Studies on the effects of edible mushroom *Grifola frondosa* on intestinal homeostasis

A dissertation

By

KMGRM KARIYAWASAM

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Dedication

To the strength of my life…

OLA,,

Abstract

Intestinal homeostasis plays a vital role in maintaining not only gastrointestinal health but also the overall systemic health. The complex interactions between epithelial cells, immune cells, stroma cells, and gut inhabitant regulate a number of diverse mechanisms in order to maintain intestinal homeostasis. A breakdown in these pathways can disrupt the homeostatic mechanisms and lead to chronic intestinal inflammation and inflammatory-related health consequences, such as inflammatory bowel disease, ulcerative colitis, and bowel cancer. Gut inflammation is frequently accompanied by imbalance in the intestinal microflora. Particularly, probiotic bacteria, that can be regarded as part of the natural human microbiota, engage in improving homeostasis through counteracting the inflammation process by stabilizing the gut microbial environment and gut barrier function and mediating pro and anti-inflammatory cytokines. Several mushroom bioactive components including polysaccharides, glycoproteins, proteins, lipids, and secondary metabolites, are known to be involved in the induction and maintenance of intestinal homeostasis. *Grifola frondosa,* commonly known as maitake, is an edible mushroom, that has been shown to possesses various biological activities against several metabolic disorders including cardiovascular disease, diabetes, hypertension, inflammatory bowel disease, and cancers of the stomach, breast, and pancreas. However, the effects of maitake on the maintenance of intestinal homeostasis are still unclear. Therefore, the purpose of our study was to identify the effects of maitake on gut homeostasis, colorectal cancer development, and growth of probiotics. The effects of maitake and the synergistic inhibitory effect of maitake when combined with whole bovine milk powder on aberrant crypt foci (ACF) formation, colonic inflammation, and suppression of colorectal cancer were evaluated

using 1,2-dimethylhydrazine (DMH)-induced mouse model of colon carcinogenesis. In addition, the impact of maitake fruiting bodies, maitake extracts, and purified fractions on the growth of colon probiotic microorganisms were assessed and specific maitake glucans were isolated and characterized.

In chapter 1, the chemopreventive properties of maitake, and the synergistic effects when combined with milk against colon carcinoma in Balb/c mice treated with DMH colon carcinogenesis were investigated. Further, the possible mechanisms by which maitake and milk may exert their actions together or individually were also studied. Animals fed with AIN-76 standard diet were used as positive and negative controls, while AIN-76 supplemented with either 10% milk (10MK), 10% maitake (10MM), 5% milk + 5% maitake (5Mix), or 10% milk + 10% maitake (10Mix) diets were used for experimental groups. Supplementation with maitake, milk, and their combinations showed significant effect on suppressing DMH-induced ACF formation compared to mice treated with DMH positive control with the highest effect observed in the 10Mix group. Cecal pH was significantly lower in the 10MM and 10Mix groups than that of control and, maitake and, its combinations were able to remarkably increase cecum short chain fatty acid levels (SCFA) which were reduced by DMH treatments. Although, no significant difference was observed among samples regarding the liver weight, the levels of thiobarbituric acid reactive substances (TBARS) in the liver which were significantly increased by DMH treatment, were remarkably lower in the dietary treatment groups, and the effect was more pronounced in the 10Mix group. Further, all the tested diets were able to modulate pro-inflammatory cytokines; TNF-α and IL16, anti-inflammatory cytokine; IL-1ra, and chemokine; IP 10. Particularly, TNF-α level was considerably lower in the 10Mix group and was comparable with the DMH

negative control showing the highest suppression of colon inflammation, induced by DMH administration. On the other hand, supplementation with these food components significantly reduced anti-apoptotic proteins Bcl-2, Bcl-x, MCL1, XIAP, and p27/Kip while increasing apoptosis-related protein p53, cytochrome c, Bad, TRAIL R2, TNF R1, and Fas in comparison to the DMH positive control, thus suggesting the involvement of apoptosis in suppression of DMH-induced colon carcinogenesis. Compared to all the other dietary treatments, the 10Mix combination treatment showed the highest protection against colon carcinogenesis. Taken together, these results suggest that maitake, dietary milk, and the combination diets can suppress colon inflammation and upregulated muted cell apoptosis to suppress ACF formation and colorectal cancer development. Importantly, the synergistic inhibitory effect of maitake in combination with milk may be an effective nutritional chemoprevention agent against colon carcinogenesis.

