MODULATION OF GUT IMMUNE AND HOST INFLAMMATORY RESPONSES BY EDIBLE MUSHROOMS

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Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
DOCTOR OF PHILOSOPHY
July, 2011

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CHAPTER I

Introduction

Infectious diseases like plague, leprosy, and small pox have been known to exist in ancient times and devastate the human population during pandemic outbreaks. Although there has been a sharp decline in the prevalence of several infectious diseases caused by bacteria, viruses and parasites after the introduction of modern chemotherapeutic agents, infectious diseases still remain a major public health problem worldwide due to emerging new pathogens that are resistance to chemotherapy (Herrera LA et al 2005).

Currently, it is estimated that at least 6 billion people around the world are infected with any one of the known infectious agents including bacteria, viruses and parasites (Mazza JJ 2010). These pathogenic organisms cause acute and/or chronic infectious diseases in susceptible or vulnerable populations. Acute infectious diseases like dengue, bacterial meningitis, septicemia, and cerebral malaria cause significant mortality whereas chronic infectious diseases devastate the economy due to long term medical care and reduction in man power (Herrera LA et al 2005).

Moreover, there is a strong relationship between certain chronic infectious diseases and the incidence of cancer, including cervical cancer, gastric and hepatocellular carcinomas

(Parkin DM 2006).Twenty percent of human cancers are directly attributed to infectious agents (Mazza JJ 2010). The worldwide increase in morbidity and mortality rates due to cancer cancer which is the second leading cause of death in the USA (CDC), pose not only challenges to medical professionals but also cause significant economic burden to the nations (WHO).

Although the etiology for each infectious disease may be different, inflammation is the key event in all infectious diseases (Robbins 2004). Inflammation is defined as the complex biological response of vascularized living tissues to harmful stimuli (Robbins 2004). Several immunological factors including cytokines, chemokines, cell adhesion molecules and antimicrobial peptides contribute to the pathophysiology of inflammation (Robbins 2004). Inflammation can be broadly classified into two categories, namely acute and chronic inflammation (Dinarello CA 1997). Acute inflammation is an immediate immune mediated defensive response for any noxius stimuli, which usually lives only a few hours to a few days (Dinarello CA 1997). In contrast, chronic inflammation is an unwanted inflammatory response that sustains for months to years.

Neutrophils are the primary immune cells involved in acute inflammation to defend against pathogens by releasing antimicrobial chemicals such as peroxides, lysozymes and defensins (Doherty NS & Janusz MJ 1994). Mononuclear leukocytes, including monocytes, macrophages, lymphocytes and plasma cells, were frequently found in tissue

section from chronic inflammation. Unlike acute inflammation, chronic inflammation has been linked to many chronic diseases, including (Santos MJ et al 2009).

Cytokines are the multifunctional and pleiotrophic molecules involved in inflammation cascades. Some cytokines are proinflammatory, whereas others are anti-inflammatory/regulatory in nature. Imbalance between these two categories of cytokines has been implicated in the pathogenesis of infectious diseases and cancer (Sanchez-Munoz F et al 2008). Defensins are the natural antimicrobial peptides active against pathogenic bacteria, fungi, and some enveloped viruses (Ramasundara M et al 2009). Leukocytes and epithelial cells are the major sources of defensins.

Defensins play many roles in the immune response including both indirect and direct antimicrobial activity, the ability to act as chemokines as well as induce chemokine production leading to recruitment of leukocytes to the site of infection and the promotion of wound healing (Ramasundara M et al 2009). Defensins such as α-defensins and β-defensins confer not only innate immunity, but also link innate immunity to adaptive immunity. Defective defensin production and secretion has been directly implicated in gastrointestinal (GI) tract diseases such as inflammatory bowel disease (Ramasundara M et al 2009).

The GI tract represents the largest mucosal surface in the body which makes it critical as most pathogens (90%) enter into the body through mucosa (Nagler-Anderson C 2001, de la Cabada 2011 and Shale M 2010). Therefore, the GI system not only plays a major role

in digestion and absorption of essential nutrients into our body but also offers protection against various pathogens through the GI immune system (Shale M 2010 and Hisamatsu T et al 2008). The GI immune system consists of mesenteric lymph nodes, Peyer's patches and lamina propria associated lymphocytes (Mowat AM 2003). The GI immune system has the constant challenge of responding to pathogens and toxins without responding to food antigens or the commensal microflora (Mowat AM 2003). Both of these challenging tasks are simultaneously performed by dendritic cells (DCs), the professional antigen presenting cells present in GI tract.

DCs orchestrate a repertoire of immune responses such as differentiation of naive T cells and initiate primary immune responses that provide resistance to infection and tolerance to self. (Ralph M 2007 and Sato K 2007). Although some microbes and tumors may hide from dendritic cells to evade immunity, DCs can be activated to recognize these camouflaged microbes and cancer cells by DC-targeted bioactive molecules. (Ralph M 2007). In addition, cytokines produced by the DCs is critical in determining the polarization of the helper T cell responses such as Th1 or Th2 or Th17 (Banchereau J 2000, Ardavín C 2004 and Yu CF 2010).

Hence, it is not surprising that cytokines produced by the intestinal dendritic cells play a major role in GI immune system (Perdigón G 2002). Although several cytokines produced by gastro intestinal DCs are involved in mucosal defense against pathogens, IL-23 is mainly involved in the defense against bacterial and fungal pathogens encountered

in GI mucosa (Ciccia F 2009, Doisne JM 2010 & Brereton CF 2011). Therefore, bioactive molecules modulating DCs induced IL-23 cytokine secretion and regulation might be a novel approach in promoting gut immunity and treating diseases (Harada N 2003, Tacken PJ 2005 and Ralph M 2007).

IL-23 is involved in the differentiation of naive CD4+ T cells into Th17 cells (Maloy KJ et al 2008) in combination with IL-6 and TGF-β1. These Th17 cells secrete IL-17 cytokine that is involved in the defense against pathogens by recruiting neutrophils to the site of inflammation (McAleer JP et al 2011). Further, the IL-23/IL-17 axis plays a major role in regulating intestinal inflammation and defensin production (Uhlig HH 2006). Therefore, IL-23/IL-17 axis is involved in destroying most GI pathogens, including Vibrio cholera, E. coli and Salmonella (Doisne JM et al 2010). Moreover, IL-23 also increases immunological memory by inducing CD4+ T memory cells' proliferation. Hence, increasing the mucosal IL-23 production is one of the novel strategies in improving mucosal immunity/immunological memory (Agrawal S et al 2010).

Although most GI tract infections have been eradicated in developed countries, Salmonellosis, Campylobacteriosis, *E. coli* infections, listeriosis, and Cholera remain major public health problems (Meyer C 2010 and de la Cabada 2011). Several antibiotics have been developed to treat GI tract infections; still there is a search for alternatives to these antibiotics due to the emergence of antibiotic resistant bacteria (DuPont HL 2005). Moreover, these antibiotics destroy or alter the commensal bacteria present in the GI tract

leading to intestinal dysbiosis. This intestinal dysbiosis is now believed to be a contributing factor to many chronic and degenerative diseases such as irritable bowel syndrome, inflammatory bowel disease, rheumatoid arthritis, and ankylosing spondylitis (Hawrelak JA 2004).

One alternative to antibiotic treatment is to increase the production of endogenous antimicrobial peptides including defensins by using natural compounds or functional foods (Cunliffe RN 2003 &Wehkamp J 2005). In this regard, several mushrooms have emerged as a functional food to stimulate innate and adaptive immunity.

Mushrooms are rich in fibers, beta glucan, and some essential micronutrients such as Se, Zn and Fe (Raymond Chang 1997). Currently, there are about 700 species of edible mushrooms being known to the world (Chang and Miles 2004). However, only handful of mushrooms such as white button (WBM), portabella (PM) and shiitake (SM) mushrooms has been marketed to the consumers (Raymond Chang 1997). Interestingly, the production of Agaricus mushrooms, including white button mushroom and portabella mushroom has increased throughout the world (USDA). The top ten countries producing Agaricus mushrooms are China, USA, Netherlands, France, Poland, Italy, Spain, Ireland, United Kingdom and Germany (USDA). Currently, the USA is the second largest producer of Agaricus mushrooms in the world. Further, the consumption of Agaricus mushrooms has increased tremendously over the past several decades in the United States (USDA).

Next to Agaricus mushrooms, SM is the second most cultivated edible mushrooms comprising about 25% of the worldwide production (Jiang T et al 2010). SM is an edible exotic mushroom scientifically known as *Lentinula edodes* native to China and cultivated in East Asia (Raymond Chang 1997). SM was considered a royal food in ancient times due to its nutritive value, delicacy and scarcity. Although SM had been cultivated mainly by traditional methods in East Asian countries, the advancement of mushroom production and biotechnology led to large scale commercial cultivation of SM worldwide (Chang and Miles 2004). Interestingly, the SM production has exceeded more than 9 million pounds per annum during 2009 in United States (USDA). Because of their widespread availability and significant reduction in price, the consumption of SM has been increased in recent years. Both fresh and dried forms of SM have been used in many East Asian cuisines for many years and have been slowly entering its way into Western cuisine in recent years (Chang and Miles 2004). Although both forms of SM are used for culinary purpose, many people prefer the dried form of SM due to its flavor and vitamin D content. Hence, SM is often sold as dried powder for commercial use. Moreover, crude and purified extracts from SM have been used in complementary and alternative medicine (CAM) therapy to treat immunodeficiency associated with viral infections and cancer (Raymond Chang 1997).

Some of the medicinal mushrooms such as maitake, shiitake, chaga, and reishi have been scientifically evaluated for their health benefits unlike Agaricus mushrooms. Moreover, the extracts/purified forms of these medicinal/exotic mushrooms have been used to

evaluate their effects rather than the culinary forms of whole mushrooms. Further, there is a paucity of data on the effect of WBM and portabello PM mushrooms, the most commonly consumed mushrooms in the World including United States. Previous report from our lab showed that the extracts from edible mushroom enhance α-defensin production in HL60, a human promyelocytic cell line (Kuvibidila S and Korlagunta 2009). Since IL-23/IL-17 axis is involved in the production of defensins in gut mucosa, we studied the effect of white button (WBM) and portabello (PM) mushrooms on IL-23 secretion both in-vitro and in-vivo models for this study. To the best of our knowledge this is the first study in investigating the effect of WBM and portabello PM mushrooms on IL-23 and gut immunity in general (Borchers AT 2008).

Although functional foods including mushrooms are gaining much attention in food and nutrition field, the term functional foods has no legal meaning in United States (ADA 2009). This is due to lack of scientific literature regarding the health benefits and safety issues associated with these functional foods (ADA). Therefore, the American Dietetic Association (ADA) supports "research to further define the health benefits and risks of individual functional foods and their physiologically active components." Surprisingly the edible wild mushroom *Tricholoma equestre* caused a rare toxicity called rhabdomyolysis in humans when they ate large quantities of these mushroom (Bedry R et al 2001). Once this report appeared in the New England Journal of Medicine in 2001, the need for examining the risk associated with edible mushrooms became imperative----. In a mice study, orally administered Shiitake mushroom at the dose rate of 9 g/ kg/ day (270

mg/mouse/day) for 5 days caused significant increase in plasma creatine kinase level. This study further supports the need for examining safety evaluation of edible mushrooms for long term consumption. Moreover, there is not much information about the adverse effects of Agaricus and Shiitake mushrooms.

Surprisingly, we observed that fortification of the AIN93 diet with 5% lyophilized SM was associated with a two fold increase in plasma interleukin-6 (IL-6) in DBA mice with collagen-induced arthritis (Lawrance Chandra et al 2010). Since IL-6 is thought to be implicated in the pathogenesis of fatty liver disease (Mclaine et al 2004), we reviewed the literature regarding the association between consumption of edible mushrooms and risk of non-alcoholic fatty liver disease (NAFLD) is a disorder of lipid metabolism characterized histologically by hepatic steatosis in the absence of excessive alcohol consumption (James et al 2010). However, we did not find much information related to SM and NAFLD.

NAFLD has drawn much attention due to its intimate association with insulin resistance, metabolic syndrome, cirrhosis, and end stage liver failure (James et al 2010). Moreover, NAFLD has emerged as a major public health problem in recent years (James et al 2010). Surprisingly, the prevalence of NAFLD in adult Japanese population with normal body mass index (BMI) was 29% which is very high compared to Western population (Jimba et al 2005). Among East Asian countries, the prevalence of NAFLD in the population with normal BMI remains high in Japan, the largest consumer of shiitake mushroom in

the world (James et al 2010). Therefore we investigated the effect of supplementation of the AIN93 diet with dry-lyophilized SM powder on fatty liver prevalence in C57BL/6 mice. To the best of our knowledge, this is the first study to evaluate chronic consumption of Agaricus mushrooms and Shiitake mushrooms on fatty liver development.

We performed two studies to test our hypotheses.

Hypothesis for study-1: a. Edible mushroom supplementation may enhance gut immunity/inflammation by up-regulating IL-23 production in the gastrointestinal tract and less so in peripheral lymphoid organs such as spleen. b. Edible mushrooms up-regulate IL-23 through dectin-1 pathway.

Objectives

1. To evaluate the effect of edible mushrooms on IL-23 secretion in DSS treated and untreated mice
2. To evaluate the effect of edible mushrooms on IL-6 secretion in DSS treated and untreated mice
3. To evaluate the effect of edible mushrooms on neutrophil infiltrations and myeloperoxidase level
4. To delineate the molecular mechanism by which edible mushrooms up-regulates the IL 23 secretion in J.744.1 cells, a mouse monocytic cell lines

Hypothesis for study- 2: Chronic consumption of edible mushrooms specifically, Shiitake mushroom will cause fatty liver.

Objectives

1. To study the effect of 5% edible Shiitake mushroom fortified diet on the prevalence of fatty liver in C57BL/6 mice.
2. To characterize the Shiitake mushroom-induced fatty changes in the liver and other vital organs

Study limitations

In our study-1, we neither isolate mouse dendritic cells from our mice nor do we perform any immunohistochemical procedures to localize these dendritic cells in the GI tract of these mice. Moreover, we did not study other closely related cytokines such as IL-17, IL-22, which are also involved in the up-regulation of defensin. In addition, we did not challenge these mice with known GI pathogens such as Salmonella, *E. coli* to see the protective effect of mushroom induced IL-23 up-regulation. Furthermore, we did not perform mucosal vaccine titer response to translate the IL-23 induced immunological memory. Finally, we did not study the dose and time response of different mushrooms on IL-23 production.

The major weakness of our study-2 is the lack of mechanistic approach to investigate how these SM caused fatty liver by using hepatocyte cell lines. Moreover, we did not have mice group fed with pure form of eritadenine, a phytochemical rich in SM. This approach would have helped us to explain some mechanism responsible for fatty liver development. In addition we did not study the dose and time response of SM in fatty liver development. Finally, we did not study the expressions of genes involved in liver fat synthesis.

Lastly, the demerits with any animal or pre-clinical study, we cannot directly extrapolate to humans because of the species difference.

CHAPTER II

Literature review

Gut immune system: Structure and function

Gut immune system is composed of extrinsic and intrinsic barriers with non-specific and specific defense mechanisms (James SP 1993). Extrinsic barriers include gastrointestinal acidity, digestive enzymes, mucus, peristalsis, and an immunological which provides a source of secretory IgA and IgM (James SP 1993 and Langkamp-Henken B et al 1992).The intrinsic barriers of gut immune system consist of aggregated and nonaggregated lymphoid follicles known as gut associated lymphoid tissues (James SP 1993 and Langkamp-Henken B et al 1992). These elements of the gut immune system function together to generate an immune response against harmful pathogens and to generate tolerance against dietary antigens and normal microbial flora (Langkamp-Henken B et al 1992). Therefore, intestinal mucosa forms a tight barrier not only against invading pathogens but also against dietary antigens and commensal bacteria. However, these tight mucosal barriers allow transport of nutrients across the epithelium. The integrity of both the intrinsic and extrinsic barriers may be influenced by environmental factors such as diet, microbes and genetic composition. Uncontrolled entry of pathogens through the gut mucosa cause not only gastointestinal infections, but also other systemic bacterial and viral diseases (James SP 1993 and Langkamp-Henken B et al 1992).

Gut immune system is distinct from other immune systems in many ways: i) presence of Peyer's patches (PP), specialized structures in the gut mucosa, where immune responses

are initiated; ii) mucosal homing of lymphoid cells, a relatively specific recirculation of lymphoid cells to the mucosa; iii) IgA producing B cells and memory T cells are predominate at mucosal surfaces and the secretory IgA is the major mucosal immunoglobulin. These features make gut immune system a unique immune system in the body (McGhee JR et al 2007 and Suzuki K et al 2010).

