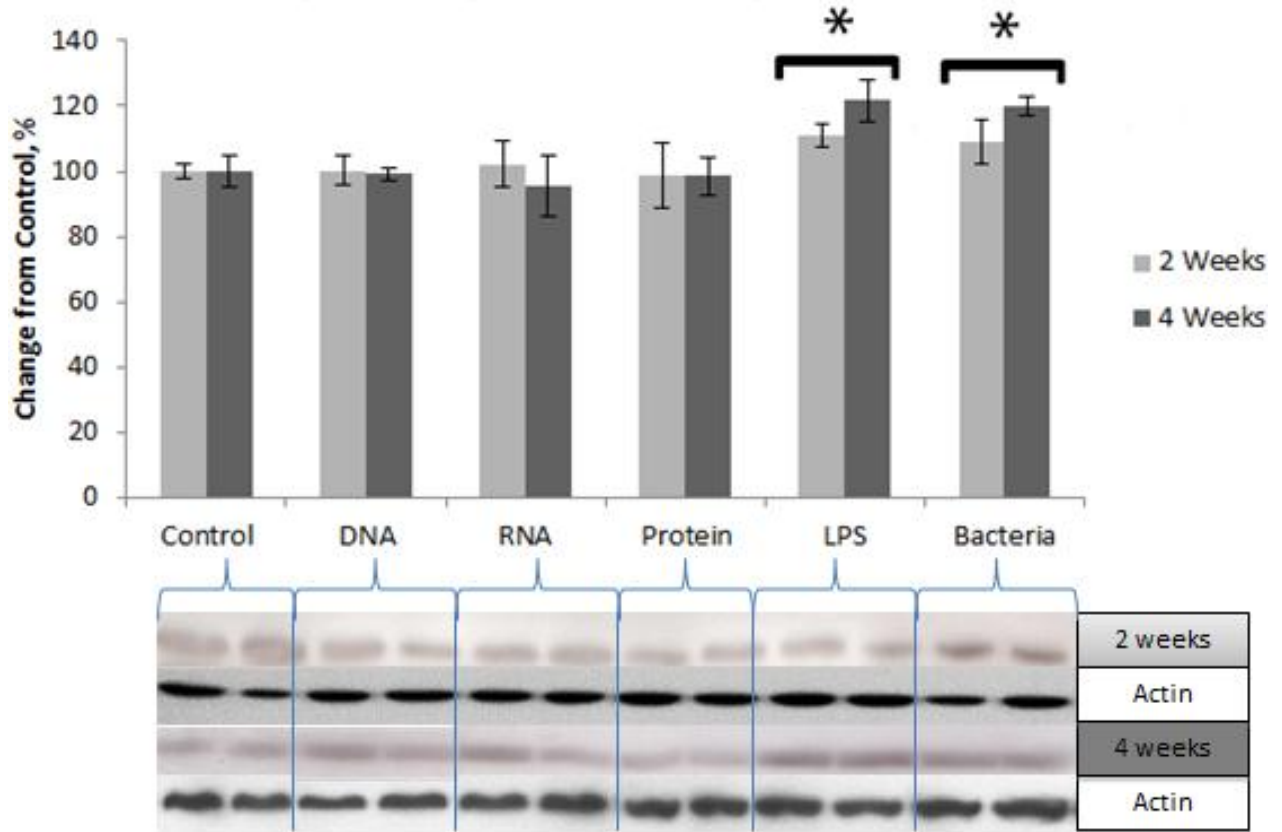
Figure 3.2. Protein expression analysis of Ape1 from spleen tissue samples for each group. Protein expression variations from mouse models representing each test group by Western Blot analysis, Bars show average (with SD) expression of the protein detected compared to the control set at 100%. Asterisks show significant increase from non-exposed controls through the analysis of data using one way ANOVA test

Table 3.2. Western blot analysis of Ape1 quantified with Image J. program. Average±SD represents the average protein expression±standard deviation, compared to the control test. Asterisks identify significant increases from non-exposed controls.