In chapter 2, the synbiotic effect of maitake, maitake extractions and further purified fractions were evaluated using *in vitro* bacterial culture models and the responsible maitake polysaccharides were identified and elucidated. Maitake glucans were isolated from fruiting bodies through subsequent boiling water, ethanol, and alkali extraction steps. The water soluble (ER1), ethanol soluble (ER2), cold alkali soluble (ER3), hot alkali soluble (ER4,) and insoluble (ER5) extracts were purified, characterized by various analytical methods and further tested for prebiotic activity. The NMR spectroscopic analysis identified the glucans in all the extracts as belonging to the β type. Evaluation of the synbiotic effect of maitake extracts showed that all extracts (ER1-ER5) facilitated the growth of seven tested colon probiotic microorganisms; *Lactobacillus. rhamnosus* (ATCC 53103), *Lactobacillus acidophilus* (JCM 1132),

Lactobacillus delbrueckii subsp bulgaricus (ATCC 11842), *Lactobacillus casei* (ATCC 393), *Lactobacillus fermentum* (ATCC 14931), *Bifidobacterium longum* (BB 536), *Bifidobacterium adolescentis* (ATCC 1275), and *Bifidobacterium animalis* subsp animalis (ATCC 1253). Among the maitake extracts, ER1 showed remarkably different growth characteristics, which were similar to that of positive control (glucose). The ER1 extract was further characterized by methylation analysis and was identified as 1,3-β D-glucan and the molecular weight as revealed by HPLC was around 2.1×10^5 Da. The ER1 extract was further purified using anion exchange chromatography and divided into three sub-fractions (ER1-F1, ER1-F2, and ER1-F3) based on the carbohydrate contents, and tested for prebiotic activity. Even though, all three sub-fractions were able to stimulate the growth of all probiotics tested, ER1-F2 and ER1-F3 showed a remarkably different effects compared to ER1-F1. The ER1-F2 and ER1-F3 sub-fractions were further divided using a molecular weight cutoff value of 10 kDa. The ER1-F2 and ER1-F3 fractions with molecular weight >10 kDa showed significant effects on the growth of *L. rhamnosus* (ATCC 53103), *B. longum* (BB 536) and *B. adolescentis* (ATCC 1275). These findings indicate that maitake can be used as potential prebiotic agent to facilitate the growth of colonic probiotic bacteria and 1,3-β D-glucan from maitake water extracts may be responsible for the observed synbiotic effect.

In conclusion, the findings of this study suggest that fruiting bodies and polysaccharide fraction of maitake may be involved in facilitating the growth of colon probiotics microorganisms, suppression of ACF development and colon carcinogenesis and regulation of intestinal homeostasis.

Key words: intestinal homeostasis, maitake, colon inflammation, colorectal cancer, ACF, probiotic, prebiotic

（和文）

腸の恒常性は，腸だけでなく，全身の健康を維持する上で重要であり、腸内の上皮細胞，免疫細胞，間質細胞，及び腸内微生物間の複雑な相互作用により維持している。恒常性の崩壊は，炎症性腸疾患，大腸癌など炎症が関与する疾患へ進展する可能性がある。腸内細菌叢の不均衡は腸の炎症誘発し、特に，プロバイオティクス細菌は，腸の微生物環境と腸のバリア機能を安定させ，炎症性と抗炎症性サイトカインのバランスを維持することにより恒常性の維持をしていると考えられる。

マイタケ *Grifola frondosa* は食用キノコでありその多糖類，糖タンパク質，タンパク質，脂質，及び二次代謝産物は，腸の恒常性の誘導と維持に関与すること報告されているだけでなく、心血管疾患，糖尿病，高血圧，炎症性腸疾患，胃がん、乳がん，膵臓がんへの効能が報告されている。しかし，腸の恒常性の維持に対するマイタケの効果はまだ不明点が多い。

本研究の目的は、マイタケが腸の恒常性の維持を介して、異常陰窩巣（ACF）形成への効果を、ミルクと組み合わせた場合のマイタケの相乗効果を、1,2-ジメチルヒドラジン（DMH）誘発マウスモデルを使用して評価し、さらに、マイタケの子実体からマイタケグルカンを分離し、これらの結腸プロバイオティクス微生物の成長に及ぼす影響を評価した。

第 1 章では、DMH 投与により誘導されるマウス大腸腺腫発症モデル系においての、食餌性マイタケならびに牛乳(ミルク)の効果について検討した。AIN-76 とし、試験食群として、10％ミルク（10MK）、10％マイタケ（10MM）、5%ミルク+ 5%マイタケ（5Mi x）ならびに 10%ミルク +10%マイタケ（10Mi x）の群を設定し。DMH は週一回ごと腹腔内へ、15mg/Kg 体重投与し、10 週間試験飼育を行った。その結果、マイタケ並びにミルクの摂取は ACF の生成を有意に抑制し、また、ミルクとマイタケの共投与群が、ACF の生成を最も抑制した。盲腸の pH は、10MM および 10Mi x グループでコントロールおよびマイタケのそれよりも有意に低く盲腸短鎖脂肪酸レベル（SCFA）を著しく増加した。肝臓のチオバルビツール酸反応性物質（TBARS）のレベルは、DMH 投与により上昇したが、マイタケ並びにミルクの投与群で著しく低く、10Mi x群において効果はより顕著であった。また、10Mi x群において　炎症性サイトカインである TNF-α および IL16 が抑制された。アポトーシス関連タンパク質については p53、シトクロム c、Bad、TRAIL R2、TNF R1 が増加し、抗アポトーシスタンパク質 Bcl-2、Bcl- x、MCL1、XIAP、および p27 / Ki pを有意に減少した。これらの結果は癌の抑制におけるアポトーシスの関与を示唆する。