The gut immune system protects the intestinal mucosa from invading pathogens by the network of immune cells including B-cells, T-cells, macrophages and dendritic cells present in the mucosa (Suzuki K et al 2010). A majority of the lymphocytes are found in Peyer´s patches, mesenteric lymph nodes (MLN) and scattered throughout the lamina propria and mucosal epithelium. The lamina propria contains large numbers of B-cells, plasma cells, macrophages, dendritic cells, and T-cells of both $CD4^+$ and $CD8^+$ subsets. Lymphocytes present within the intestinal mucosal epithelium are known as intraepithelial lymphocytes (IELs). It is estimated that 10 to 15% of the cells in the intestinal mucosal epithelium are IELs. It is also known that CD8+ T-cells constitute the major cells in intraepithelial lymphocytes population. These CD8+ T-cells express γδ-TCR (<10% in the small intestine, 40% in the colon in humans), which is yet another distinct feature of IELs. These γδ-TCR expressing CD8+ T-cells were increased in patients with active celiac disease or food allergy (Savilahti et al 1990)

Antigen presentation in the gut immune system is a complex process, carried out by professional APCs or by specialized epithelial cells known as M cells. After activation in the gut immune system, lymphocytes up regulate α4β7-integrin, a lymphocyte adhesion molecule (Hart AL et al 2010). This lymphocyte adhesion molecule helps to recirculate lymphocytes (homing) within 38 mucosal tissues by interacting with MAdCAM-1, a

mucosal vascular addressin molecule (Hart AL et al 2010). Interestingly, IELs expressing α4β7-integrin are believed to reside within the intestinal mucosa permanently (Hart AL et al 2010). Cross-talk between epithelial cells and the mucosal lymphocytes is critical for the induction of tolerance and expression of active mucosal immunity. Under normal/physiological conditions, antigen-presenting epithelial cells do not express co-stimulatory molecules (Arnett HA and Viney JL 2010). Therefore, antigen-presentation by epithelial cells leads to tolerance. However, under pathological conditions (inflammatory or infectious), activation of IELs occurs due to the expression of costimulatory molecules by the APCs (Arnett HA and Viney JL 2010).

IL-23/IL-17 axis in gut immunity

IL-23 is a cytokine produced primarily by activated DCs and macrophages and to some extent by paneth cells (Doisne JM 2010 & Ciccia F 2009). IL-23 is a heterodimeric cytokine related to IL-12 sharing a p40 sub unit (McKenzie BS et al 2006). Although both cytokines poss a strong structural relationships, these similarities not translate into functional similarity (Oppmann B 2000). In fact, these two cytokines appear to have profoundly different roles in regulating the host immune responses (Oppmann B 2000). Hue S et al 2006 observed that IL-23p19 was predominantly expressed in the intestinal mucosa rather than mesenteric LN (MLN) or spleen. These suggest that IL-23 might have primary role on gut mucosal immunity and inflammation (Hue S et al 2006).

In conjunction with IL-6 and TGF-β1, IL-23 stimulates naive CD4+ T cells to differentiate into a novel subset of cells called Th17 cells (Maloy KJ 2008). Moreover, it is required for the expansion and survival of Th17 cells that are important in intestinal, antibacterial immunity (Veldhoen, M 2006). These Th17 cells secrete a cytokine called

IL-17, which is involved in the primary and first line of defense against pathogens by recruiting neutrophils to the site of inflammation (McAleer JP 2011). Further, the IL-23/IL-17 axis plays a major role in local intestinal inflammation and production of defensin in gut mucosa (Uhlig HH 2006).

Defensins are short chain antimicrobial peptides with three characteristic pairs of intramolecular disulfide bonds and a β-sheet structure (Guaní-Guerra E et al 2010) . Defensins are classified into three major groups namely α, β and θ-defensins according to the arrangement and spacing of the disulfide bonds (Guaní-Guerra E et al 2010). Paneth cells, located at the base of intestinal crypts, are the major source of defensins in the intestinal mucosa (Bevins CL 2005). These defensins play major role in gut innate immunity (Bevins CL 2005).

Because of the IL-23/IL-17 axis is involved both directly and indirectly in destroying most GI pathogens, including *Vibrio cholera*, *E. coli* and *Salmonella* (Doisne JM 2010). IL-23 also promotes immunological memory by stimulating CD4+ T memory cells proliferation. Hence, increasing mucosal IL-23 production might improve mucosal immunity/immunological memory (Agrawal S 2010). Moreover, IL-23 augmented vaccine- induced tumor-specific CD8+ T cell responses against murine melanoma (Overwijk WW et al 2006).

Dendritic cells

Dendritic cells (DCs), recognized as professional antigen presenting cells, orchestrate a repertoire of immune responses including the differentiation of naive T cells and the initiation of primary immune responses which provide resistance to infection and

tolerance to self. (Ralph M 2007 & Sato K 2007). DCs are formed from either myeloid or lymphoid bone marrow progenitors via intermediate DC precursors that arrive at sites of potential pathogen entry, where they differentiate locally into immature DCs. (Ardavín C 2004). These immature DCs undergo a complex maturation process when they capture antigen in the presence of danger signals associated with inflammation or infection.

However, antigen capture alone in the absence of activation stimuli will not lead to maturation. The net result, in the absence of co-stimulatory signal molecules, such as CD40, CD54, CD83, B7.1 and B7.2 (CD80 and CD86), immature DCs presents antigen to the T cells that lead to T cell tolerance. Therefore, the mature DCs act as sentinels in recognizing microbes and tumors by capturing and transferring these antigens to the cells of the adaptive immune system thereby regulates the types of T cell immune responses. (Sato K 2007). Although microbes and tumors escape from these sentinel dendritic cells to evade immunity, DC can be activated to recognize those camouflaged microbes and cancer cells by DC-targeted bioactive molecule. (Ralph M 2007). Similarly, DC-targeted bioactive molecules can be used to silence DCs instigated unwanted autoimmune responses (Ralph M 2007).

Moreover, the cytokines produced by the DCs are critical in determining the polarization of the helper T cell responses such as Th1 or Th2 or Th17 (Banchereau J 2000, Ardavín C 2004 and Yu CF 2010). Th1 cell mediated immune responses targeted towards intracellular pathogens through the production of interferon gamma (IFN-γ) and IL-2. On the other hand, Th2 humoral immune response occurs through the production of IL-4, IL-5, IL-6, and IL-13. Interestingly, Th17 response targeted primarily bacterial and fungal pathogens through the production of IL-17, IL-17A and IL-22.

DC-produced IL-12 is critical in the generation of a Th1 immune response whereas IL-23 produced by DCs is critical for Th17 response (Banchereau J 2000, Ardavín C 2004 and Yu CF 2010). Similarly, DC- produced anti-inflammatory cytokines such as IL-4, IL-10 are crucial in Th2 and Treg responses. Therefore bioactive molecules targeting DCs biology and cytokines regulation are the emerging translational approach in preventing and treating illness (Harada N 2003, Tacken PJ 2005 and Ralph M 2007).

Mushroom health benefits in general

White Button Mushroom

White button mushrooms (*Agaricus bisporus*) belong to the genus *Agaricus* and are widely consumed in the world. They represent about 90% of mushrooms consumed by Americans. However, the health benefits of this strain in general are not well studied. Wu *et al* (2007) reported the immunomodulatory effects of white button mushroom by enhancing the natural killer cell activity. White button mushrooms are known to inhibit aromatase, an enzyme involved in the conversion of androgen to estrogen, thereby suppressing breast cancer cell proliferation *in vitro* (Chen S et al 2006). Further Yu et al showed mild protective effects of white button mushroom in dextran sodium sulfate (DSS) induced colitis mouse model (Yu S et al 2009), though the molecular mechanism by which WBM alters the immune response remains to be elucidated.

Portobello mushroom

Portobello mushroom belongs to the same species *Agaricus bisporus* as the WBM; however, portobello mushrooms are different from white button mushroom in terms of their brown color, large size, thick cap and stem, and a unique musky smell. Like white

button mushrooms, portobello mushrooms are also consumed widely around the world. However, to date there are no studies related to health benefits of dietary supplementation of portobello mushroom found in the scientific literatures.

Shiitake Mushroom

Shiitake mushroom (*Lentinula edodes*) is an edible mushroom consumed in Asian countries mainly Japan, China, Korea and Thailand. *Lentinus edodes* is the second most popular edible mushroom in the global market due to its medicinal properties and nutritional value (Bisen PS 2010). Lentinan, lectins and eritadenine are some of the bioactive compounds isolated from SM. (Bisen PS 2010). Unlike other mushrooms, SM has high concentration of eritadenine which exibits hypocholesterolemic properties. Lentinan, the polysaccharides found in SM consists of a beta-1,6-branched-beta-1,4-glucan linkage (Lee HH 2009). Lentinan was shown to increase the phagocytic activity of macrophages by up-regulating inflammatory cytokines. (Lee HH 2009). Lentinen was also known to have anti-tumor effect through activation of NK cells and macropahges (Kataoka H 2009). In a fish model, lentinen reduced the expression of genes involved in acute inflammatory reactions suggesting species difference in the response (Djordjevic B 2009). Antimicrobial activities of shiitake mushroom against pathogenic bacteria and fungi have recently been documented (Rao JR et al 2008). Shiitake mushroom boosted the BCG vaccine response in guinea pigs by activation of alveolar macrophages (Drandarska I 2005).

Eritadenine

Eritadenine, an adenosine compond responsible for hypocholesterolemic effects of Shiitake mushroom (Kaneda T and Tokuda S 1966). Eritadenine also known as lentinacin has been shown to reduce cholesterol levels in rats by 25% after 7 days of feeding eritadenine supplemented (0.005%) diet (Chibata I et al 1969). The hypocholesterolemic action of eritadenine is due to a modification of the hepatic phospholipid metabolism by inducing a phosphatidylethanolamine N-methyltransferase enzyme deficiency (Sugiyama K et al 1995). Further, dietary eritadenine altered the fatty acid profile of liver by suppressing the metabolic conversion of linoleic acid into arachidonic acid (Sugiyama K & Yamakawa A 1996, Sugiyama K et al 1997a and 1997b). It is also shown that eritadenine suppress the Δ^6-desaturase activity through transcriptional regulation (Shimada Y et al 2002). Dietary eritadenine supplementation counteracted the hyperhomocysteinemic effect of guanidinoacetic-acid in rats (Fukada et al 2006).

Mushrooms and dendritic cell biology

In vivo study using C3H/HeJ mice, intraperitoneal administration of polysaccharide obtained from *Grifola frondosa* stimulates dendritic cells to produce IL-12 and IL-10 production (Kodama N J 2004). This *Grifola frondosa* induced IL-12 cytokines enhanced NK cell cytotoxicity against MM-46 mammary tumor in C3H/HeJ mice (Kodama N J 2005). Similar results were observed when low-molecular-weight protein fractions of the maitake mushroom administered in colon-26 carcinoma-bearing mice by the same research group. (Kodama N J 2010). Interestingly, a novel heteropolysaccharide maitake Z-fraction (MZF) from the maitake mushroom, upregulated the markers of dendritic cell maturation such as CD80, CD86, CD83, and major histocompatibility complex (MHC) II

on bone marrow-derived dendritic cells (DCs) in BALB/c mice. In addition to this, maitake Z-fraction also increased the IL-12 and TNF-α production by DCs (Masuda Y 2010). Therefore it is clear that maitake mushrooms are capable of stimulating DC towards a Th1 response. However, the effect maitake mushrooms on Th17 pathway is still unknown.

Proteoglycan isolated from *Phellinus linteus* up-regulated the phenotypic and functional maturation markers such as major histocompatibility complex (MHC) class II and CD86 in murine bone marrow-derived dendritic cells. (Kim GY 2004). This proteoglycan activated DCs via TLR2 and/or TLR4 mediated-NFkB, ERK and p38 MAPK signal pathways. (Kim GY 2004b). Further, proteoglycan isolated from *Phellinus linteus* suppressed tumor growth in vivo through DC activated Th1 type immune response that lead to enhanced CD8+ response (Kim GY 2004a). In a recent study, extract from *Phellinus linteus* augmented mucosal vaccine induced immune response against *H5N1 influenza A* in mice (Ichinohe T 2010). Lentinan, a (1-3)-β glucan from shiitake mushroom increased the expression of MHC II, CD80/CD86, and Toll-like receptors (TLR2/TLR4), and increased production of IL-12 in spleen dendritic cells from malaria-infected mice (Zhou LD 2009). These findings suggest SM promote Th1 response during *Plasmodium yoelii* infection in mice.

Surprisingly, hemicellulase-treated *Agaricus blazei* mushroom activated BMDCs, without producing proinflammatory cytokines, such as IL-12, TNF-α, and IL-1β. (Kawamura M 2005). In contrast to the above observation, the water-soluble proteoglycan isolated from *Agaricus blazei* caused not only the maturation of murine bone marrow-derived dendritic cells, but also increased production of IL-12 (Kim GY

2005). However, in another study extracts from *Agaricus blazei* augmented NK cell activation by increasing IL-12 and IFN-γ production in mouse DCs. (Yuminamochi E 2007) Recently, the extracts from *Agaricus blazei Murill* were shown to stimulate monocyte-derived dendritic cells and up-regulate pro-inflammatory molecules including IL-8, G-CSF, TNF-α, IL-1β, IL-6 and MIP-1β. Although extracts from *Agaricus blazei Murill* increased pro-inflammatory molecules in this in-vitro study, it did not induce the synthesis of Th2 cytokines or the Th1 specific cytokine IL-12 (Førland DT 2010).

Surprisingly, water soluble extracts from *Hericium erinaceum*, a medicinal mushroom activated human DCs, without producing IL-12 (Kim SK 2010). Similarly, *Armillariella mellea* caused maturation of human dendritic cells without induction of cytokine production (Kim SK 2008). WBM activated BMDCs and up-regulated IL-12 production (Ren Z 2008). Unlike mushrooms, yeast derived β-glucan induced more IL-12 production by dendritic cells (Driscoll M 2009)

In summary, exotic mushroom compounds are capable of inducing DCs maturation of *in vitro*. Almost all exotic mushrooms have been shown to produce IL-12 production, but their effects on the Th1/Th2 polarization are variable and appear to be highly compound-specific. However, information regarding the effect of WBM and PM on DCs maturation is limited. Moreover, there is not much information about mushrooms on Th17 polarization.

Animal models of intestinal inflammation

Animal models of intestinal inflammation/inflammatory bowel disease (IBD) served as an invaluable tool to understand the pathogenesis of intestinal inflammation and design

novel treatment strategies (Blumberg RS et al 1999 and Strober W 2002). Currently, there are about 20 animal models of intestinal inflammation being employed to investigate the roles of environmental factors such as normal bacterial flora, diet and the genetic composition and their interactions on the development of intestinal inflammation (Blumberg RS et al 1999, Strober W 2002 and Wirtz S & Neurath MF 2000). Although these animal models of intestinal inflammation do not mimic the exact nature of human disease, still they are vital to answer complex research questions which cannot be carried out on humans due to ethical, legal and technical considerations (Strober W 2002 and Wirtz S & Neurath MF 2000).

Animal models of intestinal inflammation are classified into four major categories: i) inducible colitis models, ii) spontaneous colitis, iii) adoptive transfer model and iv) genetically engineered animal models (e.g., knockout (KO) models and transgenic models). In inducible colitis models, chemical agents such as acetic acid (MacPherson BR and Pfeiffer CJ 1978), dextran sodium sulfate (DSS) (Okayasu I et al 1990), trinitrobenzene sulfonic acid (TNBS) (Morris GP et al 1989 and Neurath MF et al 1995), and oxazolone (Boirivant M et al, 1998) are used to induce intestinal inflammation and tissue damage.

These chemically induced intestinal inflammation animal models are commonly used in biomedical research due to easy access, low expense, low technical demand and reproducible effects within a short period of time (Strober W 2002). In fact, DSS induced acute colitis is an invaluable tool to study the role of innate immunity involved in colitis (Jurjus AR et al 2004). It is known that oral administration of 3-5% of DSS for 5-7 days causes colonic epithelial cell lesions and acute inflammation characterized by the

presence of neutrophils and macrophages within inflamed mucosa (Okayasu I et al 1990). Therefore, DSS is a useful model to investigate the role of various leukocytes during colitis (Maisoun A et al 2006).

On the other hand, repeated DSS treatment at the rate of 0.5% to 2% leads to chronic colitis characterized by large numbers of activated T cells in the intestinal mucosa (Wirtz S 2007). Hence, DSS induced chronic colitis is a valuable tool to study the mechanisms linking colitis to colon cancer (Wirtz S 2007). Similarly, rectal administration of TNBS dissolved in ethanol promotes a severe intestinal inflammatory response (Morris GP et al 1989 and Neurath MF et al, 1995). In this model, ethanol is used to increase intestinal permeability which allows TNBS to reach the intestinal mucosa and haptenate tissue and microbial proteins. Therefore, haptenating TNBS compound-induced colitis is an invaluable model to study the T helper cell-dependent mucosal immune responses unlike DSS (Jurjus AR et al, 2004 and Wirtz S 2007). Moreover, TNBS induces Crohn's Disease-like intestinal inflammation whereas DSS induces ulcerative colitis in these animal models (Neurath MF et al, 1995 and Maisoun A et al 2006). However, both chemicals can be used to activate the mucosal immune response in GI tract (Blumberg RS et al 1999).

The striking feature of the induced inflammation model is the disruption of the epithelial cell barrier by these chemicals (MacPherson BR & Pfeiffer CJ 1978, Okayasu I et al 1990, Morris GP et al 1989, Neurath MF et al 1995, and Boirivant M et al 1998). Disruption in epithelial barrier leads to increased cellular exposure to mucosal microflora. This results in mucosal inflammation and non-specific activation of mucosal immune cells leading to release of pro-inflammatory cytokines (Strober W 2002). Although

chemically-induced intestinal inflammation models are widely used, these animal models have some disadvantages. One of the disadvantages is the leakage of these chemicals into a proximal portion of the intestine, causing inflammation similar to that seen in the colon. Moreover, these chemicals induce severe inflammation and nutrient malabsorptions. Therefore, disrupting the mucosal barrier by using electrocautery methods seems be an alternative to chemically induced intestinal inflammation (Mourad FH et al, 2010).