Test	2 Week Average ± SD	4 Week Average ± SD
Control	100.0±2.2	100.0±4.6
DNA	100.3±4.5	99.2±1.9
RNA	102.3±6.9	95.7±9.3
Protein	98.8±10.1	98.5±5.7
LPS	*111.0±3.4	*121.7±6.5
Bacteria	*109.2±6.7	*120.1±2.9
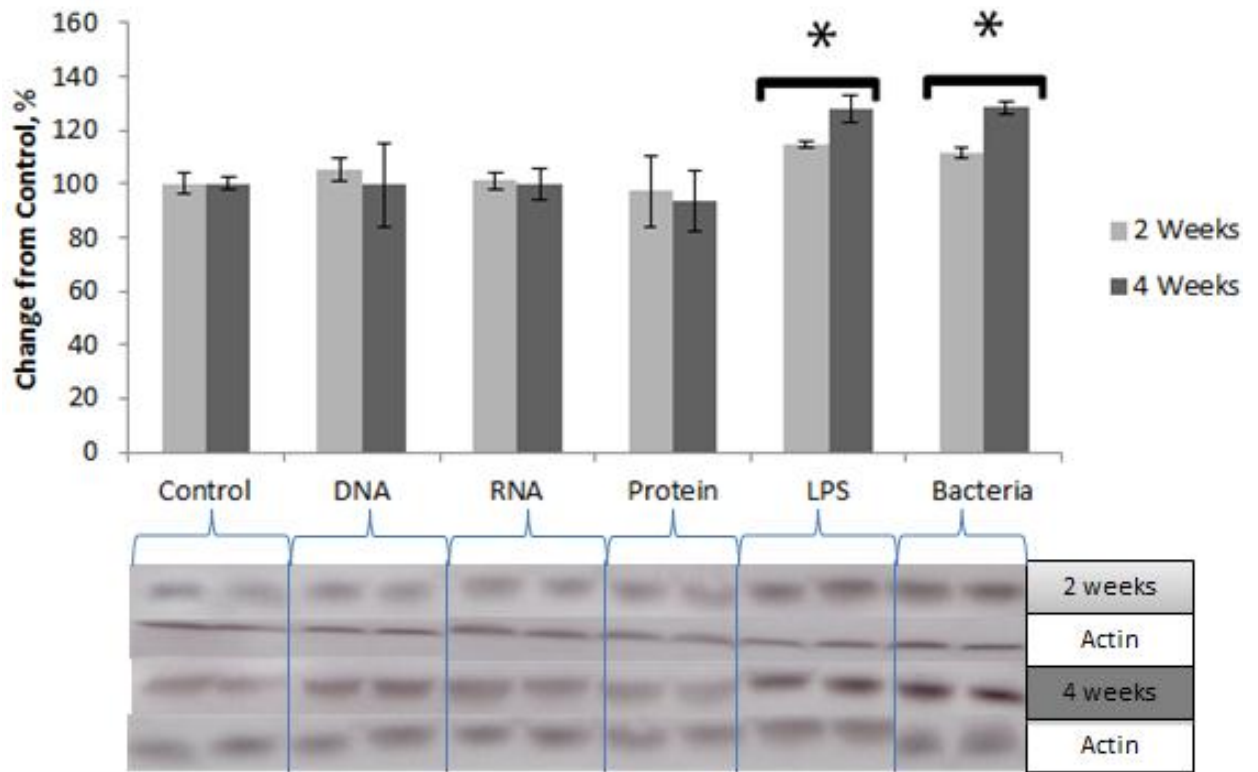
Figure 3.3. Protein expression analysis of Ku70 from spleen tissue samples for each group. Protein expression variations from mouse models representing each test group by Western Blot analysis. Bars show average (with SD) expression of the protein detected compared to the control set at 100%. Asterisks show significant increase from non-exposed controls through the analysis of data using one way ANOVA test
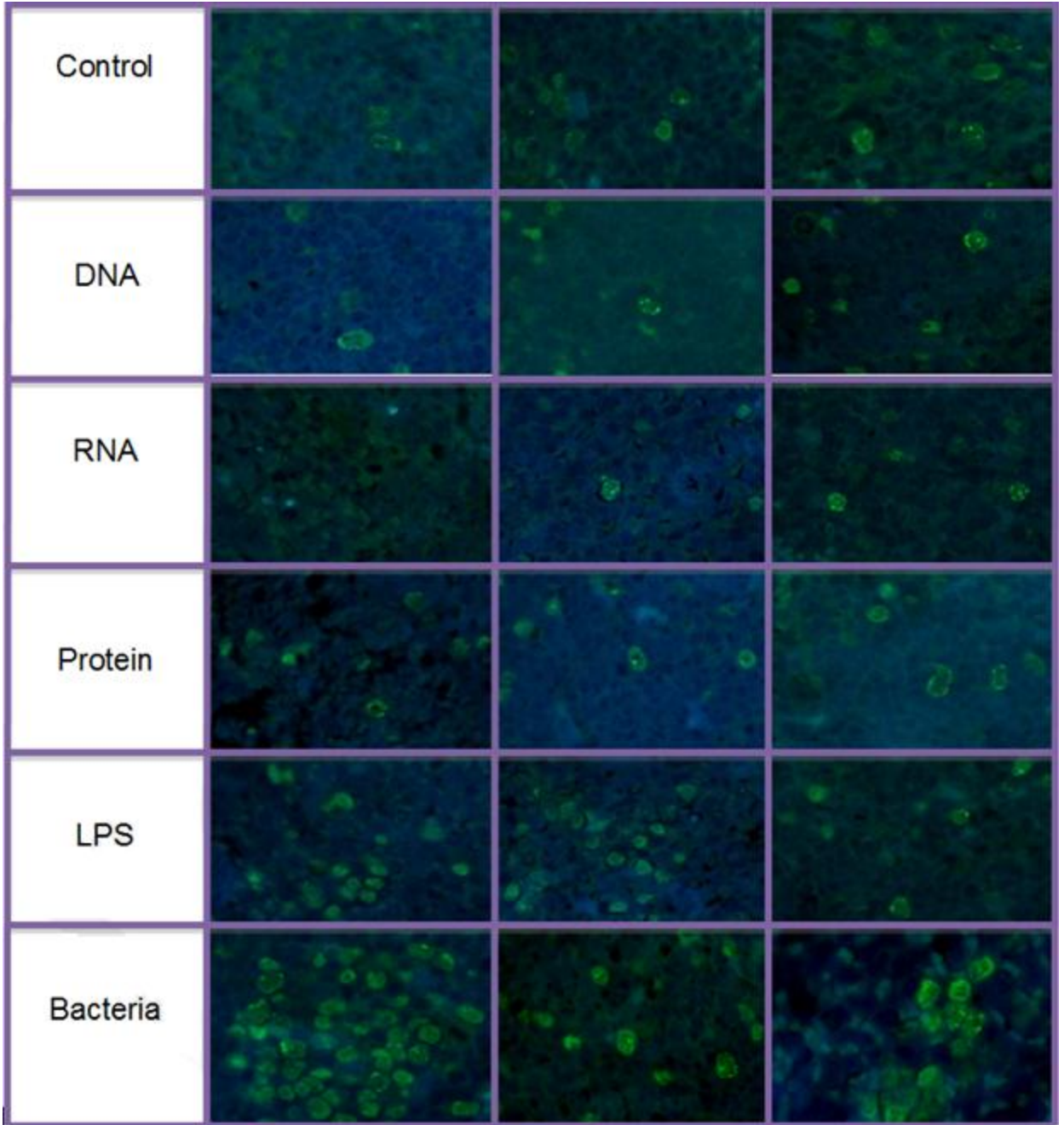
Table 3.3. Western blot analysis of Ku70 quantified with Image J. program. Average±SD represents the average protein expression±standard deviation, compared to the control test. Asterisks identify significant increases from non-exposed controls.