以上の結果より、マイタケ、牛乳、および両者の併用食が結腸炎症を抑制し、アポトーシスを上方制御して、ACF 形成および結腸直腸癌の発生を抑制する可能性示唆し、マイタケと牛乳の併用による相乗的な抑制効果は、大腸がん発症を予防する食品である可能性を示唆した。

第 2 章では、マイタケの腸内機能性を調べるために、マイタケから、溶媒分画法によって得られた多糖類の画分に対して in vitr oの腸内細菌培養モデルを使用して、その効果を検討した。　水溶性（ER1）、エタノール可溶性（ER2）、冷アルカリ可溶性（ER3）、高温アルカリ可溶性（ER4）、および不溶性（ER5）を H1－NMR を用いて解析したところすべての画分中のグルカンが β 型であった。また全画分において供試した 7 種のプロバイオティクス微生物（*Lactobacillus. rhamnosus* (ATCC 53103), *Lactobacillus acidophilus* (JCM 1132), *Lactobacillus delbrueckii* subsp bul gar icus (ATCC 11842), *Lactobacillus casei* (ATCC 393), *Lactobacillus fermentum* (ATCC 14931), *Bifidobacterium longum* (BB 536), *Bifidobacterium adolescentis* (ATCC 1275), and *Bifidobacterium animalis* subsp an imal is(ATCC 1253)）の成長を促進した。その中でも特に　水溶性（ER1）はブドウ糖を用いた陽性対照群と類似しており、この画分はメチル化分析により 1,3-βD-グルカンとして同定され、HPLC によって明らかにされた分子量は約 2.1 x 10^4 Da であった。さらにイオン交換クロマトグラフィーを用いて 3 画分に分画した。ケルろ過により（10kda カットオフ）分子量を分画したところ、、*L.ramnosus*（ATCC 53103）、*B.longum*（BB 536）、および *B. adolescentis*（ATCC 1275）の成長を有意に促進した画分が認められた。これらの結果は、1,3-βD-グルカンが観察されたシンバイオティクス効果の原因である可能性があることを示した。

結論として、この研究の結果は、マイタケの子実体と多糖類画分が、結腸プロバイオティクス微生物の成長、ACF の発生と結腸発癌の抑制、および腸の恒常性の調節に関与している可能性があることを示唆している。

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1. Introduction

Gastrointestinal tract, the largest immune interface of the human body plays a crucial role in maintaining intestinal homeostasis through controlling the balance between gut inhabitant and the host. Failure to achieve or maintain equilibrium between host and commensal microbiota can cause negative consequences for both intestinal and overall systemic health. Intestinal homeostasis depends on several biological mechanisms such as cell proliferation, differentiation, migration, and cell death which are regulated by the complex interaction among epithelial cells, immune cells, stroma cells, and gut inhabitant. Gut inhabitant regulates numerous important metabolic and physiological functions to maintain the intestinal homeostasis and host health, and the alteration, imbalance or shift of this gut inhabitant, or the disturbance in microbiota-host interaction can cause intestinal inflammation and related diseases (Carding et al., 2015). In addition, secondary metabolites produced by gut microbiota such as short chain fatty acids (SCFA), branched chain fatty acids (BCFA), aryl hydrocarbon receptor (AHR), aromatic amine, ammonia, acetaldehyde, β-glucuronidases, aglycones, and reactive oxygen species (ROS) and gases including hydrogen, hydrogen sulphide and methane also influence the diseases risk (Nicholson et al., 2012). In particular, bidirectional relationship between altered immune function and altered bacterial community with altered metabolites known to responsible for altered intestinal homeostasis and inflammation related intestinal diseases such as intestinal bowel syndrome (IBS), intestinal bowel disease (IBD), colorectal cancer (CRC), obesity, and other metabolic disorder. Knights et al., showed, increased levels of *Bacteroidetes* to *Firmicutes* ratio, an excessive abundance in *Enterobacteriaceae*, *Desulfovibrio, pasteurellaceae, Veillonellaceae* and *Fusobacteriaaceae* with decreased abundance in

Erysipelotrichales, Bacteroidales and *Clostridiales* species are associated with elevated intestinal inflammation and considered as the key factors of IBD (Knights et al., 2013). Another study revealed the involvement of *Bacteroides fragilis*, and *Streptococcus bovis* in activation of immune cells to release pro-mitogenic and pro-angiogenetic cytokines, mostly interleukin 17 (IL-17) (Wu et al., 2009). On the other hand, SCFA, particularly, butyrate showed that the inhibition of the activity of histone deacetylases (HDACs) in colonocytes to promote hyperacetylation of histone which has a crucial role in controlling inflammation through downregulating pro-inflammatory cytokines including interleukin-6 (IL-6) and IL-12 (Fung et al., 2012, Wilson et al., 2010).