Unlike induced model of colitis, spontaneous colitis develops naturally in a few mouse strains such as C3H/HeBir (Sundberg JP et al, 1994) and SAMP1/Yit (Burns RC et al, 2001) due to genetic mutation. These spontaneous mutant mouse models are useful to study the inheritable form of inflammatory bowel disease and the role of genetic composition in the development of intestinal inflammation. Interestingly, in adoptive transfer model, intestinal inflammation is transferred from immunocompetent mice to immunodeficient mice through immune reactive $CD4^+$ T cells (Powrie F et al, 1993). These adoptive transfer models are used to study the role of T cells in regulating intestinal inflammation and autoimmune diseases. Several gene-knockout mouse strains such as IL-2, IL-10 and T cell receptor (TCR) have been developed to define the role of the immune system in the development of intestinal inflammation. (Sadlack B et al, 1993, Davidson NJ et al, 1996, Mombaerts P 1993, Rudolph U et al, 1995 and Hermiston ML & Gordon JI). Unlike induced animal models, these genetically modified mice are relatively expensive and technically demanding. Moreover, these mouse strains might have developed physiological adaptation/compensatory mechanism to these targeted deletions of genes.

Cytokines and intestinal inflammation

A large number of cytokines including TNF-α, IL-6, IL-12, IL-17, IL-23 and IL-10 are known to be up/down-regulated during intestinal inflammation. Among these cytokines, TNF-α is a well-studied proinflammatory cytokine during intestinal inflammation (Rogler G and Andus T 1998). Macrophages located within lamina propria are the major source of TNF-α during intestinal inflammation (Neurath MF et al 1997) and over expression of TNF-α is the common feature of any animal model of intestinal inflammation (Rogler G and Andus T 1998). Interestingly, TNF-α knockout mice were less susceptible to TNBS induced colitis (Neurath MF et al 1997), while the other hand, TNF-α transgenic mice were highly susceptible to TNBS induced colitis (Neurath MF et al 1997). TNF-α blockade by using specific antibodies or pharmacological agents were shown to reduce intestinal inflammation (Murthy S et al 1999).

Dysregulated TNF-α production has been implicated in various inflammatory disorders including rheumatoid arthritis, IBD and psoriasis (Chatzantoni K and Mouzaki A 2006). It is known that TNF-α promotes inflammatory reactions by recruiting activated inflammatory cells to the site of injury or inflammation (Sun WY et al 2010). TNF-α activates NF-κB, signaling pathways in promoting inflammation (Escobar GA et al 2006). IL-6, a B-cell stimulator factor-2 plays an important role in the intestinal inflammation (Mudter J and Neurath MF 2007). Increased IL-6 expression is frequently reported in IBD and in animal models of intestinal inflammation (Mudter J and Neurath MF 2007). IL-6 blockade helps to reduce the severity of colitis in both experimental and clinical cases (Mudter J and Neurath MF 2007).

IL-12 is mainly produced by activated macrophages which are involved in the Th1 responses (Tozawa K et al 2003). Up-regulation of IL-12 production has been demonstrated in TNBS-induced colitis mouse model (Tozawa K et al 2003). IL-23 plays an important role in the inflammatory response against infection, and the expression is increased in IBD (Sarra M et al 2010). IL-23, together with TGFβ and IL-6 stimulate naïve CD4+ T cells to differentiate into Th17 cells, which secrete IL-17 (Sarra M et al 2010). IL-23 and IL-17 cytokines act together to participate in gut immunity and intestinal inflammation (Feng T et al 2011). Interestingly, IL-10 is known to reduce intestinal inflammation (Tomoyose M et al 1998, Paul G et al 2011 and Yanaba K et al 2011). In fact, IL-10 knockout mouse is more susceptible to colitis (Larmonier CB et al 2011).

Mushrooms and inflammation

Mushroom extracts obtained from *Inonotus xeranticus*, *Pholiota nameko* mushrooms showed anti-inflammatory effects on in-vitro and in-vivo models (Lee YG et al 2008 & Li H 2008). Isolated mushroom constituents have been shown to have beneficial effects on experimental inflammation and cancer. Many of these compounds are large polysaccharides or β-(1→6)-branched β-(1→3)-linked glucans. β-glucan is one of the active components believed to be responsible for the beneficial effects of mushrooms.

Prophylactic administration of water soluble β-glucans attenuated lipopolysaccharide induced shock in rats (Sandvik A et al 2007). Davallialactone, a polysaccharide present in

the *Inonotus xeranticus* mushrooms inhibited signaling cascade through nuclear factor kappa B (Lee YG et al 2008).

Although mushrooms are known for their high concentration of β-glucan, mushrooms also contain polyphenols and ergothioneine (Mau J 2002 & Akanmu D 1991). These bioactive compounds act as an antioxidant thereby suppressing chronic inflammation. In an *in vitro* study using human aortic endothelial cells (HAEC), the extracts of WBM reduced the IL-1β induced expression of adhesion molecules such as VCAM-1, ICAM-1, and E-selectin-1 (Martin KR 2010)

β-glucan obtained from *Agrocybe chaxingu* has been shown to inhibit LPS-induced nitric oxide (NO) and cyclooxygenase-2 (COX-2) expression in Raw 264.7 cells, a murine macrophage cell line (Lee BR, 2009). Moreover, topical application of this β-glucan reduced ear edema which is caused by an inflammatory agent 12-O-tetradecanoylphorbol 13-acetate (TPA)-TPA-induced ear edema mouse model (Lee BR, 2009). Interestingly, oral administration of proteoglycan derived from medicinal mushroom scientifically known as *Phellinus linteus* reduced the severity of collagen-induced arthritis (CIA) by suppressing pro-inflammatory cytokines, including IL-12, TNF-α, and IFN-γ (Kim GY 2003).

In an *in vivo* study, ethanolic extract of *Phellinus linteus* was shown to have anti-inflammatory effect in croton oil -induced ear edema model and acetic acid-induced writhing test mouse model (Kim SH 2004). Similarly, 70% ethanolic extracts of *Cordyceps militaris* not only showed anti-inflammatory activities in the croton oil and

carrageenin-induced edema but also anti-angiogenic and antinociceptive activites (Kim SH 2004).

Mushrooms and intestinal inflammation

Oral administration of water soluble extracts from the edible mushroom "*Pleurotus pulmonarius*" attenuated the 3.5% DSS induced acute colitis in mouse model (Lavi I 2010). The protective action of "*Pleurotus pulmonarius*" was due to its inhibitory effect on pro-inflammatory molecules including TNF-α, IL-1β and myeloperoxidase. In another study, oral supplementation of *Pleurotus ostreatus,* an edible mushroom has been shown to inhibit tumor growth in DSS induced colorectal cancer mouse model in a dose dependent manner (Jedinak A 2010). Moreover, *Pleurotus ostreatus* supplementation suppressed the expression of COX-2 and cyclin D1 in this mouse model. (Jedinak A 2010).

Han ES et al (2011) have studied the anti-inflammatory of *Cordyceps militaris* extract on DSS-induced acute colitis model. In this study, *Cordyceps militaris,* a medicinal mushroom elicited anti-inflammatory activity in both DSS-induced colitis mouse models and LPS-stimulated RAW264.7 *in vitro* cell culture. Similarly, water soluble extract of Maitake mushroom (*Grifola frondosa*) has been shown to protect TNBS-induced colitis in rat models. (Lee JS 2010). The beneficial effect of Maitake mushroom appears to be through suppression of TNF-α.

Unlike previous studies, whole mushroom powder (2% WBM) supplementation caused increased TNF-α levels in the colon and serum during DSS induced colitis. 2% WBM

supplementation protected these mice from DSS induced colonic injury in spite of their pro-inflammatory response (Yu et al 2009)

β Glucans on Immunity and Inflammation

β Glucans are naturally occurring complex homopolymers of glucose found in integral cell wall of plants and fungi. These polysaccharides have 1, 3- or 1-4 beta linkage with with or without β -1, 6-(D)-glucose side chains. β glucans have attracted much attention due to their biological response modifying (BRM) action that potentiates and modulates the immune response.

In a recent study conducted by Kim et al, authors used sparan, a 1,3-β-D-glucan isolated from a medicinal mushroom called *Sparassis crispa* for activating dendritic cells. Different mouse strains including TLR4$^{-/-}$ knock-out mice and TLR4-mutated C3H/HeJ were used to collect spleenocytes and bone marrow derived dendritic cells (DCs) (Kim et al 2010). Surface markers of dendritic cell maturation such as CD40, CD80, CD86, and MHC-I/II molecules were evaluated by flow cytometry whereas cytokines (IL-12, IL-1β, TNF-α, and IFN-α/β) secretion were assessed by ELISA method. Addition of sparan caused phenotypic and functional maturation of dendritic cells (DCs) derived from TLR4-mutated C3H/HeJ mice. On the other hand, sparan did not cause maturation of DCs generated from bone marrow cells of tlr4$^{-/-}$ knock-out mice. This clearly suggests TLR4 receptor is required for sparan action. Moreover, addition of sparan increased phosphorylation of ERK, p38, and JNK, and enhanced nuclear translocation of NF-κB p50/p65 in DCs which are the downstream of TLR4 signaling molecules (Kim et al 2010).

In another study, authors Agarwal S et al, used curdlan, an isolated β—glucan, to activate human dendritic cells. Dendritic cells were isolated from blood obtained from healthy volunteers. Cytokines secretion including IL-23, IL-1β, IL-6 and IL-12p70 were determined by ELISA. Cytotoxic T lymphocyte assay was performed against target tumor cells. In this study, the curdlan-stimulated DCs helped not only to polarize naïve CD4 cells into Th17 and Th1 cells but also induce differentiation of B cells to secrete IgG and IgA (Agarwal S et al 2010). Moreover, curdlan-stimulated DCs enhanced the cytotoxic T lymphocyte activity in vitro (Agarwal S et al 2010). These results suggest curdlan as a promising agent/ adjuvant to stimulate mucosal and systemic immune response in humans.

Unlike earlier studies, in this study Volman JJ et al, used dietary form of β--glucan rather than isolated β--glucan compounds for activating bone marrow-derived macrophages. Moreover, authors used Caco-2 cell line, a human enterocyte cell line in addition to bone marrow-derived macrophages from mice. Effects of mushroom extracts on nitric oxide production by bone marrow-derived macrophages (BMMs) from mice and on nuclear factor-kappaB transactivation in human intestinal Caco-2 cell were evaluated. All mushroom extracts containing β--glucan lowered nuclear factor-kappaB transactivation in Caco-2 cells. In this study, white button mushroom extract containing β--glucan (*Agaricus bisporus*) stimulated BMM, without activating Caco-2 cells (Volman JJ et al 2010). These results suggest white button mushroom might be considered as an adjuvant to stimulate immune response.

Non-alcoholic fatty liver disease (NAFLD)

Non-alcoholic fatty liver disease (NAFLD) is a spectrum of disease ranging from simple steatosis, to inflammatory steatohepatitis (NASH) with fibrosis that leads to cirrhosis (de Alwis NM and Day CP 2008). As the term "Non-alcoholic" infers the development of fatty liver occurs in the absence of excessive alcohol intake. NAFLD is increasingly being recognized as "hepatic manifestation of the metabolic syndrome" (Lewis GF et al 2002). This is due to its intimate relationship with obesity and insulin resistance (Sanyal AJ et al 2001). Moreover, NAFLD has been frequently observed in the obese and type 2 diabetic populations (Vuppalanchi R and Chalasani N 2009). Due to the prevalence in obesity and type 2 diabetes, NAFLD has emerged as the most common cause of liver disease in the Western world. Interestingly, NAFLD has also been observed in lean Asian population (Kausik Das et al 2010). This suggests that factors other than obesity, alteration in glucose metabolism might contribute to the development of NAFLD in this population (Kausik Das et al 2010).

Despite recent advances in elucidating the complex metabolic and inflammatory pathways involved in NAFLD, the etio-pathogenesis of NAFLD is not yet fully understood (Petta S et al 2009). Although the etiology of NAFLD may vary from populations, it is evident that NAFLD is a global public health problem (Everhart JE and Bambha KM 2010). Histopathology of liver is the gold standard in diagnosing clinical cases of fatty liver (Shifflet A and Wu GY 2009). Moreover, histopathology helps to differentiate simple fatty liver disease from NASH (Kleiner DE et al 2005). However, patients have to undergo invasive procedures such as liver biopsy for histopathology

(Shifflet A and Wu GY 2009). Hence, developing non-invasive diagnostic tests for NAFLD has become imperative (Shifflet A and Wu GY 2009).

Animal models of NAFLD

To understand the pathogenesis of NAFLD, numerous cross-sectional studies have been conducted (Vernon G et al 2011). In humans the process of NAFLD from simple fatty liver to NASH takes decades which makes it difficult to obtain the quality data and the ethical constraints in obtaining human liver tissue limit the human studies. Therefore, much attention is being focused on the development of animal models of NAFLD recently (Hebbard L and George J 2011). Moreover, these animal models can be used as reductionist approach to evaluate the role of a gene or dietary factor or combination of both in the development of NAFLD (Hebbard L and George J 2011).

Diet induced NAFLD animal models are widely used to study the pathogenesis of NAFLD. High fat, fructose, cholesterol diets and low methionine & choline diets are some examples of diet-induced NAFLD animal models (Zou Y et al 2006, Spruss A & Bergheim I 2009, Matsuzawa N et al 2007 and Rinella ME et al 2008). However, none of these animal models exhibit the complete range of histopathological, metabolic and biochemical changes associated with progression of NAFLD in humans (Hebbard L and George J 2011). This may be due to species difference in the metabolic response to fat overload. Genetically altered mice such as adiponectin null, TLR9 null in combination with high fat diet have also used to study the NAFLD (Asano T et al 2009 and Miura K et al 2010).

Cytokines and NAFLD

Proinflammatory cytokines such as TNF-α and IL-6 have been implicated in the development of obesity-associated insulin resistance and fatty liver (McClain CJ et al 1999). In animal models of obesity, increase in TNF-α were found not only in adipose tissue but also in liver suggesting the possible role of TNF-α in diet induced fatty liver (Xu H et al 2002 and Feldstein AE et al 2004). Further, *in vivo* administration of TNF-α impairs insulin action, (Peraldi P et al 1996 and Hotamisligil GS et al 1996) whereas TNF-α blockade improved insulin sensitivity in animal models of obesity (Uysal KT et al 1997). Plasma TNF-α levels and TNF-α polymorphisms are positively correlated with the degree of insulin resistance and NAFLD in humans. (Wellen KE et al 2005). It appears that TNF-α-induce insulin resistance and fatty liver through Jun N-terminal kinase (JNK) pathway (Shoelson SE et al 2006). Accumulating evidence based on experimental and clinical studies strongly supports the role of TNF-α in the inflammation, insulin signaling, and fatty liver.

Unlike TNF-α, IL-6 plays dual role in inflammation and NAFLD. Immune cells, endothelial cells, and adipocytes are the major sources of plasma IL-6 (Mohamed-Ali V 1997). It has also been reported that hepatocytes are capable of producing IL-6 (Jung BD et al 2000). IL-6 is shown to improve hepatic regeneration and repair during acute administration of IL-6 into hepatocytes (Jin X et al 2006). On the other hand, chronic exposure to excess IL-6 sensitizes the hepatocytes to apoptosis (Jin X et al 2006). Convincing in vitro and in vivo studies have been postulated that IL-6 as a contributing factor in the hepatic manifestation of the metabolic syndrome (Klover PJ et al 2002). Similar to TNF-α, IL-6 is also elevated in plasma, adipose tissue and liver in both

experimental and clinical NAFLD (Polyzos SA et al 2009). In clinical NAFLD, increased IL-6 expression in hepatocytes and Kupffer cells is positively correlated with NAFLD progression (Dogru T et al 2008). In summary, the the elevated plasma IL-6 might be the surroagte biomarker for fatty liver disease.

CHAPTER III

Manuscript-1

Supplementation of edible mushrooms up- regulates IL-23 secretion in both in-vivo and in-vitro studies in C57BL/6 mice and murine macrophage cells.

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Short running title: Mushrooms and IL-23 secretion

Source of funding: The Mushroom Council/USDA, Oklahoma State University start-up funds, and OCAST (Oklahoma Center for Advancement of Sciences and technology).

Abstract

Background: IL-23, a novel cytokine produced primarily by dendritic cells, is involved in host defense against gut pathogens such as *Vibrio cholerae* and *E. coli* and promotes gut immune function through the IL-23/IL-17 axis. Although certain exotic mushrooms have long been used in Asia for treatment and/or prevention of infection due to their immuno-stimulatory properties, there is a paucity of data on the effect of white button (WBM) and portabello (PM) mushrooms on IL-23 and gut immunity.

Hypothesis: Since the IL-23/IL-17 axis is involved in the production of defensins in gut mucosa, we hypothesized that edible mushroom supplementation may enhance gut immunity by up-regulating IL-23 production both *in vivo* and *in vitro.*

Methods: To test our hypothesis, we conducted *in vivo* and *in vitro* studies using C57BL/6 mice and a murine macrophage cell line

Two-month-old female C57BL/6 mice were fed either AIN93-control diet or the AIN93 diet supplemented with 5% WBM, PM or shiitake mushrooms (SM) (n=16/group) for six weeks. To assess *in vivo* cytokine secretion, 8 mice in each group received 3% dextran sodium sulfate (DSS) in their drinking water during the last 5 days of the 6-week feeding period. Upon euthanasia, blood was collected, centrifuged, and plasma was aliquoted for cytokine studies. The length of colon tissue was assessed to determine the shortening of colon during inflammation. The severity of colon inflammation and number of lymphoid follicles were assessed by histopathology. Sections of colon were also homogenized, centrifuged, and supernatants were collected and processed for subsequent determination of cytokine expression.