Test	2 Week Average ± SD	4 Week Average ± SD
Control	100.0±3.9	100.0±2.3
DNA	105.3±4.5	99.7±15.5
RNA	101.2±2.9	100.3±5.91
Protein	97.4±13.2	93.5±11.3
LPS	*114.7±1.1	*128.0±5.32
Bacteria	*111.8±2.2	*128.6±2.3
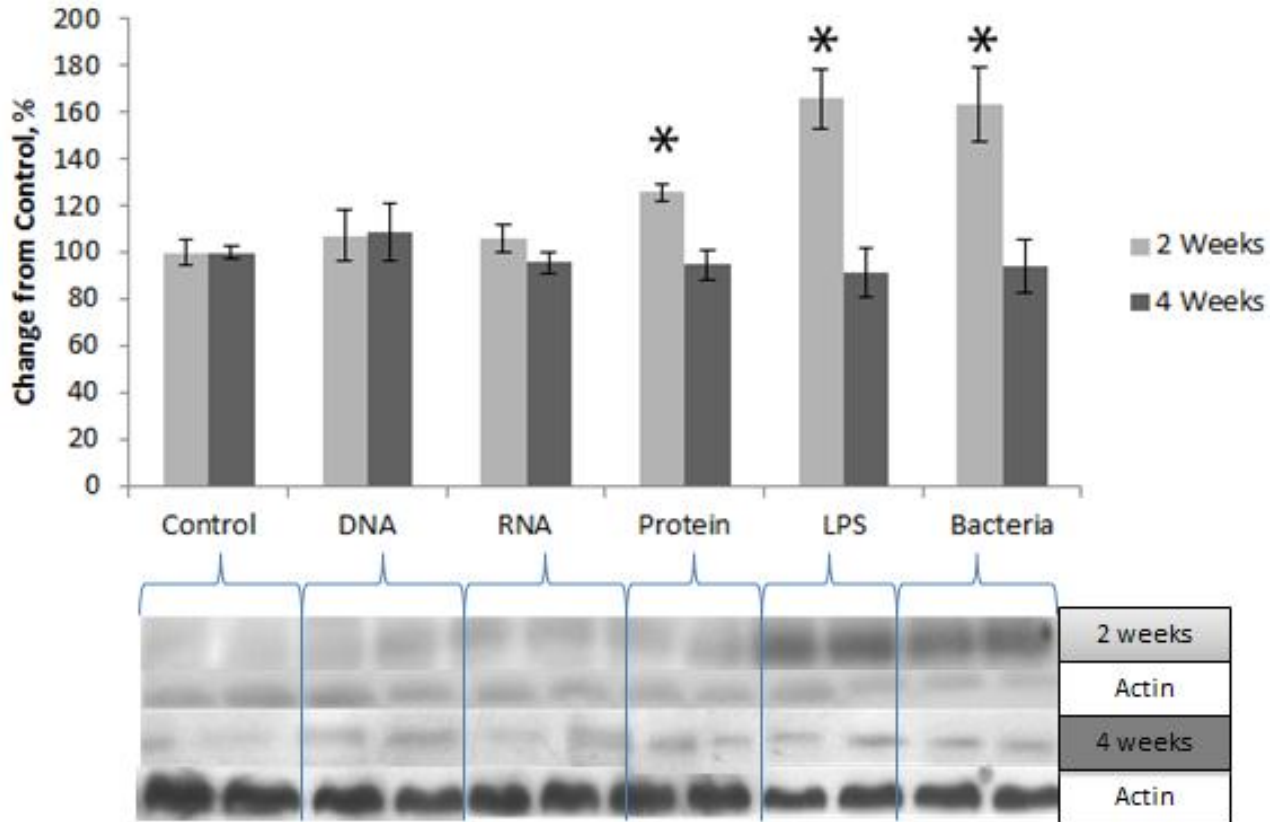
Figure 3.4. Protein expression analysis of Dnmt1 from spleen tissue samples for each group. Protein expression variations from mouse models representing each test group by Western Blot analysis. Bars show average (with SD) expression of the protein detected compared to the control set at 100%. Asterisks show significant increase from non-exposed controls through the analysis of data using one way ANOVA test

Table 3.4. Western blot analysis of Dnmt1 quantified with Image J. program. Average±SD represents the average protein expression±standard deviation, compared to the control test. Asterisks identify significant increases from non-exposed controls.

| Test	2 Week Average ± SD	4 Week Average ± SD
Control	100.0±5.3	100.0±2.5
DNA	107.5±10.6	108.9±12.4
RNA	105.9±6.0	95.8±4.4
Protein	*125.8±3.51	94.8±6.5
LPS	*165.9±12.7	91.8±10.5
Bacteria	*163.5±16.3	94.5±11.2
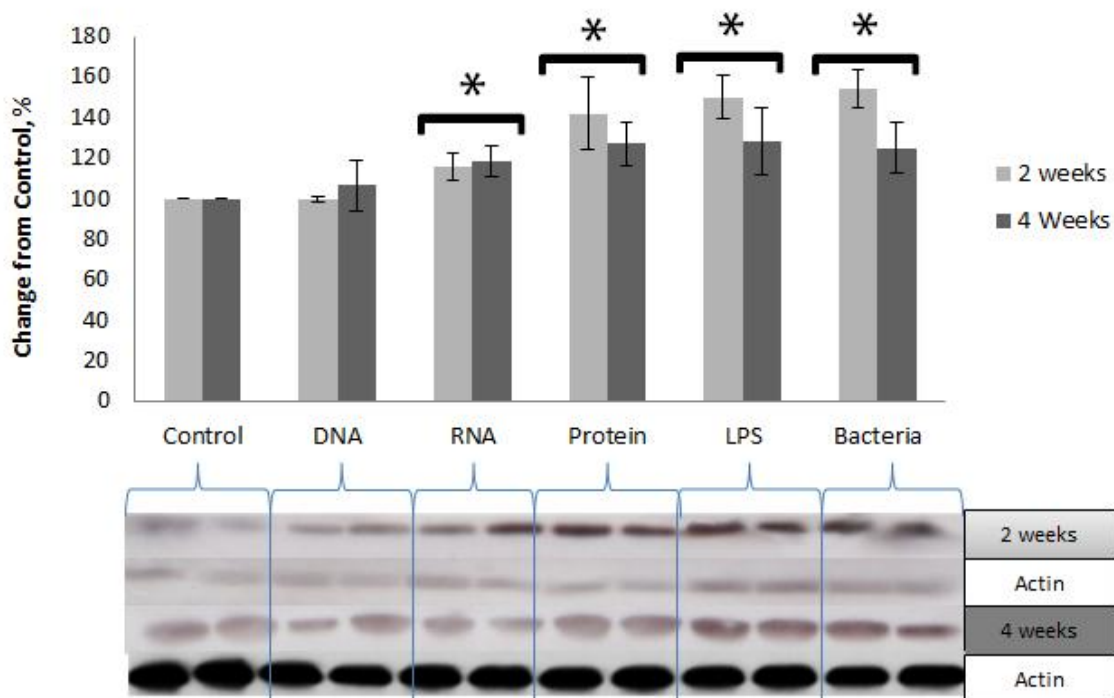
Figure 3.5. Protein expression analysis of MecP2 from spleen tissue samples for each group. Protein expression variations from mouse models representing each test group by Western Blot analysis. Bars show average (with SD) expression of the protein detected compared to the control set at 100%. Asterisks show significant increase from non-exposed controls through the analysis of data using one way ANOVA test