Chronic inflammation in intestine is a well-established risk factor of CRC (Arthur et al., 2012). Although, the mechanism by which chronic intestinal inflammation leads to CRC is still unclear, numerous experimental studies suggest that inflammatory cells and their associated mediators such as IL-6, IL-12, tumor necrosis factor–α (TNF-α), IL-23, and ROS facilitate the development of CRC, presumably by enhancing DNA damage in epithelial cells (Danese and Mantovani, 2010, Lin and Karin, 2007, Ullman and Itzkowitz, 2011). CRC is a malignant tumor with high morbidity and mortality rate, and estimated to be the fourth most common cancer that is responsible for almost 700,000 cancer deaths in 2018, and is more common in developed countries (Center et al., 2009). In Japan, around 10% of men and 8% of women are diagnosed with CRC during their lifetime causing 53,500 deaths in 2018 (National Cancer Center Japan). Although, several risk factors both modifiable and non-modifiable are associated with CRC risk, life style risk factors are considered as modifiable risk factors that play a crucial role in the development of bowel cancer. According to the American Cancer Society, over 47% of cases are theoretically preventable through modifying these

lifestyle risk factors such as diet and food habits, physical inactivity, high alcohol consumption and tobacco smoking (Boyle & Langman, 2000, Johnson & Lund, 2007). Diet and food habits are strongly associated with CRC and changes in dietary habits might reduce up to 70% of cancer burden (Willett, 2005). The development of CRC is a multistep process accompanied by multiple genetic and epigenetic alterations. Morphologically, it is characterized by the progression from normal colon tissue to cancer via the earliest neoplastic lesions called aberrant crypt foci (ACF) to precancerous adenomas (mostly polyps) to adenoma-containing carcinomas and adenocarcinomas followed by malignant transformation (Orlando et al., 2008, Yang et al., 2009). However, the progression from a precursor lesion to CRC takes around 10 to 20 years, allowing time for effective intervention and prevention. Currently, several chemo-preventive agents such as celecoxib, aspirin, satin, sulindac and metformin are in use, most of which are associated with detrimental side effects including gastrointestinal toxicity (Algra et al., 2012, Half et al., 2009). In addition, currently available drugs are monotargeted which make them less efficient in suppressing CRC since growth and progression of cancer is a multiple-step process which include dysregulation of multiple cell signaling pathways (Xu et al., 2010).

Extensive research has revealed that most naturally occurring compounds, could modulate multiple cell signaling pathways and thus are multi-targeted naturally (Xu et al., 2010). Particularly, phytochemicals and secondary metabolites such as phenolic compounds, flavonoids, carotenoids, polysaccharides, saponins, reseratols, spingolipids, folic acid, vitamin D, butyrate, calcium, compounds contain nitrogen and organosulfur in fruits and vegetables are known to have pleiotropic anticancer effect and are able to modulate various cell signaling pathways to prevent, and suppress CRC

development (Rajamanickam et al., 2008). Mushroom, is a source of bioactive compounds such as β-glucan, mannan, xylose, glycosides, vitamin D and precursors, alkaloids, glucosylceramides, volatile oils, tocopherols, phenolic compounds, flavonoids, carotenoids, folates, ascorbic acid enzymes, and organic acids that are responsible for conferring anti-cancer potential (Chen and Seviour, 2007). These bioactive ingredients are known to regulate multiple and different cell signaling pathways in order to suppress the development of CRC (Patel et al., 2012). Moreover, mushroom polysaccharides, in particular, β-glucan is a well-known potential prebiotic agent which has the potential to suppress colon inflammation. Brown et al., stated that purified β-1-3-glucans retain their bioactivity and can work at cellular levels to maintain the intestinal homeostats through modulating the growth of probiotics (Brown and Gordon, 2001).