Single cell suspensions were prepared from spleens of DSS treated and untreated mice fed mushroom-fortified and unfortified diets. To assess the effect of mushroom and DSS treatments on cytokine secretion by non-gut immune cells, 2 $x10^6$ spleen cells/ml were incubated with Concavalin A, Lipopolysaccharide (LPS) (2.5 µg/ ml) or culture medium (baseline) for 72 h. Cells were then centrifuged and the supernatants were collected and stored for cytokine assays.

To further assess the mechanisms by which mushrooms alter cytokine secretion the murine macrophage cell line, J.744.1 cells were incubated with curdlan, a dectin-1 agonist (100 µg/ml), LPS (100 ng/ml), WBM, PM, and SM (100 µg/ml, each) alone or in combination with curdlan or 1 mg/ml laminarin (a dectin-1 antagonist) for 3 and 6 days for cytokine assay. Cytokines IL-23, IL-6, TNF-α, and myeloperoxidase (MPO), an indicator of neutrophil infiltration were measured in plasma, colon homogenate and spleen cell supernatant by using ELISA kits.

Results: In DSS-untreated mice, mushroom-supplemented diets increased plasma IL-23 (2 to 15-fold); but decreased IL-6 (45%-98%; $p<0.05$). In DSS-treated mice, mushroom-supplemented diets increased IL-6 (1.4 - 3.7 fold) and IL-23 (10-50 fold) ($p<0.05$). DSS alone increased plasma IL-6 and IL-23 by$\geq$ 4 fold ($p<0.05$), with the highest increase occurring in mice fed WM-diet. Similarly, colonic IL-6 and MPO expression or productions were elevated after DSS administration. Mushroom-supplementation did not elicit an increase in *ex vivo* IL-6 secretion by activated spleen cells from these DSS treated mice suggesting the main role of mushrooms on gut immune cells activation.

After DSS treatment, all the mice irrespective of their dietary treatments developed inflammation and infiltration of neutrophils and mononuclear cells which are evidenced by colon histopathology and high inflammation scores. Among dietary treatments, WBM had massive infiltration of neutrophils and mononuclear cells with high mean inflammation score (3.6 WBM vs 1.8 Control) followed by PM, SM and control. Similarly, the WBM treated mice had elevated colonic MPO (64.7 ng/mg of protein in WBM versus 51.2 ng/mg of protein in Control) and decreased colon length. After DSS treatment, only mice fed the control diet showed increased number of lymphoid follicles compared to those fed mushroom fortified diets. PM extracts, but not other mushrooms, up-regulated IL-23 secretion by J.744.1 cells on the day 3 of incubation. All mushroom extracts potentiated curdlan-induced IL-23 secretion. Further, WBM, PM induced IL-23 secretion was completely blocked by the dectin-1 antagonist, laminarin.

Conclusion: Data suggest that mushroom consumption may increase gut immunity and provide resistance against certain pathogens through the up-regulation of IL-23. These findings suggest the Dectin-1 pathway is the major pathway in WBM and PM induced IL-23 production.

Introduction:

The gastrointestinal (GI) tract is vital organ in nutrient absorption and host defense against pathogens (Nagler-Anderson C 2001). As much as 90% of pathogens enter into the body through the mucosa (Nagler-Anderson C 2001). The GI immune system is composed of mesenteric lymph nodes, Peyer's patches and lamina propria associated lymphocytes (Mowat AM 2003). Unlike other immune systems in the body, the GI immune system has the constant challenge of responding to pathogens without responding to food antigens or the commensal microflora (Mowat AM 2003). Cytokines produced by the intestinal dendritic cells play a major role in orchestrating the innate and adaptive arms of the immune system (Perdigon G et al 2002). Although several cytokines are involved in mucosal defense against pathogens, IL-23 is crucial in the fight against bacterial and fungal pathogens encountered in GI mucosa (Doisne JM et al 2010& Brereton CF et al 2011).

IL-23 is a cytokine produced primarily by dendritic cells and to some extent by paneth cells (Doisne JM et al 2010 & Ciccia F et al 2009). In conjunction with IL-6 and TGF-β1, IL-23 stimulates naive $CD4^+$ T cells to differentiate into a novel subset of cells called Th17 cells (Maloy KJ & Kullberg MC 2008). These Th17 cells secrete IL-17, a cytokine which is involved in the primary and first line of defense against pathogens by recruiting neutrophils to the site of inflammation (McAleer JP& Kolls JK 2011). Further, the IL-

23/IL-17 axis plays a major role in local intestinal inflammation and production of defensin in gut mucosa (Uhlig HH et al 2006).

Therefore, the IL-23/IL-17 axis is involved both directly and indirectly in combatting most GI pathogens, including *Vibrio cholera, E. coli* and *Salmonella* (Doisne JM et al 2010). IL-23 also promotes immunological memory by stimulating $CD4^+$ T memory cells proliferation. Hence, increasing mucosal IL-23 production might improve mucosal immunity/immunological memory (Agrawal S et al 2010).

Although most GI tract pathogens have been eradicated, food borne diseases such as Salmonellosis, Campylobacteriosis, *E. coli* infections, listeriosis, and Cholera are still major public health problems (Meyer C et al 2010&de la Cabada Bauche J, Dupont HL 2011). Several antibiotics have been developed to treat GI tract infections, including the above mentioned food borne diseases; however, a search for alternatives to these antibiotics is necessary due to the emergence of antibiotic resistant bacteria (DuPont HL 2005). Moreover, these antibiotics can destroy or alter the commensal bacteria present in the GI tract which leads to the condition called intestinal dysbiosis. It is now believed that dysbiosis is a contributing factor to many chronic and autoimmune diseases such as irritable bowel syndrome, inflammatory bowel disease, rheumatoid arthritis, and ankylosing spondylitis (Hawrelak JA,Myers SP 2004).

One alternative to antibiotic therapy is to increase the production of endogenous antimicrobial peptides such as defensins by using natural compounds or functional foods (Cunliffe RN 2003 &Wehkamp J et al 2005). In this regard, several mushroom varieties have emerged as a functional food to stimulate the gut innate and adaptive immune

system (Brochers AT et al 2008). Although medicinal mushrooms such as Shiitake have long been used in Asia for treatment and/or prevention of infection due to their immuno-stimulatory properties, there is a paucity of data on the effect of white button (WM) and portabello (PM) mushrooms on IL-23 and gut immunity in general (Brochers AT et al 2008).

Previously, we found that the extracts from edible mushroom enhance α-defensin production in the human promyelocytic cell line HL60 (Solo Kuvibidila& Kiranmayi Korlagunta). Since the IL-23/IL-17 axis is involved in the production of defensins in gut mucosa, we hypothesized that: (a) the edible mushroom supplementation may enhance gut immunity/inflammation by up-regulating IL-23 production in the gastrointestinal tract and to a less extent in peripheral lymphoid organs such as spleen; and (b) mushrooms up-regulate IL-23 through the dectin-1 pathway.

Experimental design and methods:

Two types of studies were conducted: a mouse model study and a murine macrophage cell line study.

i. Animal study:

*Animal feeding***:** The protocol was approved by IACUC of Oklahoma State University and all the experimental procedures were strictly followed as per the approved protocol. Two-month-old female C57BL/6 mice (n=64) were purchased from Jackson Laboratories and acclimatized to our laboratory for a week. After this acclimation, mice were fed either AIN93-control diet or the same diet supplemented with 5% WM, PM or SM (n=16/group) for six weeks. To assess *in vivo* and *in vitro* cytokine secretion, 8 mice in

each group received 3% dextran sodium sulfate (DSS) in drinking water during the last 5 days of the 6-week feeding period. All mice were sacrificed immediately after DSS treatment.

Tissue collection and Histopathology:

Upon euthanasia, blood was collected for plasma cytokine analysis and spleen was collected from each mouse in a sterile manner and weighed. Thymus was also removed and weighed for each mouse. In order to measure the length of colon, the entire portion of large intestine from cecum to anus was removed and the length was evaluated as previously described (Okayasu I et al 1990). After measuring the colon length, a small piece from the distal part of the colon from each mouse was collected in a vial containing 10% buffered formalin for histopathological analysis. The remaining portion of colon was frozen for colon homogenate. The severity of colon inflammation and the presence of number of lymphoid follicles were evaluated in a blinded manner by the study pathologist. The inflammation score was based on the extent of infiltration of inflammatory cells, mucosal injury, loss of crypts and surface epithelium on a scale from 0 to 4 (0–4: none, basal 1/3 damaged, basal 2/3 damaged, only surface epithelium intact, entire crypt and epithelium lost) (Vowinkel T et al 2004).

Colon homogenate for cytokines and MPO assay:

Colons were homogenized in 1 ml ice-cold lysis buffer (Radio-immunoprecipitation assay, RIPA buffer) by using a tissue homogenizer (VWR PowerMax Advanced Homogenizing System 200 987556). The buffer contained 1% protease inhibitor cocktail and 1% phosphatase inhibitor cocktail. The lysate was centrifuged (Microcentrifuge 5415

R) for 15 min at 1400 × *g*, 4°C and the supernatant transferred to Eppendorf tubes for cytokines and MPO assay (In-Ah Lee et al 2010).

*In vitro cytokine secretion by spleen cells***:**

Spleen single cell suspensions from various mice were prepared by filtration through a cell strainer (Becton Dickinson) as previously described (Kuvibidila SR et al 1998). Cells were centrifuged at 290 *x g*, and red blood cells were lysed by ice cold deionized water followed by centrifugation.Cell membrane and viability were determined by trypan blue exclusion test. Spleen cells (2 x 10^6 cell/ml, 24-well plates) were cultured in complete RPMI 1640 medium supplemented with 100 ml/L FBS, mmol/L HEPES, 2 mmol/L glutamine, 100 U/L penicillin and 100 mg/L streptomycin (Invitrogen). To assess the *ex vivo* cytokine secretion cultured-splenocytes were incubated with LPS, (2.5 μg/ml), Con A (2.5 μg/ml) or culture medium only for 72 h. At the end of incubation period, plates were centrifuged (Eppendorf Centrifuge 5810) at 290 *x g* for 10 min at 4°C. Supernatants were collected and aliquots of 500 μl were immediately frozen at -80ºC until used for cytokine assays by enzyme-linked immunosorbent assay (ELISA).

ii. Murine macrophage study using J.744.1 cells:

In order to assess the mechanisms by which mushrooms alter IL-23 secretion and/or other cytokines, we used a mouse cell line J.744.1 (ATTC, Manassas, VA 20108, USA). Under certain conditions, these cells can be induced to differentiate into dendritic cells (Jiang X et al 2008). J.744.1 cells were cultured in DMEM supplemented with 100 ml/L FBS, 2 mmol/L glutamine, 100 U/L penicillin and 100 mg/L streptomycin (Invitrogen), 1.5 g/L $NaHCO_3$ and 1.5 ml/L β-mercaptoethanol (Schwarzbaum S,Diamond B 1983). Cells

were cultured in a humidified atmosphere of 95% air and 5% CO_2. For cytokine secretion, 5×10^5 cells were seeded into a six-well tissue culture plate (2 ml/well) and were incubated with either curdlan (100 μg/ml), LPS (100 ng/ml), WBM (100 μg/ml), PM (100 μg/ml), SM (100 μg/ml) alone or in combination with curdlan (Sigma: C7821; Curdlan from *Alcaligenes faecalis*), a known dectin-1 agonist (100 μg/ml) or with laminarin (Sigma: L9634; Laminarin from *Laminaria digitata*), a dectin-1 antagonist (1 mg/ml) for 3 days and 6 days. The supernatant from this cell culture were collected and stored immediately in -80^o C until used for cytokine analysis.

Cytokine detection:

Commercially available ELISA kits (R&D Systems, Minneapolis, MN) were used to determine the levels of IL-23, IL-6, TNF-α and MPO in serum, colonic homogenates and the supernatants from spleen cells and J.744.1 cell cultures.

Statistical analysis:

Statistical analyses were performed using SPSS or Microstatistical program. Descriptive statistics were evaluated. Data were expressed mean ± SEM. Post-hoc multiple comparisons were made when the F value in ANOVA reached significance. P values of ≤ 0.05 were considered as significant.

Results:

Effect of dietary treatment on body weight & thymus weight

After DSS administration, all mice showed significant loss in body weight and thymus weight that varied from 25% to 28% as compared to those without DSS (Fig 1A). None

of the dietary interventions prevented thymic atrophy associated with DSS treatment. (Fig 1B)

Effect of mushroom supplementation on plasma IL-23 with or without DSS challenge

In DSS-untreated mice, PM-supplemented diet significantly increased plasma IL-23 ($p < 0.05$; Figure 2A) compared to the control, WBM and SM. In DSS-treated mice, however, all three types of mushrooms significantly ($p < 0.05$) increased plasma IL-23 compared to control group. Among dietary treatment WBM had the highest plasma IL-23 followed by the PM and SM groups ($p < 0.05$; Figure 2B).

Effect of mushroom supplementation and with or without DSS treatment on plasma IL-6 levels

DSS alone increased plasma IL-6 by≥ 3.91 fold ($p<0.05$), with the highest increase occurring in mice fed WM-diet (Figure 3B). Similarly PM supplementation also significantly ($p < 0.05$) increased the plasma IL-6 levels after DSS challenge. However SM did not increase plasma IL-6 levels.

Effect of mushroom supplementation on IL-6 secretion by spleen cells

LPS-activated spleenocytes obtained from mice fed mushroom supplemented diets and treated with DSS did not show any increase in IL-6 secretion (Figure 4A). Moreover, none of the dietary treatments produced alteration in IL-6 that reached the level of statistical significance.

Effect of mushroom supplementation on colon IL-6 and MPO concentration

Regardless of dietary treatment, DSS administration significantly increased MPO levels in the colon homogenates relative to untreated mice (Figure 5A, $p < 0.05$). While WBM supplementation increased colon IL-6 levels, unlike PM, SM decreased them (Figure 4B).

Effect of mushroom supplementation on colon length

There was a significant reduction in colon length following DSS treatment in all mice group (Figure 5B, $p < 0.05$). Mice fed WBM-fortified diet had 35% reduction in colon length as compared to those fed the control diet that showed only 25% reduction in colon length. SM and PM had 28% and 27% reduction in colon length respectively.

Effect of mushroom treatment on colon histology

After DSS treatment, all mice regardless of their dietary treatments, developed inflammation and infiltration of neutrophils and mononuclear cells which are evidenced by colon histopathology and high mean inflammation scores. Among dietary treatments, WBM had massive infiltration of neutrophils and mononuclear cells with high mean inflammation score (Figure 6 (histology) & Figure 7 (inflammation score)) followed by PM, and control (Figure 6 & Figure 7). After DSS treatment, only mice fed the control diet showed hyperplasia of lymphoid follicles (Figure 6 & Figure 7) compared to those fed mushroom-supplemented diets.

Effect of mushroom extracts on in vitro IL-23 secretion by the murine J.744.1

After three days of incubation, only PM extracts significantly induced IL-23 secretion by J.744.1 cells (Figure 8). IL-23 was undetectable in all other cultures. PM induced IL-23 secretion was blocked by addition of laminarin, a dectin-1 antagonist. Following six days

of incubation, IL-23 was detectable in all cultures including control, WBM, PM, WBM+curdlan, PM+curdlan, SM+curdlan. Further, WBM- and PM- induced IL-23 secretion was completely blocked by the dectin 1 antagonist, laminarin. In addition, all mushrooms augmented the curdlan induced IL-23 secretion. Interestingly as observed with the 3 day-cultures, SM did not cause any upregulation of IL-23 production, but dectin-1 blockade lead to production of IL-23.

Discussion:

In this study we used 3% DSS oral administration to investigate the effect of edible mushrooms on gut immunity with respect to innate immunity and acute inflammation. The DSS induced acute colitis model is the accepted model to study innate immunity, acute inflammation and neutrophilic function (Okayasu I et al 1990 &Wirtz S et al 1990). Interestingly, 5% mushroom dietary supplementation significantly increased the plasma IL-23 production in mice challenged with 3% DSS and PM up-regulated the IL-23 production in mice without DSS challenge. Although all mushroom supplementation caused significant increases in IL-23 secretion in DSS treated mice, there was a difference in IL-23 response among mushroom diets. This suggests that there may be differences in immuno stimulatory action of certain varieties of mushrooms on gut immunity.

Since macrophage/dendritic cells are the major source of IL-23 production, we used J.744.1, a mouse monocyte/macrophage cell line to further probe the mechanism through which mushroom extracts up-regulate IL-23 production. In our *in vitro* study, the addition of mushroom extracts to J.744.1 cells caused increased IL-23 secretion. Interestingly PM

up-regulates IL-23 secretion on the 3rd day of treatment unlike other mushrooms. This supports our in-vivo observation regarding PM on IL-23 production in normal mice.

All mushroom extracts potentiated curdlan, a Dectin-1 agonist increase in IL-23 secretion suggesting the Dectin-1 mediated mechanism. Further, WBM, PM induced IL-23 secretion was completely blocked by laminarin, a Dectin-1 antagonist. These findings suggest Dectin-1 pathway is involved in the WBM and PM induced IL-23 production. Surprisingly SM alone was not associated with any up-regulation of IL-23 production. In fact, Dectin-1 blockade lead to the production of IL-23 suggesting Dectin-1 independent mechanism that has yet to be investigated.

Therefore the observed difference in the IL-23 secretion of mice by various mushroom supplementations might be due to the difference in their mechanism of action. Our *in vitro* and *in vivo* findings suggest that edible mushrooms might activate the macrophages and dendrtic cells present in the GI immune system. Mushrooms are the rich source of beta glucan, which is the natural ligand for the Dectin-1 receptor present in the macrophages and dendrtic cells (Brown GD et al 2002 & Taylor PR et al 2002). In a recent study, curdlan, an isolated β glucan and dectin1 agonist showed an increased production of IL-23, IL-6, and IL-1 β in dendritic cells isolated from healthy humans (Agrawal S et al 2010) Moreover, the curdlan-stimulated DC helped to differentiate naïve CD4 cells into Th17 (Agrawal S et al 2010).