Table 3.5. Western blot analysis of MecP2 quantified with Image J. program. Average±SD represents the average protein expression±standard deviation, compared to the control test. Asterisks identify significant increases from non-exposed controls.

| Test	2 Week Average ± SD	4 Week Average ± SD
Control	100.0±2.6	100.0±2.2
DNA	99.5±1.4	106.4±12.5
RNA	*115.7±12.3	*118.1±10.2
Protein	*141.9±18.0	*127.3±10.7
LPS	*150.0±10.4	*128.5±19.2
Bacteria	*154.4±2.3	*124.8±12.6
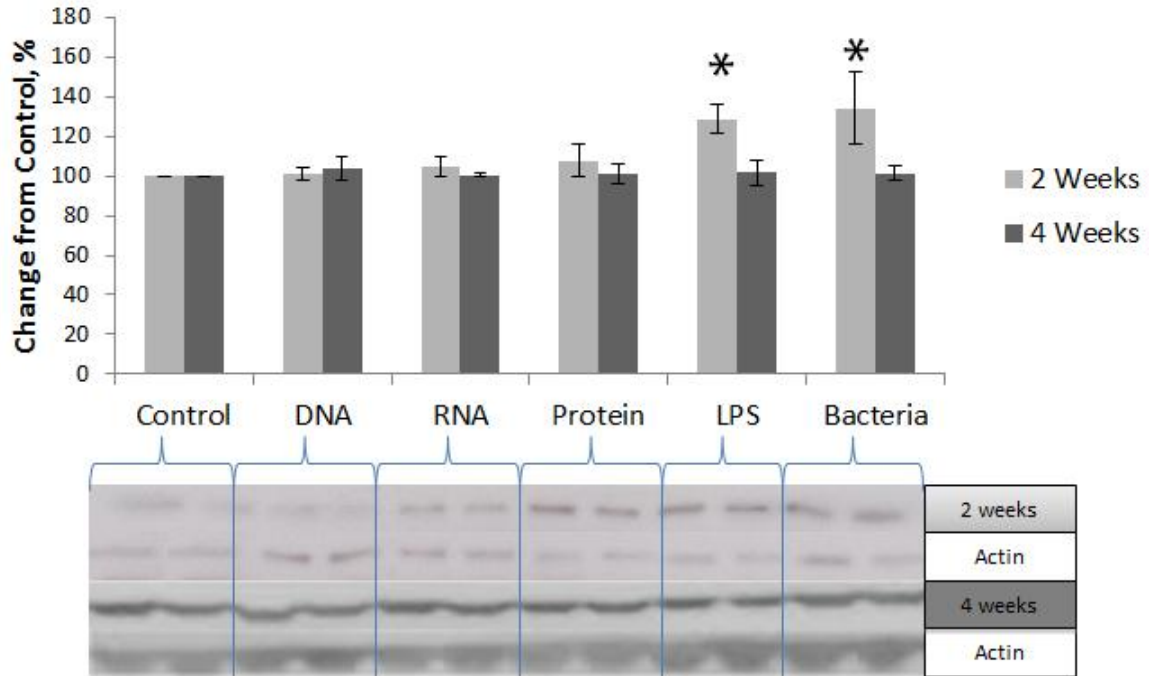
Figure 3.6. Protein expression analysis of Dnmt3A from spleen tissue samples for each group. Protein expression variations from mouse models representing each test group by Western Blot analysis. Bars show average (with SD) expression of the protein detected compared to the control set at 100%. Asterisks show significant increase from non-exposed controls through the analysis of data using one way ANOVA test

Table 3.6. Western blot analysis of Dnmt3A quantified with Image J. program. Average±SD represents the average protein expression±standard deviation, compared to the control test. Asterisks identify significant increases from non-exposed controls.

| Test	2 Week Average ± SD	4 Week Average ± SD
Control	100.0±1.3	100.0±4.3
DNA	101.2±2.9	103.6±6.1
RNA	104.8±4.8	100.5±1.1
Protein	107.8±8.4	101.3±5.1
LPS	*128.7±7.4	101.7±6.4
Bacteria	*134.1±18.4	101.3±3.5
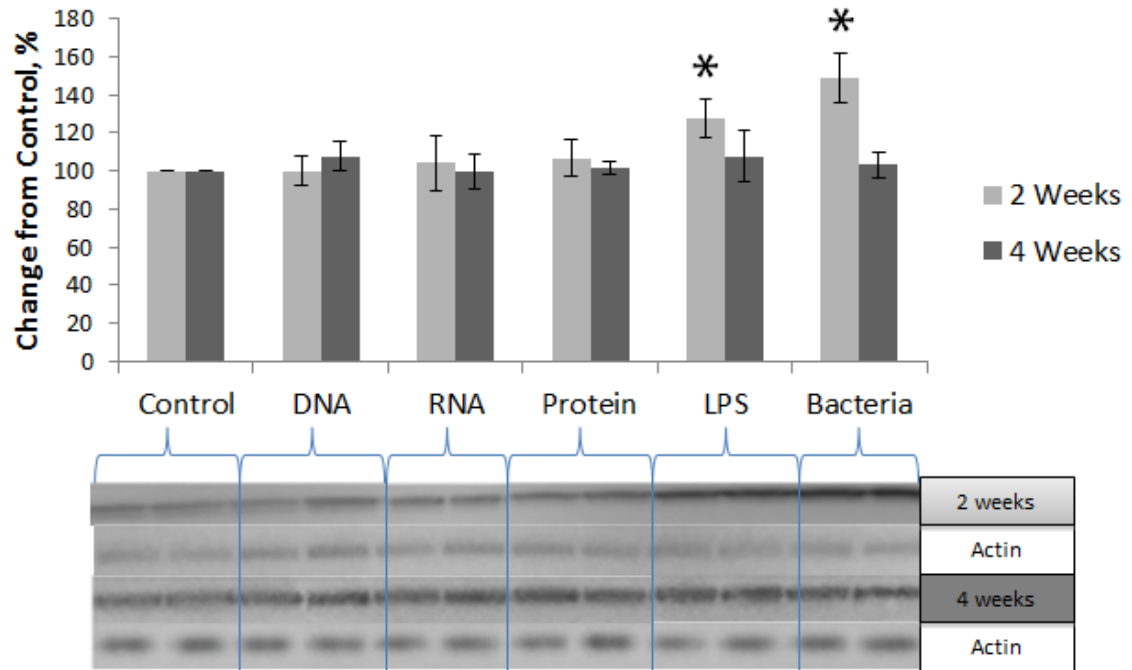
Figure 3.7. Protein expression analysis of Dnmt3B from spleen tissue samples for each group. Protein expression variations from mouse models representing each test group by Western Blot analysis. Bars show average (with SD) expression of the protein detected compared to the control set at 100%. Asterisks show significant increase from non-exposed controls through the analysis of data using one way ANOVA test

Table 3.7. Western blot analysis of Dnmt3B quantified with Image J. program. Average±SD represents the average protein expression±standard deviation, compared to the control test. Asterisks identify significant increases from non-exposed controls.