Probiotic microorganisms are living microorganisms which have a beneficial effect on human health. These microorganisms mostly belong to the bacterial genera *Lactobacillus, Bifidobacterium,* and *Lactococus, Streptococcus, Enterococcus,* and fungal genus *Saccharomyces* (Simon et al., 2005). Human probiotics maintain the proper balance between beneficial and pathogenic bacteria through the production of anti-microbial substances, inhibition of bacterial toxin production, competition with pathogens for adhesion to the epithelium, and immunomodulation in order to maintain the intestinal homeostasis (Brandao et al., 1998, Isolauri et al., 2001, Guillo, 2003, Vandenbergh, 1993). Prebiotics are the non-digestible food ingredients that beneficially affect the host health by stimulating the growth and activity of probiotics (DeVrese and Schrezenmeir, 2008). Consumption of prebiotics helps to modulate the composition of the intestinal microbiota and their metabolic activities, especially through modulation

of lipid metabolism, enhanced absorbability of calcium, modulation of immunological system and modification of bowel functions (Van Loo et al., 2005). *In vitro* studies on human L97 and HT29 cell lines demonstrated that inulin, a prebiotic caused a significant inhibition of growth and induction of apoptosis in human colorectal carcinoma (Munjal et al., 2009). Combination of both pro-and prebiotics generally known as synbiotics had been developed to overcome the possible survival difficulties of probiotics (Cencic and Chingwaru, 2010). Studies stated that synbiotics are more effective than probiotics or prebiotics in reducing concentration of undesirable metabolites, inactivation of the nitrosamines and cancerogenic substances, increasing levels of SCFAs, ketones, carbon disulphides and methyl acetates which potentially affect host's health (Manigandan et al., 2012). One study, in which 52 adults fed with a synbitoic product containing a blend of probiotics (*Lactobacillus casei, Lactobaillus rhamnosus, Streptococcus thermophiles, Bifidobacterium breve, Lactobacillus acidophilus, Bifidobacterium longum, Lactobacillus bulgaricus*) with fructo-oligosaccharides (FOS) for 28 weeks showed inhibition of Nuclear factor $_{K}$B (NF-$_{K}$B) and reduced production of TNF-α and TNF-α induced inflammation (Eslamparast et al., 2014). In another study, dietary administration of *Bifidobacterium longum* and oligofructose and inulin inhibited the formation of pre-neoplastic lesion development in 1,2- dimethyl hydrazine (DMH) colon carcinogenesis induced rats (Kaur and Gupta 2002).

Grifola frondosa, commonly known as maitake, is an edible mushroom, rich in a variety of nutrients similar to other edible mushrooms, but the corresponding content is higher (Aoki et al., 2018, He et al., 2017). The polysaccharide fraction of *Grifola frondosa* contains natural oligofructoses, FOS, lactulose, galactomannan, and indigestible

polydextrose, mannan, xylose, indigestible dextrin and β-glucan as polysaccharides (Jayachandran et al, 2017). Particularly, β-glucan in *Grifola frondosa* with unique and complex structure containing both 1, 6 main chain with 1, 3 branches and 1, 3 main chain with 1, 6 branches might be the responsible ingredient of higher bioactivity of maitake (Mayell, 2001). The β-glucan fraction of maitake, known as grifolan has the ability to stimulate the growth of cultured *Bifidobacterium* and *Lactobacillus* while suppressing the growth of *Salmonella*. This suggests that grifolan may have the prebiotic effect on intestinal microbiota. In addition, one study showed that maitake has the ability to inhibit hepatocellular carcinoma in Hep3B hepatoma cells by inhibiting proliferation, inducing cell cycle arrest, and inducing apoptosis (Lin et al., 2016). Further, triterpene components, low molecular weight substances and ergosterol (vitamin D2) in maitake are also known to have anti-cancer effect against several cancer types (Mayell, 2001). In another study, the effect of chemically sulfated polysaccharide derived from water-insoluble polysaccharide of maitake against human gastric carcinoma cells showed anti-cancer activity through apoptotic induction and the effect was accelerated in combination with 5-fluorouracil (Shi et al., 2007).

The main objective of this study was to identify the effects of *Grifola frondosa*, on intestinal homeostasis and colon inflammation. To achieve this objective, we evaluated the effects of maitake and the synergistic inhibitory effect of maitake when combined with milk on aberrant crypt foci (ACF) formation, colonic inflammation, and suppression of colorectal cancer development using mouse model of colon carcinogenesis. In addition, to identify the effect of maitake on the growth of colonic probiotic microorganisms, *in vitro* bacterial culture were used and the responsible maitake glucans were isolated and characterized.

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2. Literature review

2.1 Gastro-intestinal homeostasis

2.1.1 Intestinal homeostasis

The intestine is the largest mucosal surface of the body, and is the single biggest compartment of the immune system which constantly expose to large numbers of dietary and microbial antigens. Thus, complex and highly regulated immune responses are required by different mucosal cell types to induce and maintain intestinal homeostasis. However, if the protective immune responses are generated against dietary proteins or commensal bacteria other than the response to pathogenic bacteria or dietary antigens, chronic inflammation, and inflammatory disorders such as celiac disease and Crohn's disease (CD) can occur (Mowat et al., 2003). Therefore, regulation of intestinal homeostasis is important in maintaining not only the gastrointestinal health but also the overall systemic health (Mowat et al., 2003). Intestinal homeostasis depends on the number of diverse mechanisms, which are regulated by the complex interaction between epithelial cells, immune cells, stroma cells, and gut inhabitant. Since the turnover rate of the intestinal epithelium is very high, the tight regulation of the processes such as proliferation, differentiation, migration and cell death are crucial to ensure homeostasis.