In our study, mushroom dietary supplementation also increased plasma IL-6 production. IL-23 in conjunction with IL-6, IL-1 β and TGF-β1, have been shown to stimulate naïve $CD4^+$ T cells to differentiate into a novel subset of cells called Th17 cells which secretes

IL-17 (Dong C 2008,Manel N et al 2008& Cunin P et al 2011). This IL-17 cytokine is involved in the recruitment of neutrophils, the first line of body defense against bacterial pathogens to the site of inflammation (McAleer JP, Kolls JK 2011).

After DSS treatment, all mice, regardless of their dietary treatments developed inflammation and infiltration of neutrophils and mononuclear cells, which is evidenced by colon histopathology and high inflammation scores. Among dietary treatments, WBM had massive infiltration of neutrophils and mononuclear cells with a high mean inflammation score, followed by PM, SM, and control. In addition, the WBM treated mice had elevated colonic MPO level, an indication of an enhanced neutrophilic response. These results suggest edible mushroom dietary supplementation might shift immune response toward a Th17 paradigm through IL-23/IL-17 axis.

Interestingly, mice fed the control diet showed hyperplasia of lymphoid follicles after DSS treatment. However, mushroom supplemented groups showed massive neutrophillic infiltration. Lymphoid hyperplasia is frequently found in the histopathology of colonic tumors (Masaru Kojima et al 2009). These colonic histological findings suggest that mushroom supplementation promotes acute inflammatory response rather than chronic inflammatory response, which may lead to malignancy (Masaru Kojima et al 2009).

Eventhough DSS treated mice lost significant body weight and thymus weight, none of the dietary intervention influenced the body weight and thymus weight. This may be due to short duration of mushroom dietary treatment as we sacrificed the mice immediately after 5 days of 3 % DSS administration.

In summary, mice fed with mushroom diets for six weeks showed an increase in the plasma IL-23, IL-6, colon MPO, colon IL-6, and inflammation score when they were challenged with 3% DSS. Although all mushroom up-regulated IL-23, PM up-regulated IL-23 secretion on the 3rd day of *in vitro* treatment and mice without DSS challenge unlike other mushrooms. All mushroom extracts potentiated curdlan induced IL-23 secretion and mushroom induced IL-23 secretion was completely blocked by laminarin. These findings clearly suggest Dectin-1 is the major pathway in WBM and PM induced IL-23 production. Difference in mushrooms action on IL-23 could partly be explained by their mechanism of action on Dectin-1 pathway. To the best of our knowledge, this is the first report documenting the possible health benefits of WBM and PM, through up-regulation of innate immunity/ inflammation through IL-23 pathway.

Acknowledgements

We would like to thank Ms Anita Pere for her help in proof reading and grammar.

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Funding sources: This project was supported by Mushroom Council/USDA, OSU startup funds, and OCAST.

Figure1 Effect of dietary treatment on final body weight and thymus weight

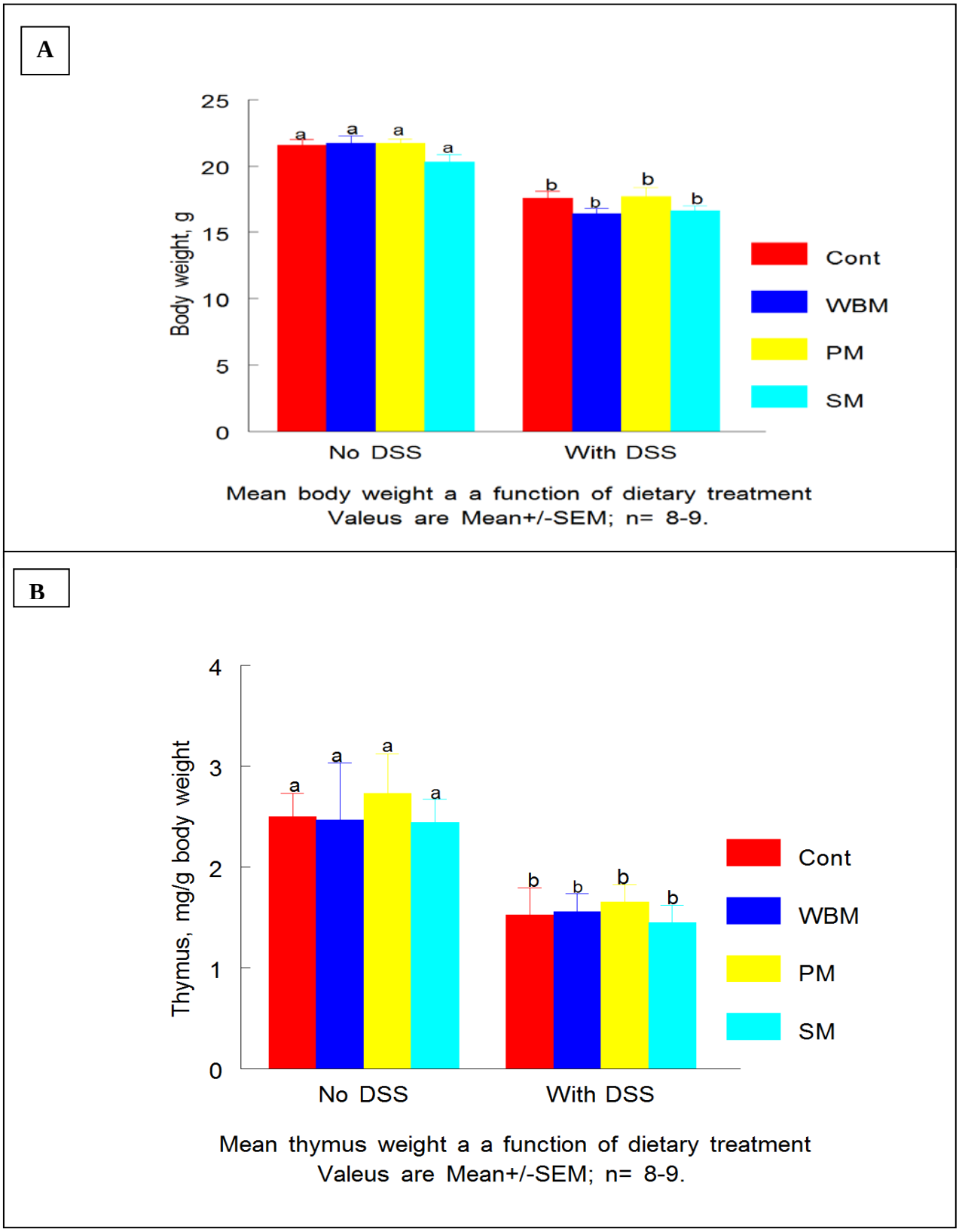


Figure 2 Effect of mushroom supplementation on plasma IL-23 with or without DSS challenge

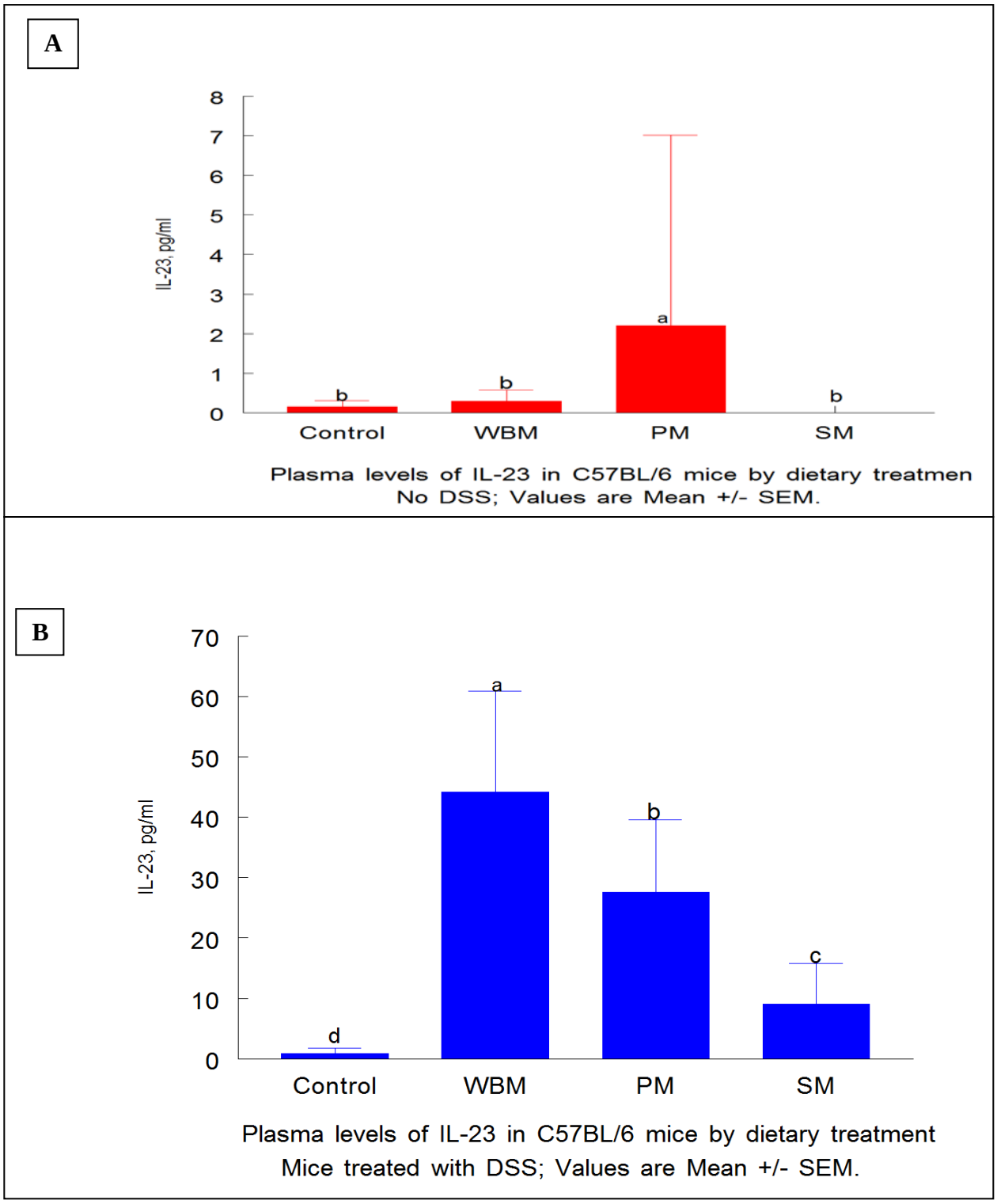


Figure 3 Effect of mushroom supplementation and DSS treatment on plasma IL-6 levels

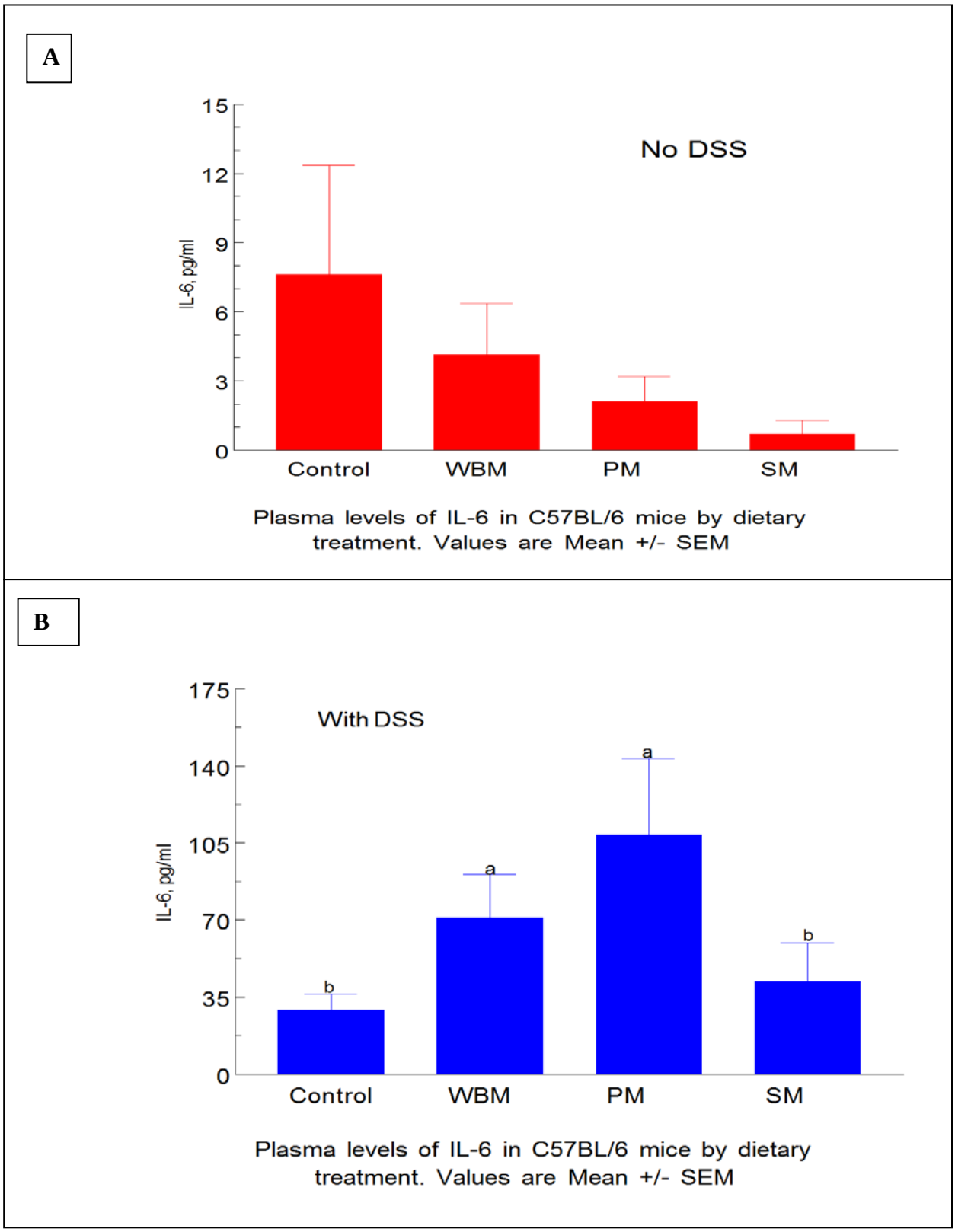


Figure 4 Effect of mushroom supplementation on IL-6 secretion by spleen cells

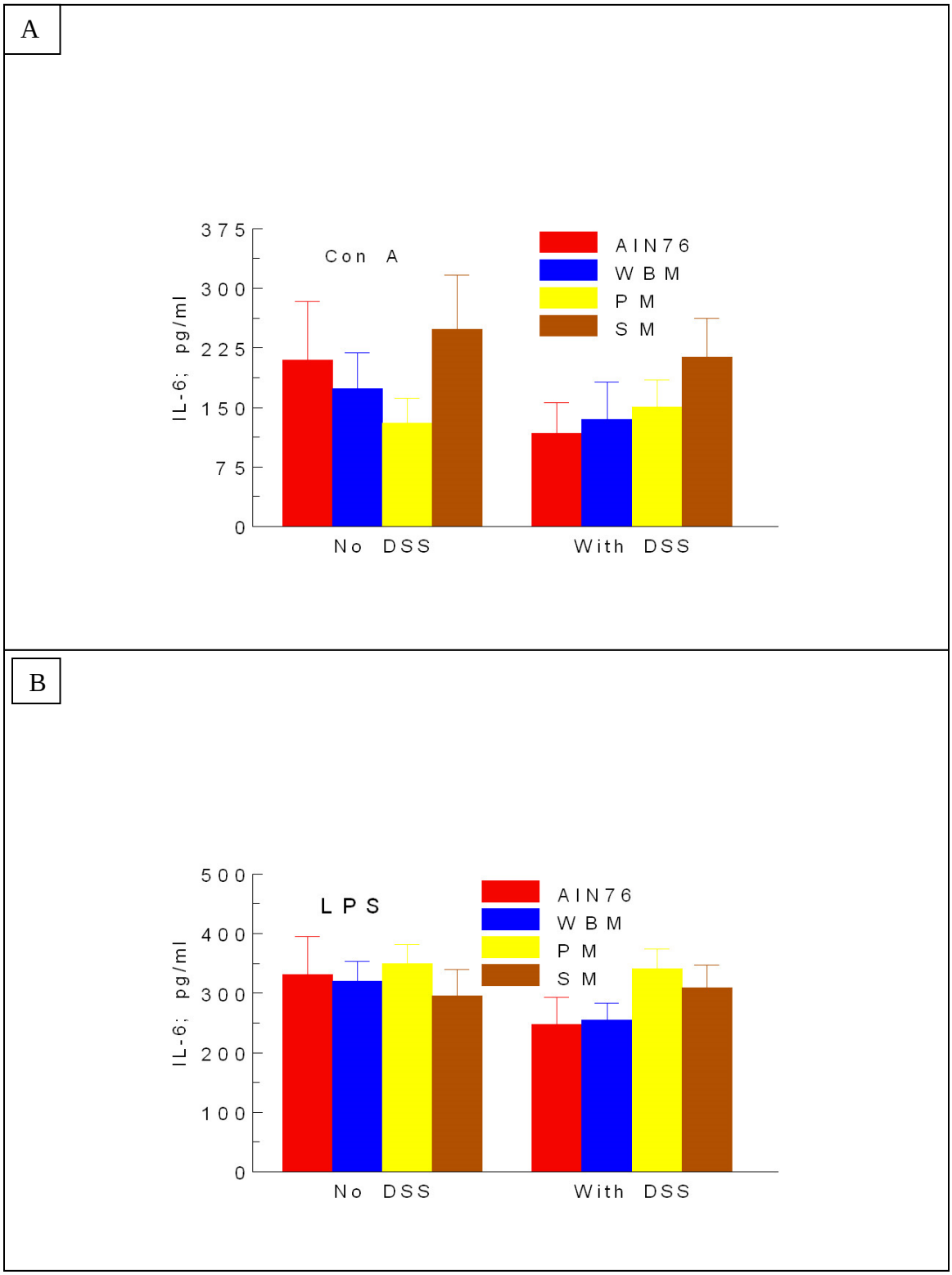


Figure 5 Effect of mushroom supplementation on colon MPO concentration and length