Test	2 Week Average ± SD	4 Week Average ± SD
Control	100.0±2.1	100.0±4.8
DNA	106.4±6.2	106.2±7.1
RNA	96.5±0.2	97.8±4.7
Protein	103.2±8.8	102.7±7.4
LPS	125.8±4.9	104.8±11.1
Bacteria	157.8±6.3	97.7±11.5

Chapter 4. Effects of LPS on direct exposure to intestinal tissue via water contamination

Abstract

The gastrointestinal mucosa is in contact with all ingested liquids and is in direct contact with external antigens on a daily basis. Bacteria inducing inflammation of the intestinal tissue are known to induce an immune response (either local or systemic). Exposure to an LPS rich solution from the membrane of Gram-negative bacteria induces an inflammatory response and an increase in PCNA expression within multiple cell lines of intestinal tissue.

4.1. Introduction

Rural areas containing a large number of livestock facilities have been identified as problematic areas for contamination caused by run off from fields into the water source producing large numbers o f gastrointestinal illnesses (Gannon *et al.,* 2004). One bacterial strain identified as a contaminant in the food/water supply is *Escherichia coli* O157:H7. *E. coli* O157:H7 has been recognized for decades as a pathogen responsible for several disease outbreaks in North American and throughout the world (Karmali *et al.,* 1985; Karmali, 1989) . These bacteria have been identified as a contaminant associated with severe cramping, watery/bloody diarrhea, inflammation of the intestine, Hemolytic-uremic syndrome (HUS), thrombotic thrombocytopenic purpura and potential death (Griffin *et al.,* 1988).

Cases of infections have been identified to be a preventable cause to many forms of malignancies worldwide, but mechanisms responsible for the oncotransformation of the cells have yet to be identified (Lax and Thomas, 2002). In rural Canada, where a large number of livestock farms are located, contamination of surface water has occurred frequently and has propelled the Government of Canada to introduce and impose multiple boil water advisories across the country (Gannon *et al.,* 2004). Boiling of water containing bacterial contaminants results in apparent neutralization of its pathogenic abilities, resulting in clean, safe water for consumption. Nevertheless, recent research has identified that the consumption of bacterial water contaminants can induce genomic instability of naive tissues (Koturbash *et al.,* 2009) possibly by the phenomenon termed the bystander-like effect. Epidemiological analysis of bacterial outbreaks identifies a

possible correlation with infections and onset of gastrointestinal cancer (Falk *et al.,* 2000).

A possible reason for the continuous influence is that the remaining components of the bacteria could induce a response from the tissue. Lipopolysaccharides (LPS) are a major component of the Gram-negative bacterial cell wall and have the capability of surviving a heat-shock treatment that would neutralize the pathogenic bacteria (Gao *et al.,* 2006). It is hypothesized that LPS is a bacterial component that induces genome instability and produces inflammation of the tissue. LPS binds to the receptors on the exterior of eukaryotic cells, stimulating TLR4 pathways, interacting with NF-κB of infected cells. The NF-κB pathway is a protein complex that is involved in transcription of DNA and is known to act in response indigenous cytokines and to viral and bacterial antigens, and stress caused by chemical agents such as free radicals, cytokines and bacterial/viral antigens (Gilmore, 2000). NF-κB plays a key role in regulating the immune response to infection and, incorrect regulation of NF-κB has been linked to the onset of inflammation and in some cases, cancer (Albensi and Mattson, 2000).

The purpose of the study is to analyze alteration in the expression of PCNA protein in the intestine of mice upon direct contact with whole heat-killed bacteria or its components (DNA, RNA, protein and LPS).

4.2. Methods

Please refer to Chapter 2 for a complete explanation of the methodology. For
these experiments, the analysis was conducted on intestinal tissues samples from the mice
experimental groups.

4.3. Results

Intestinal tissue from animals exposed for two weeks to DNA, RNA and protein
extracts from heat-killed bacteria remained unaltered in the structure or expression of
PCNA, compared to the control animals. In contrast to the previous test groups, the LPS
and whole heat-killed bacterial exposure groups showed increased levels of PCNA
protein. A 1.40 and 1.42 fold increase in LPS and whole bacteria groups, respectively
was identified for the two week exposure group. Protein analysis identified a continual
increase in expression of LPS (1.34 fold increase) and whole heat-killed bacteria (1.34
fold increase) for the four week samples. The analysis also identified that the four week
exposure group of animals that consumed proteins from these bacteria also had PCNA
levels significantly increased by 1.33 fold (Figure 4.1, Table 4.0).

4.4. Discussion

The gastrointestinal mucosa is in continuous contact with external antigens from
food and water consumed by an individual. Increasing population and demand on our
resources will increase the requirement to ensure that consumables are pure and
knowledge about contaminants is expanded. Increased mobility and mortality warrants
further research and alteration to preventative measures in order to protect individuals

who consume bacterial contaminated water. Further analysis of the pathogenic effects bacterial agents is required to facilitate treatment of patients as well as to establish preventative treatments.

Ingestion of LPS extracts caused an alteration to the principal structure of cells within the intestinal tissue of mice. Figure 4.0 A and B identify the presence of PCNA; however intestinal tissue is continuously sloughed off, therefore a high level of proliferation is expected within these tissue samples (Bullen *et al.,* 2006). Both small and large intestines are composed of four distinct cell layers: the mucosa, the submucosa, the muscularis externa and the serosa (Lloyd and Gabe, 2007). The mucosa consists of a layer of epithelium, and below lies the lamina propria, a connective tissue layer containing blood vessels, lymphatics, and some lymphoid tissue. Within portions of the intestinal tract, the mucosa forms villi (finger-like structures). Each villus contains a dense capillary network and blood vessels, which drain into lymphatics forming a plexus in the lamina propria (Leedham *et al.,* 2005). These crypts contain intestinal epithelial stem cells, which allow repopulation and repair of the small intestinal mucosa. Scattered throughout the lamina propria and submucosa of the small intestine, predominantly the ileum, are visible aggregates of lymphoid tissue known as Peyer's patches with flattened mucosa overlapping. The lamina propria is separated from the submucosa by a thin inner circular layer and an outer longitudinal layer of smooth muscle known as the muscularis mucosae (Tennyson *et al*., 2005).