2.1.2 Signaling pathways involved in intestinal homeostasis

Despite the diversity of cellular responses these processes are controlled by several signaling pathways such as Wnt, Notch, TGFβ/BMP, and Hedgehog pathways (Radtke et al., 2006). Substantial amounts of evidence have suggested that the Wnt pathway is

essential for maintenance of the cell-to-cell communication and maintenance of the proliferating transient amplifying cells within the crypt compartment (Kuhnert et al., 2004, Pinto et al., 2003). In the absence of Wnt signaling, β catenin, which is secreted as the result of activation of frizzled receptor complexes (Bhanot et al., 1996, Pinson et al., 2000, Tamai et al., 2000, Wehrli et al., 2000), is retained in the cytoplasm in a multi-protein complex comprising the tumor suppressor adenomatous polyposis coli (APC gene), the scaffold protein axin (Ikeda et al., 1998, Kishida et al., 1998), caseine kinases-1 (CK-1), (Polakis, 2002) and glycogen synthase kinases-3β (GSK-3β) (Behrens et al., 1998). Furthermore, mutation of APC gene results in hyperpoliferation of the epithelium which eventually progresses into development of adenomas (Nagase and Nakamura, 1993, Oshima et al., 1995). The Notch pathway is involved in cell differentiation (Artavanis et al., 1999) and proliferation (Van et al., 2005). Loss of CSL/RBP-J, the transcription factor that mediate Notch signaling of all Notch receptors within the crypt compartment leads to complete loss of proliferation and amplification of epithelial cells as well the conversion into postmitotic goblet cells (Van et al., 2005). In addition, recent studies show that not only Wnt signaling pathway but also the Notch pathway are simultaneously involved in profiling adenomas (Van et al., 2005), thus, Notch and Wnt signaling pathways appear to work together and loss of either signaling cascade results in the loss of crypt compartment. In contrast to signaling components of the Wnt and Notch pathways, TGF-β receptor and ligands are expressed in the differentiated compartment of the gut (Barnard et al., 1993, Winesett et al., 1996). The TGF-β family protein: TGF-β isomers, bone morphogenic proteins (BMPs), and activins, regulate different processes such as embryonic development, differentiation, proliferation, adhesion, wound healing, and inflammation (Blobe et al., 2000, Dunker et al., 2000). Cell culture experiments showed that TGF-β signaling not only exert

growth inhibitory properties (Kurokawa et al., 1987) but also has tumor promoting roles, in particular, during tumor invasion (Derynck et al., 2001, Massague et al., 2000). The Hedgehog signaling pathway is another evolutionary conserved pathway involved in maintaining gut homeostasis. In general, in vitro cell culture experiment suggested the indian gene (Ihh) can repress β-catenin mediated Wnt signaling (An den Brink et al., 2004) and the Hedgehog interacting protein (HhIP) can enhance Wnt target gene expression suggesting that Hedgehog signaling in the intestine may antagonize Wnt signaling (Madison et al., 2005).

2.1.3 Role of gut microbiota on intestinal homeostasis

Colon, the most densely populated organ in the human body, harbors over 100 trillion of microbes (Bäckhed et al., 2005) that belong to more than 1000 phylotypes at species level. In adults, *Bacteroidetes* and *Firmicutes* usually dominate the intestinal microbiota, whereas *Actinobacteria*, *Proteobacteria* and *Verrucomicrobia* are in considerably minor proportion. This microbial community regulates several important metabolic and physiological functions in the body such as those related to energy expenditure, satiety and glucose level, in order to maintain intestinal homeostasis and host health (Cani et al., 2013, Reigstad et al., 2013). In particular, the mucus layer residing bacteria decide whether the host cellular homeostasis is maintained or whether inflammatory mechanisms are triggered either through direct contact with host cells or through indirect communication via bacterial metabolites (Carding et al., 2015). Alteration in the microbiota can result from exposure to various environmental factors such as diet, toxin, drugs and antibiotics, and pathogens. The alteration, imbalance or shift in the microbiota composition and function, and the amendment of microbiota-host interaction known to trigger both local and systemic inflammation causing several