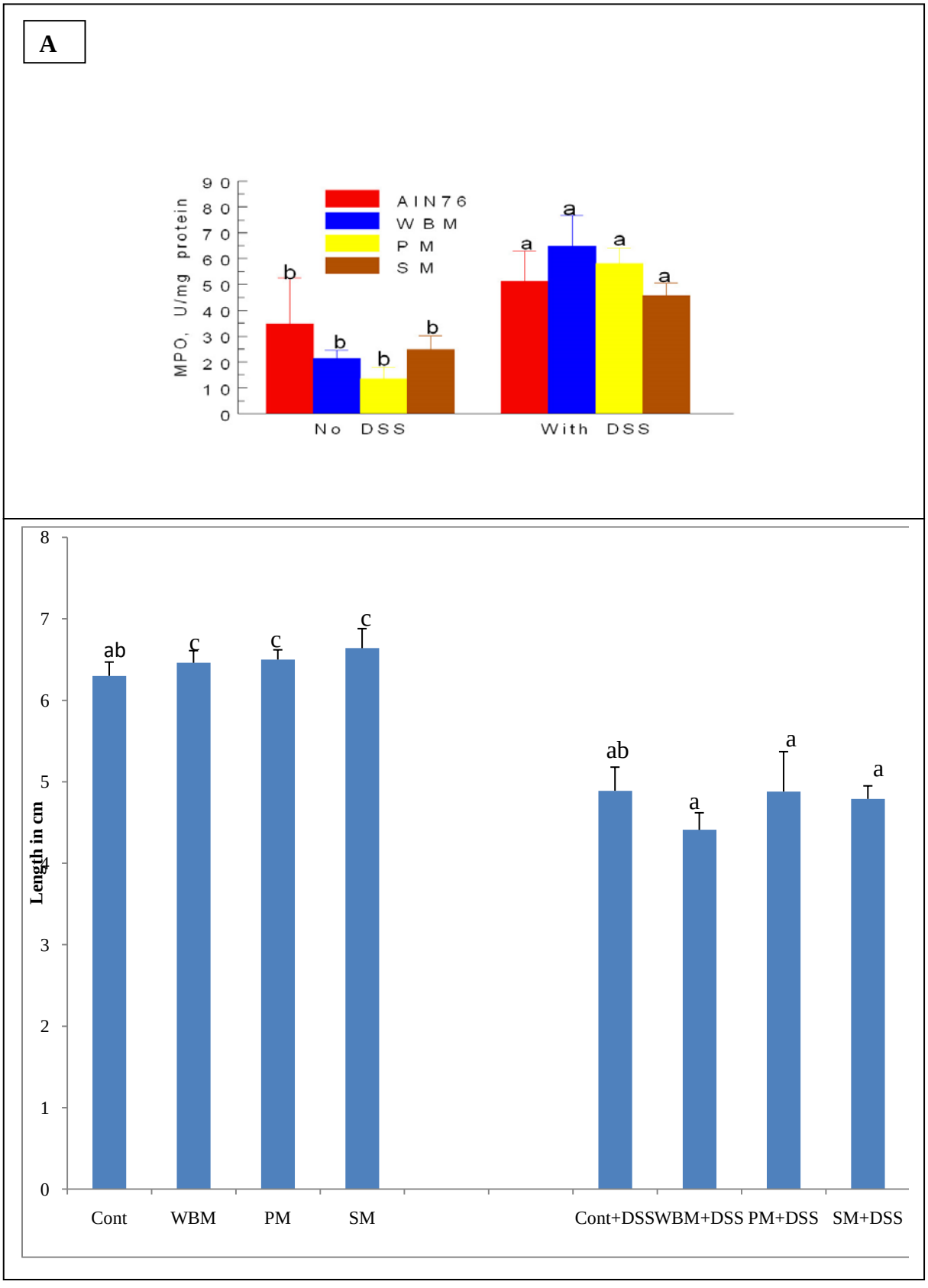


Figure 6A Effect of mushroom supplementation on colon histology

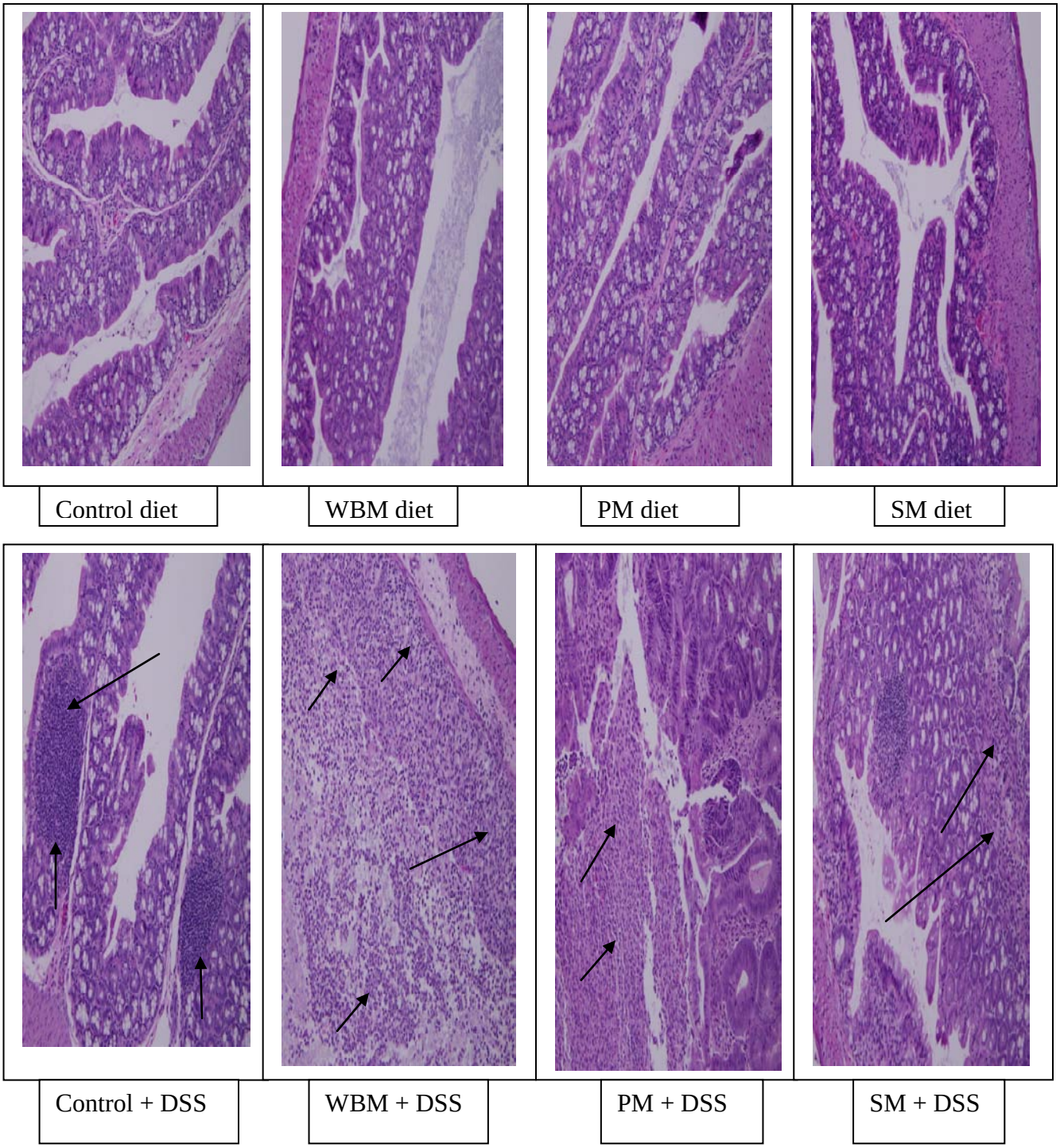


Figure 6B Effect of mushroom supplementation on colon inflammation score

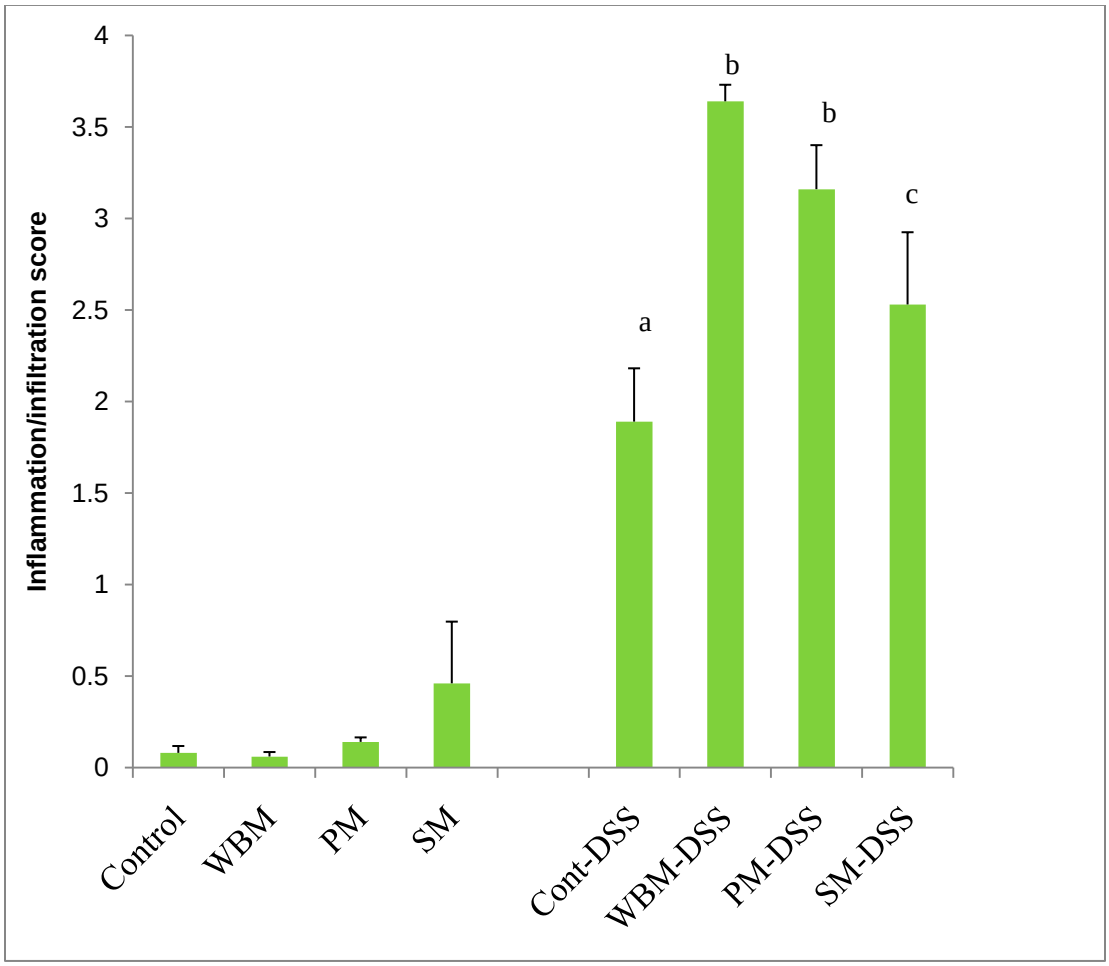
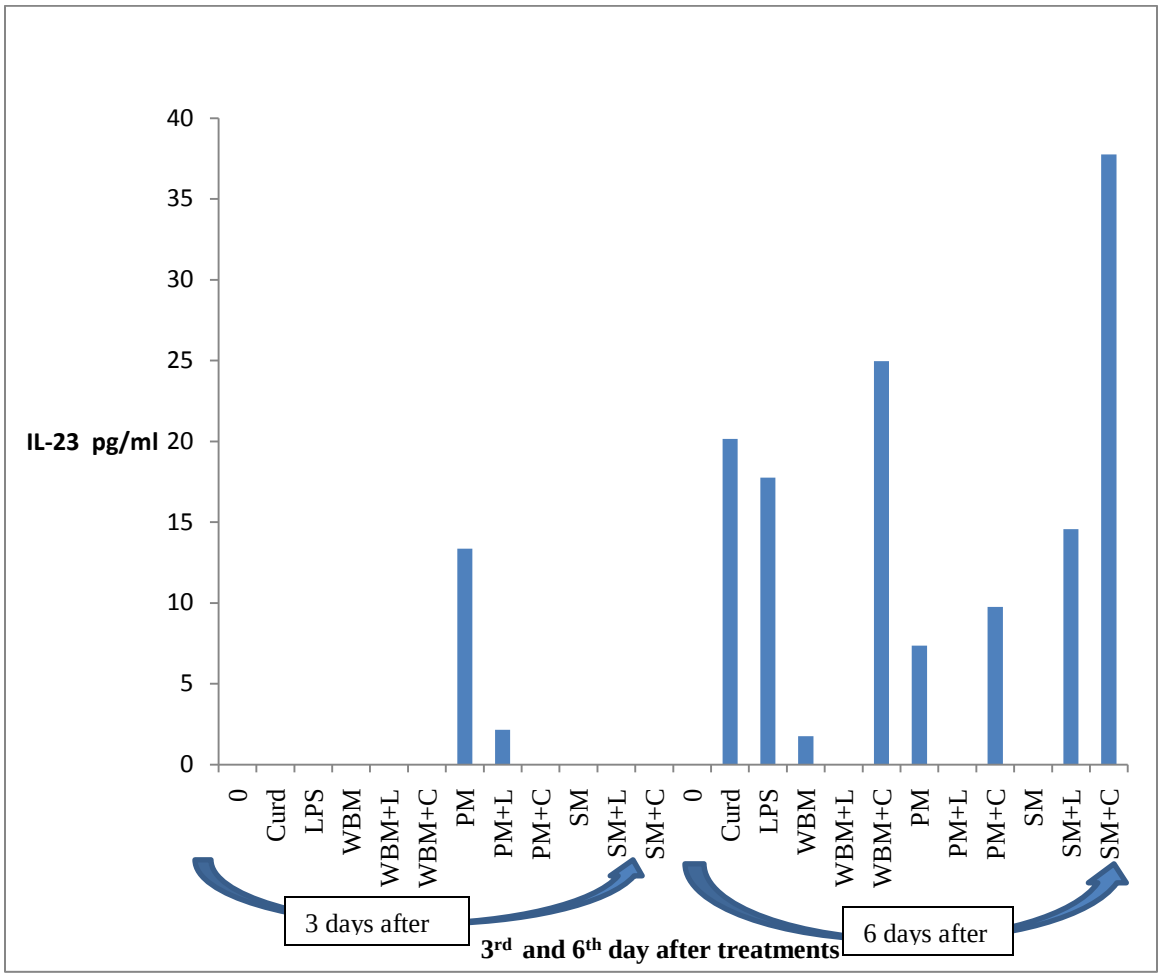


Figure 7 Effect of mushroom extracts on *in vitro* IL-23 secretion by the murine J.744.1**.**



CHAPTER IV

Manuscript-2

Differential effects of shiitake and white button mushroom fortified diets on the development of reversible hepatic steatosis in C57BL/6 mice.

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Source of support: Mushroom Council, USDA (grant #), and Oklahoma State University startup funds.

Key words: fatty liver, inflammation, fibrosis, glycogen, triglycerides

Footnotes:

NAFLD: non-alcoholic fatty liver disease

Abstract:

Shiitake is a medicinal mushroom that has long been used in Asia for treatment and/or prevention of chronic diseases and hypercholesterolemia in humans. Previously, we observed supplementation of the AIN76 diet with 5% lyophilized shiitake elevated plasma IL-6 levels by two-fold in DBA arthritis mice. Considering that high plasma IL-6 has been implicated in fatty liver disease pathogenesis, we hypothesized that consumption of a shiitake supplemented diet would lead to transient hepatic steatosis. Two studies were conducted. In study 1, eight-week old C57BL/6 female mice were randomly assigned to the following groups for 6 weeks: the AIN-93 diet; 5% shiitake (SM), and 5% white button mushroom (WBM) supplemented diets (12 mice/group). In Study 2, mice were fed either the control or SM (20/group). After 6 weeks, 13 mice fed SM diet were given the AIN93 diet for 8 or 15 days. Unlike mice fed the AIN93 and WBM diets, mice fed the SM diet developed fatty liver (mean histopathology score 4.5 vs <1 in the other groups; $p < 0.001$) without fibrosis and inflammation after 6 weeks of supplementation. Fat droplets were detected only in the liver and not other organs. SM moderately reduced liver glycogen content. Fifteen days post withdrawal of SM completely normalized liver histology. Unlike our previous studies, SM supplementation did not increase plasma IL-6 in C57BL/6 mice suggesting a strain difference. Plasma triglycerides were 42% higher in mice fed SM diet for 6 weeks and switched to control diet for 8 days ($p < 0.05$). Data suggest that SM induces simple reversible hepatic steatosis. This is the first time that SM are shown to increase liver fat content and the mechanism of increased liver steatosis warrants further investigation.