In our experiments, intestinal tissue expressed an increase in PCNA detection within the two and four week samples. The intestinal tissue did not recover from the initial two week treatment after a two week recovery period. This delay in recovery

indicates that exposure to *E. coli* (even after heat treatment) induced alterations within the cells in direct contact with whole bacteria or the LPS component of their outer cell membrane. Interesting to note is the fact that even though intestinal tissue is identified as a highly proliferating tissue, there was a substantial increase in PNCA production in the exposed intestinal cells. Exposure to the protein extract from the bacteria resulted in an increase in expression of PCNA, but had no effect on the primary structure of the intestinal cells themselves. It has been hypothesized that these changes could be suppressed by blocking the endotoxin activity using a monoclonal CD14 antibody or a CD18 peptide that could neutralize LPS binding, thus inhibiting leukocyte infiltration into the inflamed tissue (Chan *et al*., 2009).

A.
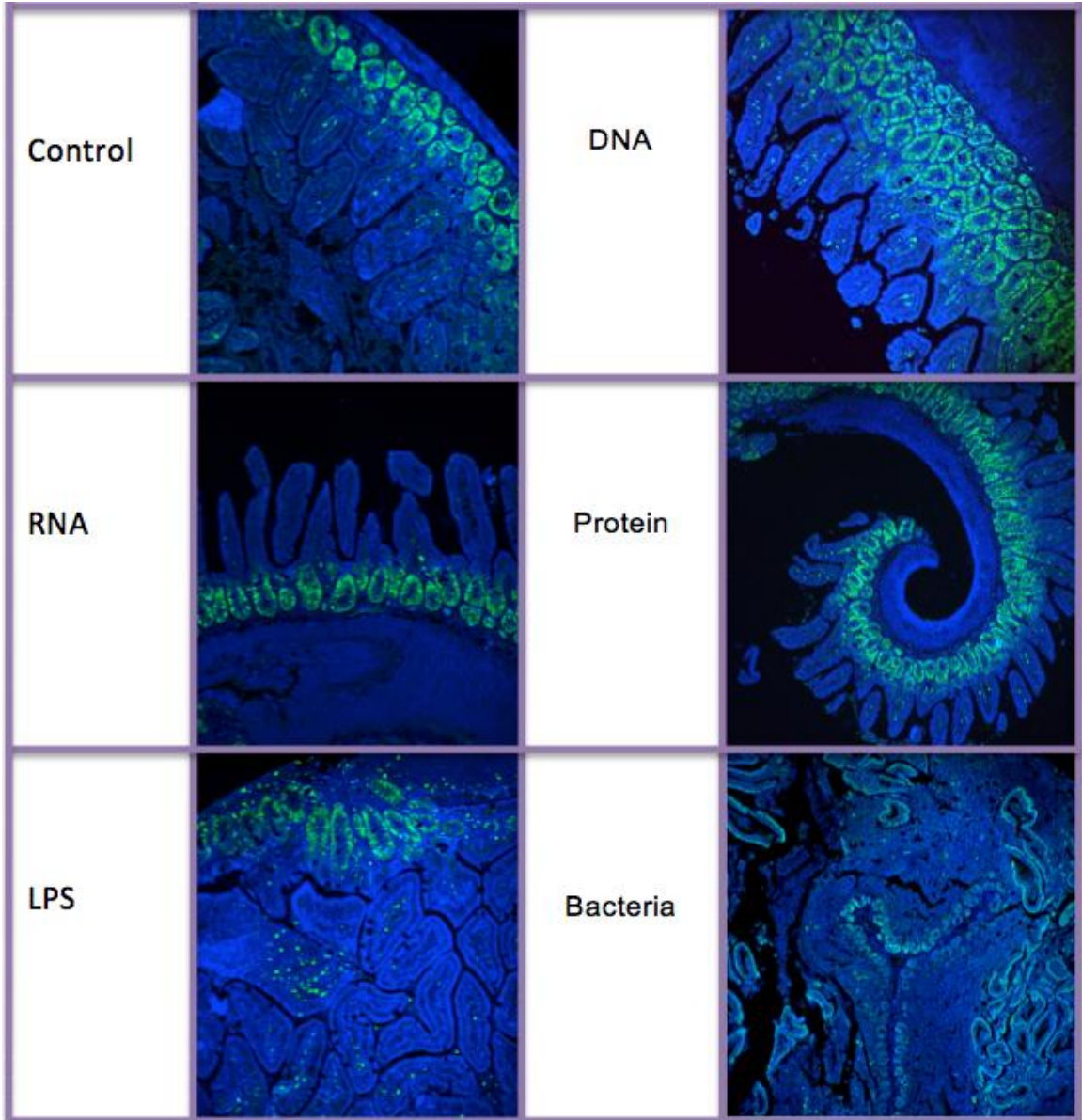
B.
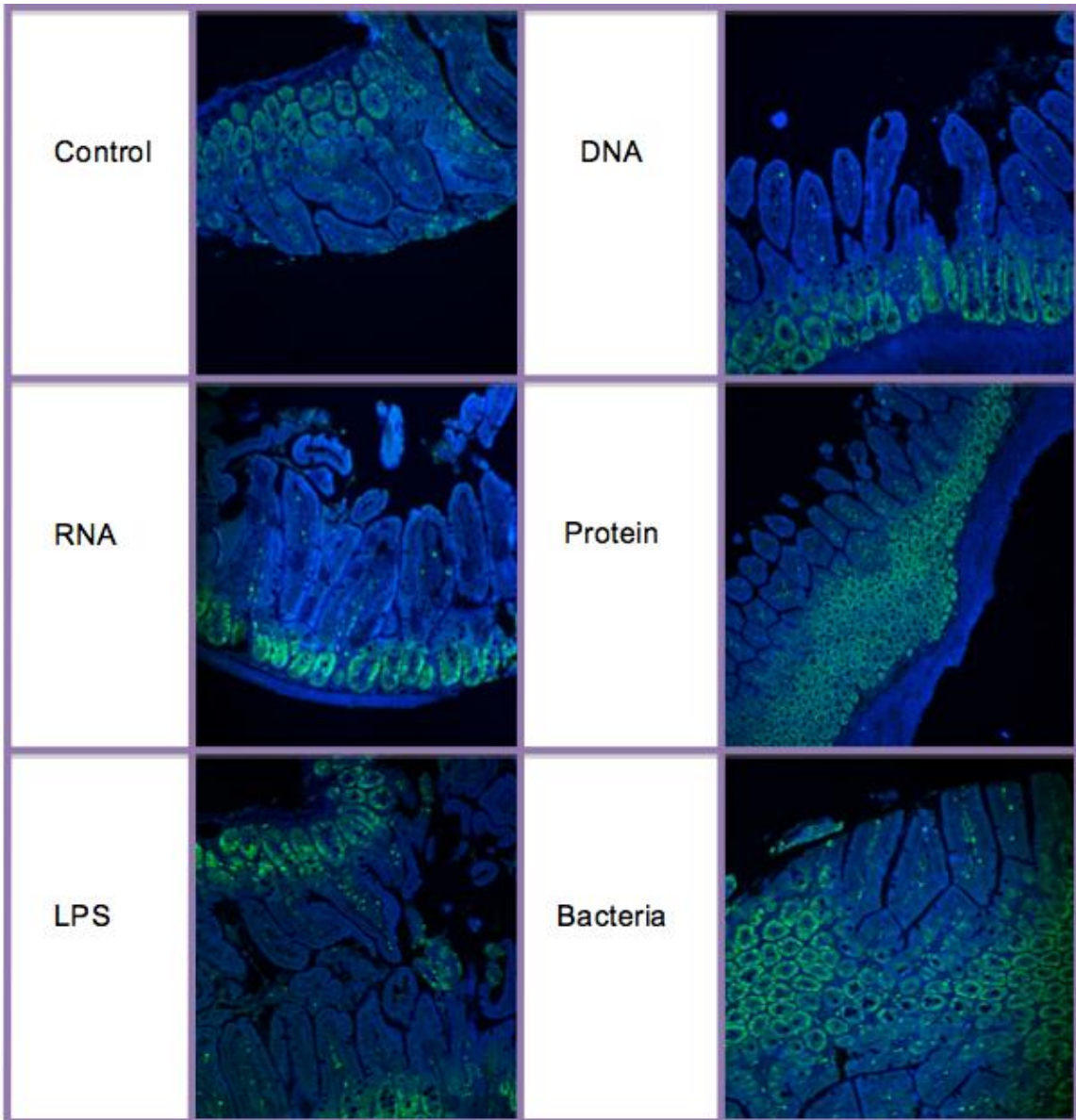
C.
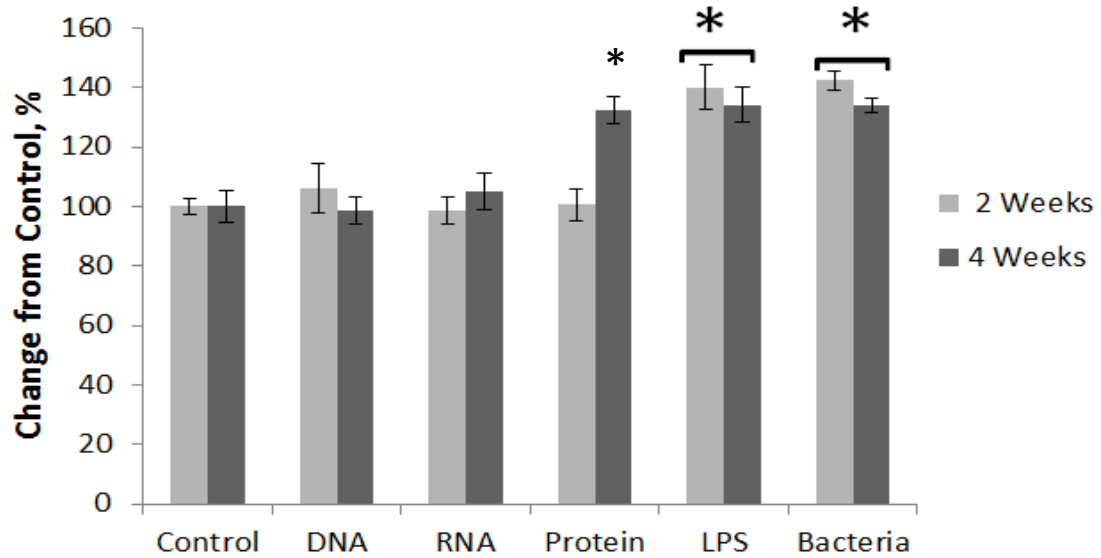
Figure 4.0. Immunohistochemical images of intestinal tissue samples stained with DAPI and Green Fluorescent antibody for PCNA. A, Intestinal tissue samples taken from individuals within test Group A. **B,** Intestinal tissue samples taken from individuals within test Group B. Quantification of PCNA-positive cells. **C.** Bars represent the average (with SD) number of PCNA cells. Asterisks show significant differences from control.