metabolic disorders (Aziz et al., 2013, Wu et al., 2013) particularly, IBD, CRC and IBS. Moreover, dysbiosis can cause extra-intestinal pathologies, such as allergies, diabetes, obesity and metabolic syndrome, rheumatic disease, and degenerative processes (Ebel et al., 2014, Leaph et al., 2013, Yeoh et al., 2013). In addition, secondary metabolites produced by bacteria, that are in close contact with the cell can affect metabolic phenotype of the host and influence the risk of diseases (Nicholson et al., 2013). Particularly, fermentation of undigested carbohydrates and protein results in the production of a range of metabolites including SCFA, BCFA, ammonia, amines, phenolic compounds and gases including hydrogen, hydrogen sulphide and methane. Furthermore, gut microbiota involves in production of vitamins, bioactive compounds such as isoflavanoids and plant lignans, the conversion of inactive-drugs into active forms, and the transformation of bile acids and xenobiotics (Blaut et al., 2007, Marchesi et al., 2007).

2.1.4 Intestinal homeostasis, health and diseases

As the gut microbiota and their metabolites has a well-established role in intestinal homeostasis, several highly prevalent gastrointestinal diseases have been associated with shift or imbalance in gut microbiota composition and function, and with altered secondary metabolites. In particular, bidirectional relationship between altered immune function and altered bacterial community with altered metabolites are known to be responsible for altered intestinal homeostasis and inflammation related intestinal diseases (Guarner, 2008). Inflammatory bowel disease which can eventually developed into CRC, and characterized by chronic relapsing inflammation affect the intestinal mucosa are divided into two forms as Crohn's disease and ulcerative colitis (UC). Although, the aetiology is unknown, there is increasing evidence of the pathogenic

implication of the host microbiota in IBD (Baumgar and Carding, 2007). A deregulated immune response against commensal gut bacteria may contribute to the onset or perpetuation of IBD. Several *in vivo* and *in vitro* studies have demonstrated qualitative and quantitative changes in composition and function associated with IBD, with a shift towards an inflammatory-related microbiome (Kostic et al., 2014). The intestinal microbiota with increase *Bacteroidetes* to *Firmicutes* ratio, an excessive abundance in *Enterobacteriaceae*, *Desulfovibrio, pasteurellaceae, Veillonellaceae* and *Fusobacteriaaceae and* decreased abundance in *Erysipelotrichales, Bacteroidales* and *Clostridiales* species have been described in IBD (Rowan et al., 2010).

CRC and development of colorectal adenoma strongly depends on genetic components, but environmental factors also play a vital role in CRC (Castells et al., 2009). The altered microbial community, and its metabolites have been proposed as contributing factors to the development of CRC, however, the mechanisms of this association remain unknown (Zhu et al., 2013). Predominantly, chronic intestinal inflammation has been associated with the development of adenoma and CRC, and it can result from an aberrant ratio of tolerogenic to aggressive (pro-inflammatory, pro-tumorigenic) microbiota (Terzic et al., 2010). *Bacteroides fragilis*, and *Streptococcus bovis* are known to activate immune cells to release pro-mitogenic and pro-angiogeneic cytokines, mostly interleukin 17 (IL-17) (Wu et al., 2009). An increased diversity of *Clostridium leptum* and *Clostridium coccoides,* and decreased complexity in composition are linked to colitis-associated CRC (Scanlan et al., 2008, Uronis et al., 2009). In addition, *Bacteroides fragilis, Bacteroides vulgatus, Bifidobacterium longum, Clostridum butyricum*, *Mitsuokella multiacida, Escherichia coli, Enterococcus faecali*, and *Streptococcus bovis* are also known to associate with the development of CRC (De

Palma et al., 2010, Shen et al., 2010, Terzic et al., 2010,). Other than that, some biological activities of the gut microbiota are presumed to generate metabolites involved in CRC carcinogenesis such as secondary bile salt transformation, production of hydrogen sulfide, desullfuration of bile acids, production of aglycones from inactivated harmful compounds, bacterial β-glucuronidases, production of aromatic amines by azoreductases and nitroreductases, generation of acetaldehyde, and generation of reactive oxygen species (Azcarate-Peril et al., 2009). A number of studies have addressed the role of the host innate immune system in regulating carcinogenesis and stated the involvement of Nod-like receptors (NLR) and the Toll-like receptors (TLR) (Zhu et al., 2011). Using a mouse model of Colits-associated cancer (CAC). Chen et al., demonstrated the increased tumor incidences in Nod1-deficient mice. (Chen et al., 2008). Further the results suggest that depletion of the gut microbiota using the antibiotic treatments can suppress the tumor development in Nod1-deficient mice. In addition, TLRs are known to play a critical role in gut homeostasis, intestinal inflammation and colitis-associated tumorigenesis (Gong et al., 2010, Kadota et al., 2010, Wald et al., 2003, Xiao et al., 2007). For an instance, TLR4 promotes the development of CRC in the Azoxymethane/Dextran sodium sulfate (AOM/DSS) models (Fukata et al., 2007). In a model of spontaneous colitis, $IL10^{-/-}TLR4^{-/-}$ mice displayed accelerated development of disease, as early as 8 weeks of age compared to $IL10^{-/-}$ and $IL10^{-/-}TLR9^{-/-}$ animal which did not develop colitis by 8 months (50 in Zhu). These results highlighted the complex relationship between the gut homeostasis, gut microbiota and state of inflammation and CRC. Consequently, modulation of intestinal microbiota and secondary metabolites using probiotics and/or prebiotics could influence the development of tumors.