Introduction

The shiitake mushroom (SM) is an edible medicinal mushroom, scientifically known as *Lentinula edodes* that native to China and cultivated in East Asia (Raymond Chang 1996). SM was considered a royal food in ancient times due to its medicinal value, delicacy and scarcity. Although SM had been cultivated mainly by traditional methods in East Asian countries including Japan, China and Korea before 1982, the advancement of mushroom production and biotechnology led to large scale commercial cultivation of SM worldwide (Chang & Miles 2004). Presently, SM is the second most cultivated edible mushroom comprising about 25% of the worldwide production followed by the white button mushroom (WBM) (Jiang T et al 2010). In the United States, the SM production has exceeded 9 million pounds per year during 2009 (National Agricultural Statistics Service 2009).

Because of their widespread availability and significant reduction in price, the consumption of SM has increased in recent years. Both fresh and dried forms of SM have been used in many East Asian cuisines including vegetarian "miso soup" of Japan and has been slowly incorporated into western cuisine in recent years (Chang & Miles 2004). Although both forms of SM are used for culinary purposes, some people prefer the dried form of SM due to its flavor and vitamin D content (Chang & Miles 2004). Hence, SM is often sold as a dried powder for commercial use.

In a few uncontrolled clinical studies, dried powder of SM has been used in complementary and alternative medicine (CAM) therapy to treat hypercholesterolemia and immunodeficiency associated with viral infections and cancer (Raymond Chang

1996& Bisen PS et al 2010). Interestingly, we recently observed that fortification of the AIN76 diet with 5% lyophilized SM was associated with a two-fold significant increase ($p \leq 0.05$) in plasma interleukin-6 (IL-6) in DBA mice with collagen-induced arthritis (Chandra L et al 2011). Activated leukocytes, endothelial cells, and adipocytes are the primary sources of circulating IL-6(Pradhan AD et al & Fasshauer M 2003), which usually elevated patients with fatty liver disease (McClain CJ et al 1999). Peripheral blood monocytes from these patients also secrete higher levels of IL-6 in *vitro* (McClain CJ et al 1999). Thus IL-6 is has been implicated in the pathogenesis of fatty liver disease (McClain CJ et al 1999), a review of the literature revealed very limited information available regarding the association between consumption of edible mushrooms and risk of non-alcoholic fatty liver disease (NAFLD).

NAFLD is a disorder of lipid metabolism characterized histologically by hepatic steatosis in the absence of excessive alcohol consumption (James E et al 2010). NAFLD has drawn much attention among gastroenterologists, endocrinologists, cardiologists, physicians and surgeons due to its intimate association with insulin resistance, metabolic syndrome, cirrhosis and end stage liver failure (James E et al 2010). Moreover, the prevalence and health burden associated with NAFLD has increased globally in recent years (James E et al 2010). Although obesity was found to be the major contributing factor in the development of NAFLD in Western populations, the prevalence of NAFLD also occurs in people with relatively low BMI and waist circumference in East Asian countries suggesting other possible factors might be involved in the development of NAFLD in these populations (Kausik Das et al 2010).

Surprisingly, the prevalence of NAFLD in adult Japanese population with normal BMI was 29% even though the exact NAFLD prevalence among the general population remains unknown (Jimba S et al 2005). Among East Asian countries, the prevalence of NAFLD in the population with normal BMI remains high in Japan, the largest consumer of shiitake mushroom in the world (James E et al 2010). Based on our observation of increased IL-6 plasma levels in mice fed SM-fortified diet, and the high prevalence of NAFLD among Japanese, we hypothesized that SM may contribute to the development of fatty liver. We used an animal model to investigate the effect of supplementation of the AIN93 diet with dry-lyophilized SM powder on fatty liver prevalence in C57BL/6 mice.

Materials and Methods

***Chemicals & Reagents*:** WBM and SM were a generous gift from JM Mushrooms in Miami (OK). An endotoxin assay kit was purchased from GenScript (Piscataway, NJ) and AIN93 diet from Teklad (Indianapolis, IN). Triglyceride assay kits were purchased from BioVision Research Products, (Mountain View, CA).

Methods: All the procedures were approved by the IACUC of Oklahoma State University (OSU, Stillwater). Two studies were performed. In the first study, 8-week old female C57BL/6 mice (n = 36; body weight 15-18 g) were purchased from Jackson Laboratories (Bar Harbor, ME). Upon arrival, they were housed at the OSU laboratory animal research facility, 5 mice per cage, and were acclimated to our laboratory while being fed the AIN93 for 7 days. At the end of the adjustment period, mice were randomly assigned to one of the following treatment groups: AIN93 diet; and AIN93 supplemented with 5%

lyophilized white button mushrooms (WBM), or shiitake mushrooms (SM). The nutritional composition of the three different diets is summarized Table-1. All diets were formulated to be isocaloric and normal fat diets.

In the second experiment, 40 eight-week old C57BL/6 female mice were randomly assigned into two dietary treatment groups, the AIN93 (control group) and SM group. After 6 weeks of feeding, mice that received SM were divided in three sub-groups: SM-I continued to receive the SM supplemented diet; SM-II, received the control diet for 8 days; and SM-III, received the control diet for 15 days. During the feeding protocols, all mice had free access to their diets and deionized water, and they were housed under standard 12 h light to dark cycle conditions.

Histopathological analysis: At the time of euthanasia, mice were subjected to CO_2 inhalation for about 60 sec. After they were weighed, the liver, heart and kidneys were collected and fixed with 10% buffered formalin. Tissues were embedded in paraffin, and 5 mm sections were cut and stained with hematoxylin and eosin, periodic acid-Schiff, or Masson's trichrome according to standard techniques. Histopathological changes were scored in a blinded manner by using a modified method adopted from Kleiner DE et al (Kleiner DE et al 2005).

***Measurement of plasma triglycerides, IL-6, endotoxins and aflatoxins*:** Plasma IL-6 was measured by using ELISA kit (R&D Systems, Minneapolis, MN). Triglycerides and endotoxins were assayed by colorimetric methods and the methods provided with the kits ( ) following the manufacturers guidelines or procedure. Samples were sent to Oklahoma Animal Disease Diagnostic Laboratory for aflatoxins estimation in the diets.

Statistical analysis: Descriptive statistics (mean ± SEM), analysis of variance (ANOVA) and/or Student's t test, were performed by Microstatistical Program (Microsoft Inc, Indianapolis, IN). The level of significance was set at $p \leq 0.05$.

Results

Effect of mushrooms on food intake, body weights, and organ weights. All three diets are isocaloric with similar macronutrient (fat, carbohydrates, proteins) and micronutrients except for the 50 g of sucrose that were substituted by lyophilized mushrooms per kg diet (Table 1). Table 2 describes the effect of different dietary treatments on food intake and body weight and vital organs, including the liver. Under the experimental conditions, supplementation of the AIN93 diet with 5% lyophilized SM and WBM did not significantly affect the mean food intake or the mean body weights. Moreover, mushroom supplementation did not affect absolute or relative mean weights of the liver, kidneys, or heart.

Effect of different dietary treatment on organ histology and histochemistry. Compared to mice fed the control (AIN93) diet (Fig 1A), all mice fed SM-fortified diet exhibited signs of hepatic steatosis (Fig 1C and Fig 2). In contrast to SM supplementation, WBM supplementation did not induce fat droplets, and in fact showed normal liver histology and fatty liver score (Fig 1B and Fig 2). While SM reduced liver glycogen content as evidenced by PAS reaction, WBM had no effect on PAS reaction (Fig 1D-F). However neither SM nor WBM induced fibrosis and/or excessive inflammation as assessed by Trichrome staining (Fig 1G-I). Furthermore, SM supplemented diet did not induce histopathological changes in heart and renal tissue sections and suggests that the fat

accumulations are specific to the liver (Figure 3 D-I). We performed our second study to see whether the SM induced hepatic steatosis are reversible or not. Interestingly, as little as 8 days of withdrawal of SM supplemented diet were sufficient to reverse and normalize liver fat content (Fig 3 B & C). By 15 days of feeding the control diet, liver structure was not different from that of mice that received the control diet throughout the study period (Fig 3C).

Effect of SM on plasma triglycerides and IL-6. Contrary to what we expected, mice fed SM supplemented diet for 6 weeks, thereafter given the control diet for 8 days showed elevated serum triglycerides levels compared to those fed the control (Figure 4). WBM had no effect on serum triglyceride levels. Moreover, SM did not elevate plasma IL-6 levels in these mice as compared to control and WBM diets (data not shown)

Discussion

In our first experiment, all mice supplemented with SM alone developed simple hepatic steatosis without showing any inflammatory or fibrotic changes in the liver. Moreover, SM group mice were fed isocaloric and normal fat diet, but not with high fat or fructose diet during SM supplementation. Therefore, the results of the present study suggest that SM can induce moderate hepatic steatosis even within the content of an otherwise adequate diet. However, SM- induced fatty changes in liver histology were normalized within two weeks of SM withdrawal from the diet as observed in the second experiment. This cross over design of our second experiment confirmed that dried powder of SM in the diet contributed, at least in part; to the development of fatty liver observed in SM group mice and the SM induced histological changes in the liver were reversible. Based

on the mild-to-moderate reduction of liver glycogen content, it appears that SM induced the conversion of dietary carbohydrates to fat rather than glycogen, but this was not the case with WBM. This possible induction of fat synthesis in the liver will be the focus of our future studies.

It has been previously shown that both endotoxin and aflatoxin can induce fatty liver (Harte AL et al 2010& Newman SJ 2007). To rule out the possibility that increased hepatic steatosis was due to low level toxins in the mushrooms, we analyzed the dried mushroom powder and diet for the presence of these compounds. However, the levels were barely detectable suggesting that the increased fat content was due to another component in the SM. Moreover, none of the mice from SM showed toxicological changes in the liver, kidneys or heart.

SM-induced fatty changes were confined to the liver, sparing heart and kidneys suggesting liver is the target for SM. One possible or likely explanation for lack of effect of SM is that neither the heart, nor the kidneys are major sites for glycogen storage or fatty acid biosynthesis. Unlike other hepatotoxic agents such as aflatoxin and endotoxin**,** SM did not appear to induce inflammation, zonal necrosis or fibrosis based on histopathological analysis. Moreover, SM induced steatosis did not show any zone specific pattern in liver histology, which further suggests that SM may be inducing the conversion of carbohydrates to fat rather than glycogen.

Since the control diet (AIN93) was used to prepare SM and WBM supplemented diets, and none of the mice fed with WBM diet developed signs of fatty liver, our data suggest some unique components present in the SM might be responsible for the development of

fatty liver. It has been previously shown that SM has a high concentration of eritadenine, a compound that has a hypocholesterolemic effect (Sugiyama K &Yamakawa 1996). Rats fed a diet supplemented with eritadenine showed altered hepatic phospholipid metabolism and decrease in phophatidylcholine/ phophatidylethanolamine ratio (Sugiyama K et al 1995). Therefore it is possible that the eritadenine might be contributing for the fatty liver development in mice fed SM supplemented diets.

Since SM is known to improve lipid profile and atherosclerosis in many animal studies (Sugiyama K &Yamakawa 1996, Sugiyama K et al 1995,Takashima et al 1973 &YamadaT 2002), we hypothesized that SM might reduce the serum triglycerides by accumulating in them in the liver. However, we found elevated serum triglycerides in mice fed SM for 6 weeks then given the control diet for 8 days. This lead to us to examine the SM health benefits against lipedemia and atherosclerosis. In recent years, Liver X receptor (LXR) agonists have emerged as promising drug candidates due to their role in promoting reverse cholesterol transport, promoting action for the treatment and prevention of atherosclerosis in animal models (Venkateswaran A et al 2000). LXR agonists control cholesterol homeostasis by inducing ABCA1 (ATP-binding cassette) cholesterol efflux transporter gene (Calkin AC et al 2010). Moreover, LXR agonists have been shown to have exibit anti-inflammatory effect on rheumatoid arthritis animal model (Park MC et al 2010). We also observed and reported the anti-inflammatory effect of SM on rheumatoid arthritis animal model by reducing TNF-α (Chandra L et al 2011). In addition, SM induced fatty accumulation did not cause inflammatory or fibrotic changes in the liver histology. Taking all this information together, we hypothesize that SM might show its effects through LXR mediated mechanism. The exact molecular

mechanisms by which SM and its bioactive compound cause fatty liver are under investigation.

Based on our experiment, it is evident that 5% SM supplementation can lead to simple reversible fatty liver under isocaloric and low fat diet. In this study we used 5% SM fortification instead of 2% (which is close to the normal fresh mushroom consumption in humans based on 2000 calories/day), because we observed SM health benefits on arthritis animal model at this dose in our earlier study (Chandra L et al 2011). Moreover, SM is often being used as dried powder in the form of soup rather than whole mushroom, which contains 90% water content, leading to high dietary intake. Further, high dose of SM supplement is used in complementary and alternative medicine therapy to treat cancer patients (Boon H et al 2010).

Since non-alcoholic fatty liver disease (NAFLD) has emerged as a major public health problem, our in-vivo observation that SM induced fatty liver has public health importance. There are the two main histological patterns being described in non-alcoholic fatty liver disease namely simple fatty liver and steato-hepatitis (NASH) (James E et al 2010). Unlike simple fatty liver, NASH is the silent killer disease that can lead to cirrhosis and end stage liver failure (James E et al 2010). Invasive procedures such as biopsy and liver histology are required to differentiate simple liver disease from NASH. In our study supplementation SM caused simple fatty liver not NASH.

In summary, the main findings of the current study are: (I) an increase in liver fat content in mice fed SM supplemented diet, but not WBM supplemented diet; (II) a resolution of hepatic steatosis within two weeks of stopping SM diet; (III) lack of effect of mushrooms

on hepatic inflammation. Determining the mechanism of increased fat liver content and mild-to-moderate reduction of glycogen is the focus of our next studies.

Acknowledgements: We would like to thank Ms.Anita Pere for her help in proof reading and grammar; Mr. D. Traore and Miss C. French for their assistance.

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Table 1: Composition of study diets

Ingredients; gram/kg	Control diet	5 % SM fortified	5 % WBM fortified
Casein	200	200	200
DL-methionine	3	3	3
Sucrose	499.99	450	450
Corn starch	150	150	150
Corn oil	50	50	50
Fiber (cellulose)	50	50	50
Mineral mix	35	35	35
Vitamin mix	10	10	10
Choline bitartarte	2	2	2
Ethoxyguin	0.01	0.01	0.01
Mushroom powder	0	50 g	50 g
Aflatoxin	0 ppb	0 ppb	0.3 ppb
Endotoxin	0.02 EU	0.02 EU	0.02 EU

Table 2: Effect of different diet on body weight, food intake, liver weight and liver weight to body weight ratio.

Measurement	Control (AIN76) diet	5 % SM- fortified diet	5 % WBM fortified
Body weight, g	21.58±1.22	20.28±1.71	21.57±1.68
Liver, mg	1088.91±135.34	1032.09±134.24	1087.11±145.11
Relative liver weight mg/g body weight	49.86±4.56	50.90±5.04	50.12±5.26
Kidney, mg	266.13±37.59	253.17±42.27	267.84±18.77
Relative kidney weight, mg/g body weight	12.31±1.33	12.45±1.81	12.40±1.12
Heart, mg	119.29±23.40	111.68±25.69	95.22±29.48
Relative heart weight, mg/g body weight	5.53±1.02	5.51±1.24	4.40±1.25
Food intake, g/day	4.64±0.32	4.8±0.33	4.7±0.30

Values are mean ± SEM; n = 12 (Study 1 only). No significant differences were observed among groups.

Fig. 1. Effect of control, SM and WBM supplemented diet on liver histology (H&E- A, B & C) and histochemistry (PAS- D, E & F and Trichrome staining- G, H & I) (imges representative of the each dietary treatment group under 20x magnification placed as control A,D&G, WBM B,E&H and SM C,F&I).