Table 4.0 Imunohistochemical analysis of PCNA protein expression quantified. Average±SD represents the average protein expression±standard deviation, compared to the control test detected via analysis of images. Asterisks identify significant increases compared to controls.

Test	2 Week Average ± SD	4 Week Average ± SD
Control	100.0±2.6	100.0±5.3
DNA	106.2±8.2	98.6±4.6
RNA	98.6±4.3	105.1±6.0
Protein	100.5 ±5.3	*132.5±4.6
LPS	*140.1±7.6	*134.2±5.7
Bacteria	*142.3±3.2	*133.9±2.4

5.0 GENERAL DISCUSSION AND CONCLUSION

Bacteria, even with the lack of molecular evidence, have been hypothesized to be the cause of millions of carcinomas worldwide (Parsonnet *et al.,* 1991). Originally, based on epidemiological studies, research today continues to gradually produce evidence to identify molecular pathways that suggests that bacteria can induce instability in the genome. Pathogenic *E. coli*, are a common bacterial cause of intestinal infection worldwide, and are slowly being accepted as a Gram-negative bacteria responsible for a large numbers of carcinomas in humans (Lax and Thomas, 2002).

Heat-inactivation of bacterial contaminated water is used by health officials in Canada to treat water in order to allow the water to be safely consumed. However, recent research from Koturbash *et al.* (2009) identified that bacteria induce alterations in intestinal tissues. This effect was identified in distal tissues in the form of changes in the expression of various proteins involved in cell proliferation, DNA repair and DNA methylation. Finally, filtered water (0.45µm filter to ensure whole bacteria are removed) induced the same alteration to distal cells identifying a potential for bacterial components to induce genomic instability. This phenomenon identified as a bystander-like effect influences the alteration in distal naive cells of organs such as the liver and spleen; however the exact method of inducing these alterations is not yet identified.

The results presented in the previous chapters of the thesis provide support to our hypotheses that LPS as a component of the heat-killed *E. coli* could induce genome instability. These alterations in the expression of DNA repair proteins and an increase in γH2AX, indicating the presence of a bystander-like effect, had an influence on the

genome stability of naive cells. Accumulation of DNA damage caused by the infection of distal tissues could disrupt cellular regulation and contribute to genome instability of the tissue. Also, alteration in the expression of proteins involved in DNA methylation indicates presence of an epigenetic influence on the distal cells of the organism.

The purpose of this study was to identify which bacterial component could induce genome instability in intestinal tissue, and transmit the same reaction via a bystander-like effect in the distal organs, liver and spleen.

5.1. Major Findings:

1. Exposure to whole heat-killed bacteria influenced an alteration in protein expression in exposed and distal cells with the bystander-like effect being a potential mediator to transmit the effects.
2. Identification of lipopolysaccharides (LPS) as one of the major components causing bacterial effects on the immediate target organ – intestine and on the distal non-target organs spleen and liver. not identified to target the hypotesis
3. LPS influence bystander-like effects on distal naive tissue such as spleen and liver. These effects are (and may not exclusive to) increased proliferation, DNA damage and alteration in the methylation of the genome.
4. Total protein extracts from heat-killed *E. coli* O157:H7 also has an influence on the host cells in direct contact of proliferation of the intestinal cells and the state of the (normal or healthy) spleen and liver cells.

6.0. Future Directions

As mentioned earlier, this field of study is relatively new, and extensive research is being conducted to support the hypothesis that bacteria may influence genomic stability and may be tied to carcinogenesis. The results presented, identify LPS as a major component of the bacteria that influences the host cells in the same manner as the whole bacteria. However, the results also imply that LPS may not be the only component of the bacteria that could induce alterations to the stability of the genome. Studies are required to understand the complete mechanistic approach of bacterial influences on genome stability. The following are suggestions of possible future studies in this area of research.

The expansion into the human cell line analysis is essential to the understanding and the implementation of this research/field for the creation of preventable steps or treatment of water contamination. Another important question to consider is: <u>Do LPS induce genome instability and inflammation in human intestinal tissue similar to murine samples, and what molecular mechanisms are influenced by the endotoxins?</u> With new technology (e.g., three dimensional cell line analysis), it is possible to purchase human intestinal cell lines (multiple cell lines structurally arranged as seen in nature) from MatTek Corporation (Walle *et al.,* 2005) and to perform similar studies on human tissue. Also, a new detection method, HEK-BlueTH LPS Detection Kit from InvivoGen allowing the research to detect LPS and ensure quantity of the LPS extract.

1. PCNA is an essential component of the DNA replication machinery functioning as the accessory protein for DNA polymerase δ (Pol δ), required for processive

chromosomal DNA synthesis and DNA polymerase ε (Pol ε) (Kelman, 1997). However, evidence suggests that PCNA plays a different role in replication, than in repair of the genome. The link between PCNA and DNA damage is the tumour suppressor protein, p53, which is a transcription factor that inhibits replication of the genome during unfavourable conditions by regulating cell cycle progression and cell viability (Levine, 1997). p53 has been identified to mediate the expression of PCNA protein for the control of cellular division as PCNA directly binds two p53-inducible proteins , GADD45 and p21 (Xiong *et al.,* 1992). These interactions may regulate, PCNA dependant DNA replication (Waga *et al.,* 1994). p53 may directly control DNA replication and repair by modulating levels of PCNA in cells. The aforementioned connection identifies the possibility that PCNA expression could correlate with the response to DNA damage. In order to decisively identify proliferation alteration within exposed or distal tissues, further analysis with Ki-67 protein must be performed. Ki-67 protein is thought to be an indicator of cells actively proliferating (Alison, 1995). Ki-67 protein is expressed during all active phases of cell cycle (G_1, S, G_2 and mitosis), but is absent during the resting stage (G_0) of the cell cycle, making it an ideal marker to identify cell proliferation (Scholzen and Gerdes, 2000). The question of "Does exposure to a LPS rich solution induce proliferation in distal cells through a bystander-like effect" has been analysed in this thesis, but further tests must be conducted to verify the aforementioned results.

2. *E. coli* O157:H7 are known to produce toxins that promote its pathogenic effects in host cells. These toxins are called "Stx". *E. coli* O157:H7 produce Stx that fall

under two main categories. The first category identified as Stx1, consists of toxins such as Stx1c (Friedrich *et al.,* 2003), and Stx1d (Burk *et al.,* 2003). The second group is identified as Stx2 and consists of a range of toxins such as Stx2c2 (Jelacic *et al.,* 2003), and Stx2g (Leung *et al.,* 2003). These toxins are capable of inducing damage to cells, and could transmit their effects to other distal cells though a bystander-like effect or could travel themselves through the bloodstream. Analysis of distal tissue with real-time PCR rather than DNA based methods could identify genes responding to these toxins (Kuezius *et al.,* 2004). These toxins produced by the bacteria, have been analysed, but no extensive research has focused on the potential to influence distal cells by bystander-like signalling. The toxins could be the factors involved in triggering alterations in methylation and DNA damage. The question that needs to be considered is: <u>Do the toxins produced by EHEC have any influence, either on the directly exposed or distal cells in response to heat-killed *E. coli* O157:H7?</u>

3. The bystander-like signalling needs to be further researched as well as other components of the bacteria that may induce genome instability. A possible explanation is that the intestinal cells may release inflammatory cytokines into the bloodstream. Therefore, any tissue with a high vascular flow will accumulate these circulatory inflammatory cytokines, influencing their genomic stability. Although cytokines have been identified as potential signalling mechanisms for the bystander effect, there is a list of molecules that could influence the distal cells, and may include (but is not limited to) the production of reactive oxygen species (ROS) and short RNAs (Koturbash *et al.,* 2007). <u>The bystander-like</u>

effect needs to be further researched by looking at of the inflammatory response mounted by the host immune system, and its contribution to alteration in genomic DNA/expression of naive cells.

7.0. References

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