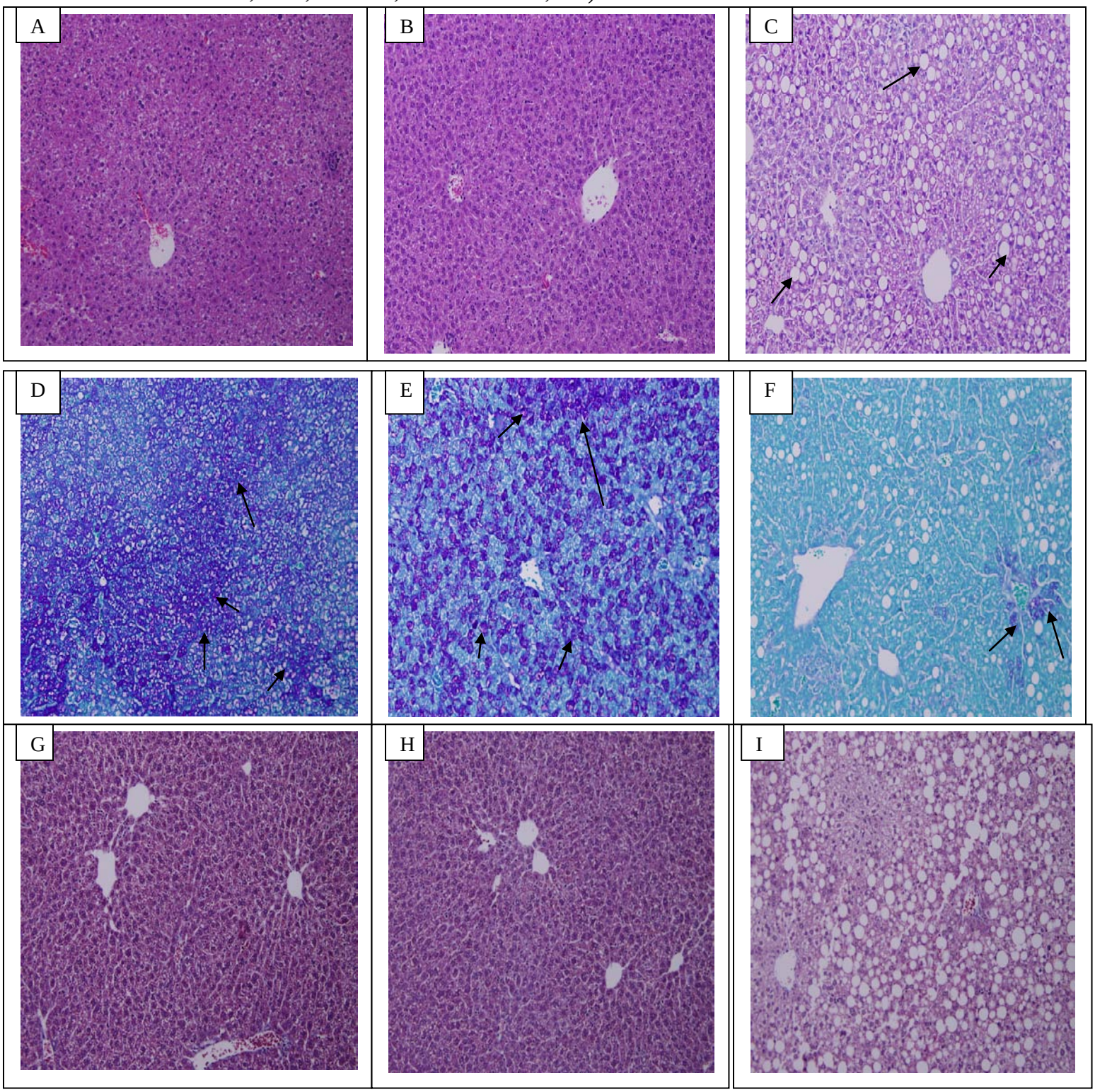


Fig. 2. Effect of control, SM and WBM supplemented diet on fatty liver scores in C57BL/6 mice. Values are means ± SEM; n = 7-9. Bars followed by different superscript letters are significantly different: a > b; p <0.001.

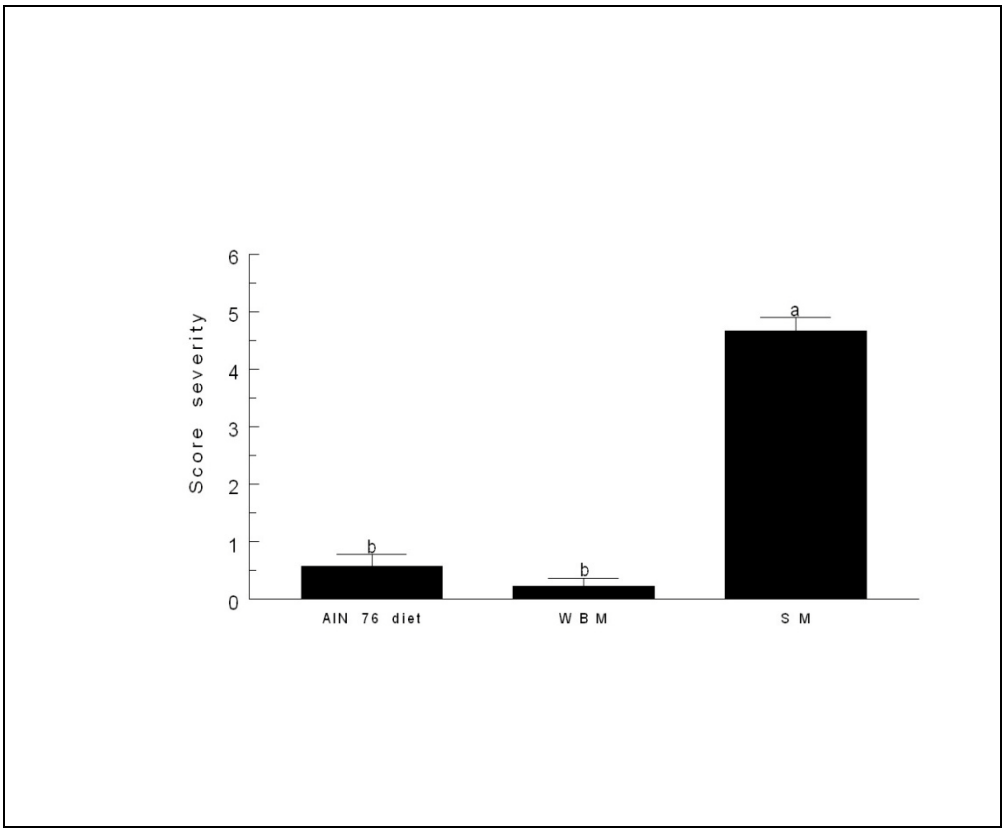


Fig. 3. Effect of SM withdrawal from the diet showing liver histology (H&E A-SM diet, B-8 days post withdrawal & C-8 days post withdrawal) and effect SM supplemented diet showing normal heart (H&E D, E & F) and Kidney histology (H&E G, H & I) (imges representative of the each dietary treatment group under 20x magnification placed as control D&G, WBM E&H and SM F&I).

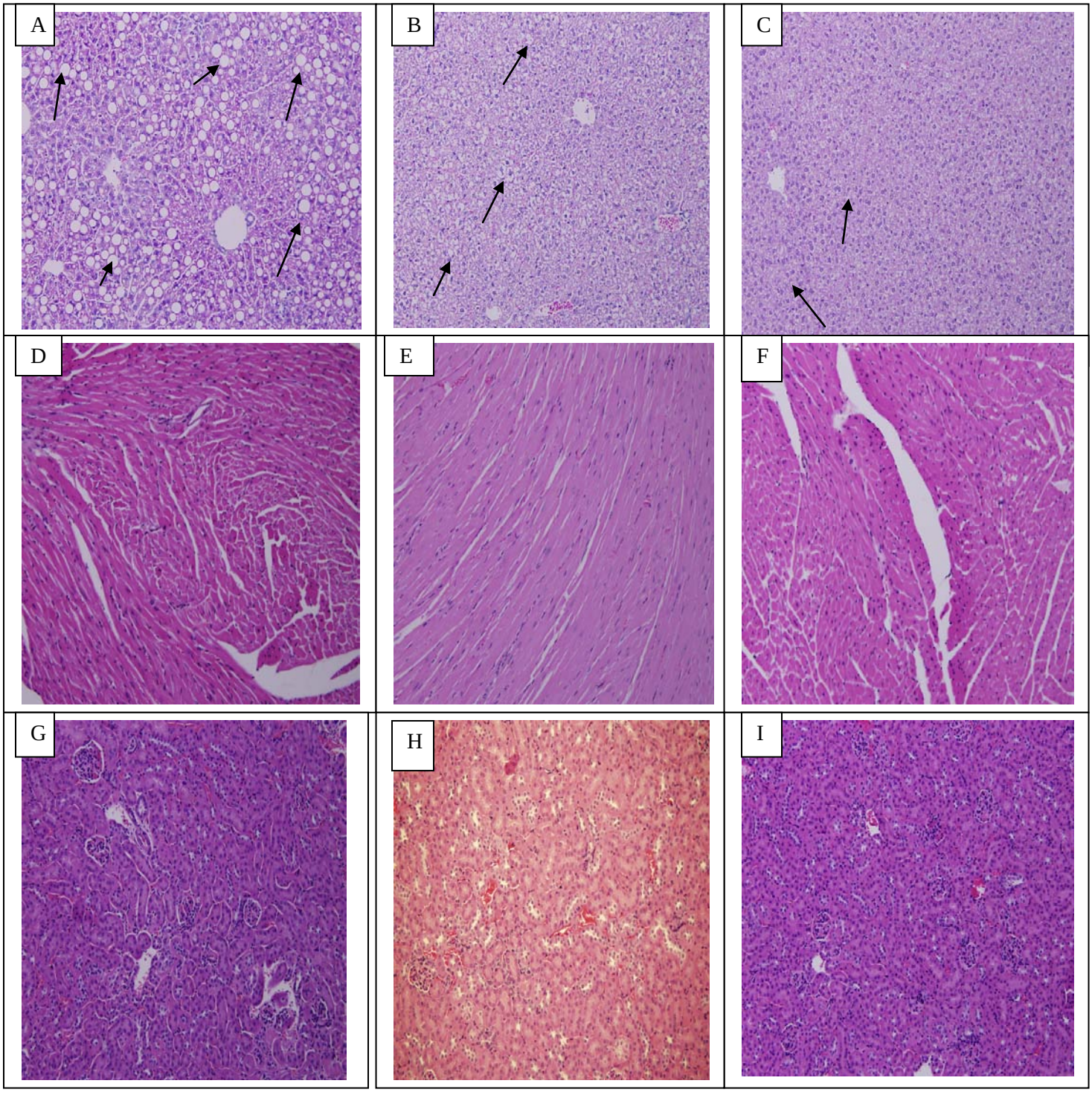
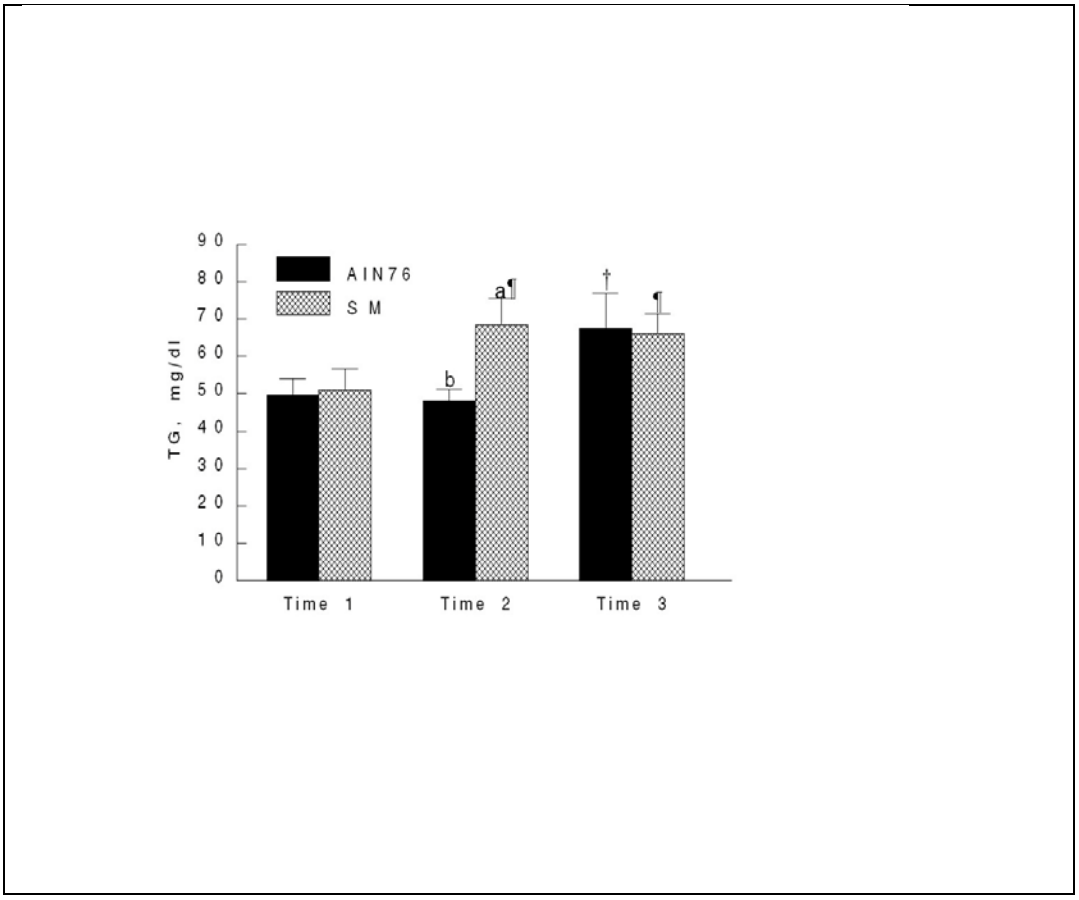


Fig. 4. Effect of Shiitake mushroom on serum triglycerides. Values are mean ± SEM; n = 6-7/group. Time 1= 6 weeks of feeding either the AIN93 or shiitake mushroom-fortified (SM) diets; Time 2 = mice fed SM diet then given the AIN93 diet for 8 days; Time = mice fed SM diet then given AIN93 diet for 15 days. Bars followed by different super-script letters or signs are significantly different; a >b; $p < 0.05$, Shiitake versus AIN93 diet; † AIN93 at time 3 compared to time 1, $p < 0.05$; ¶ SM at time 2 & 3 compared to time 1, $p < 0.05$.



CHAPTER V

Conclusions

In our study-1, all mice fed with mushroom diets for six weeks showed an increase in the plasma IL-23, IL-6, colon MPO, colon IL-6, and inflammation score when challenged with 3% DSS. Although all mushroom up-regulated IL-23, PM up-regulated IL-23 secretion on the 3rd day of in-vitro treatment and mice without DSS challenge unlike other mushrooms. These findings suggest mushroom supplementation promotes IL-23/IL-17 axis in improving gut immunity against certain GI pathogens. Our *in-vitro* findings indicate the Dectin-1 pathway is the major pathway in WBM and PM induced IL-23 production. Difference in the IL-23 secretion response among these mushrooms was observed in both in-vitro and in-vivo models. This difference could partly be explained by the mushrooms action on Dectin-1 pathway.

In our study-2, mice fed the SM diet developed fatty liver unlike mice fed the AIN93 and WBM diets. This study suggests the observed difference in the fatty liver development among mushrooms could be due to their mechanism of action on fatty acid metabolism. SM can induce fatty liver in normal/low fat diet. Moreover SM diet induced hepatic

steatosis was resolved within two weeks of stopping SM diet. In addition, SM induced fatty liver did not lead to hepatic inflammation or hepatic fibrosis.

CHAPTER VI

Recommendations for future research

Study-1:

Future research should be directed to evaluate the protective effect of WBM & PM on animal models of GI infectious diseases such Salmonellosis, Campylobacteriosis, *E. coli* infections, listeriosis, and Cholera. Studies investigating the immunostimulatory effects of WBM & PM on oral mucosal vaccines could be an exciting area of future research. Considering mushroom consumption patterns among humans such as average weekly consumption, frequency of consumption, realistic animal studies using 2% or less mushroom supplementation with 2-3 times per week are warranted . More mechanistic studies using primary dendritic cells and genetically modified mice might be useful to establish the molecular targets for these mushrooms.

Study-2:

Future studies should be directed to investigate how these SM caused fatty liver by using hepatocyte cell lines. Studies looking at the dose and time response of SM in fatty liver development are also needed. Future research should also be focused to investigate the effect of SM on high fat diet induced fatty liver animal models. Human studies evaluating the benefits versus risk of SM supplementation are warranted.

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VITA

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Candidate for the Degree of

Doctor of Philosophy

Thesis: MODULATION OF GUT IMMUNE AND HOST INFLAMMATORY RESPONSES BY EDIBLE MUSHROOMS

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Major Field: Nutritional Sciences

Scope and Method of Study:

The Gastro Intestinal (GI) tract represents the largest mucosal surface in the body responsible for digestion, absorption of essential nutrients and protection against various pathogens entering through GI tract. Dendritic cells (DCs) in the GI tract plays major role in gut immune response through capturing and transferring antigens to T-cells and releasing cytokines. Although several cytokines produced by DCs are involved in gut immunity, IL-23 is involved in the defense against most pathogens in the GI tract through up-regulation of defensins. Previous report from our lab showed that the extracts from edible mushroom enhance α-defensin production in in-vitro cell line The aim of this study was to investigate effect of white button (WM) and portabello (PM) mushrooms on IL-23 secretion both in-vitro and in-vivo models. We also assessed the side effects of these mushrooms by using histopathology. We used C57B6 mice for studying IL-23 secretion and toxicological evaluation. We used 3% DSS to induce acute inflammation because DSS induced inflammation is localized to the GI tract rather than systemic inflammatory response. Moreover, we are interested to study the effect of mushrooms on intestinal dendritic cell response. We also used J.744.1 a mouse monocytic cell line to study the molecular mechanism by which edible mushrooms up-regulate IL-23 secretion. We used spleen cell culture for ex-vivo cytokine secretion assay. Cytokines were measured by using ELISA.

Findings and Conclusions:

In DSS untreated mice, the plasma IL-23, IL-6 levels were low compared to DSS treated mice. All the mice fed with mushroom diets for six weeks showed an increase in the plasma IL-23, IL-6, colon MPO, colon IL-6, and inflammation score when they challenged with 3% DSS. Although all mushroom up-regulates IL-23, PM up-regulates IL-23 secretion on the 3rd of day of in-vitro treatment and mice without DSS challenge unlike other mushrooms. Data suggest that mushroom consumption may increase gut immunity and provide resistance against certain pathogens through up-regulation of IL-23. These findings also suggest Dectin-1 receptor pathway is the major pathway in WBM and PM induced IL-23 production. Difference in the IL-23 secretion response among these mushrooms was observed. This difference could be due to their mechanism of action on Dectin-1 receptor. Similarly, mice fed the SM diet developed fatty liver unlike mice fed the AIN93 and WBM diets. This also suggests difference in the mechanism of action of these mushrooms in the development of fatty liver.

ADVISER'S APPROVAL: Dr. Solo Kuvibidila