Probiotics and their secondary metabolites mainly SCFA contribute to the development of the mucosal immune system by influencing the innate inflammatory response and reducing intestinal inflammation, and as well as suppress the development of colon tumors. Kim et al., assessed the anti-cancer activity and bacterial enzyme (β-glucuronidase, β-glucosidase, tryptophanase, and urease) inhibition of *Bifidobacterium adolescentis* SPM0212 using human colon cancer cell lines: HT-29, SW 480 and Caco-2. The authors, stated that the strain inhibited TNF-α production and changes in cellular morphology in dose dependent manner (Kim et al., 2008). Perdigon et al., found that yogurt supplementd with live probiotic cells inhibited tumor growth in DMH treated Balb/c mice (Perdigon et al., 1998). In another study the effects of bacteria *Bifidobacterium lactis* and "resistant starch" (RS) and their combination (synbiotic) against CRC in murine model were evaluated, and found out that *B. lactis* utilizes the RS, and up-regulates the acute apoptotic response against carcinogen in the colon. Further, rats fed with RS in combination with *Bifidobacterium lactis* showed a significantly lowered incidence and multiplicity of colonic neoplasms compared with the control group (Le Leu et al.2010). Lara-Villoslada et al., reported that short-chain-fructooligosaccharides (SC-FOS) increased cecal *Lactobacilli* and *Bifidobacteria* as well as SCFA production in both healthy rats and colitic rats. In addition, SC-FOS feeding caused a decrease of MPO activity, leukotriene B4 (LTB4) production and iNOS expression (Lara-Villoslada et al., 2006)

One of the main beneficial metabolite of probiotics, SCFA which includes mainly acetate, propionate and butyrate, are known to have anti-inflammatory properties that influence not only host health but also intestinal homeostasis (Louis et al., 2014). Intracellular butyrate and propionate inhibit the activity of histone deacetylases

(HDACs) in coloncytes to promote hyperacetylation of histone which has a crucial role in controlling inflammation (Fung et al., 2012, Wlison et al., 2010). In particular, hyperacetylation of histone downregulate pro-inflammatory cytokines such as IL-6 and IL-12 (49 in louis) in colonic macrophages. Another recent study shows butyrate driven signaling interaction that involve G protein-coupled receptor 109A (GPR109A) might be involved in the anti-inflammatory action by promoting differentiation of regulatory T (T_{reg} cells) cells and IL-10 producing T cells, and by blocking activation of nuclear factor-κB (NF-κB) and induction of apoptosis (Singh et al., 2014, Thangaraju wt al., 2009). Butyrate has the ability to inhibit cell proliferation and selectively induce apoptosis of CRC cells possibly through HDAC inhibition and G-protein-coupled receptor interactions (Buda et al., 2003, Clarke et al., 2008, Fung et al., 2012, Haner et al., 2008, Wilson et al., 2010). In another study, a mouse line that is genetically susceptible to CRC showed low concentration of butyrate which might promote CRC risk by stimulating the colonic epithelial cell proliferation (Louis et al., 2014).

2.2 Colorectal cancer

2.2.1 Prevalence of CRC

Cancer is one of the major human diseases and is the cause of considerable suffering and economic loss worldwide. CRC is a malignant tumor with high morbidity and mortality rate and estimated to be the fourth most common form of cancer in both men and women, and responsible for almost 700,000 cancer deaths in 2018 and is more common in developed countries (Center et al., 2009). In the USA alone, an estimated 145,600 cases of CRC were predicted to occur in 2019, among which 51,020 deaths were expected, accounting for 9% of all cancer deaths (American Cancer Society, 2018). In Europe, 417,000 people were diagnosed with CRC and among them 228,000 died in

2018. It has been of the highest incidence and the second-deadliest after lung cancer (Europacolon, 2011). In Japan, around 10% of men and 8% of women are diagnosed with CRC during their lifetime causing 53,500 deaths in 2018 (National cancer center Japan). The global rates of CRC have been increasing largely in economically developing countries (Jemal et al., 2011). CRC, also known as bowel cancer, is the cancer caused by uncontrolled cell growth in the colon or rectum of the gastrointestinal tract (American Cancer Society, 2010). CRC start in the lining of the bowel; most are benign as colorectal polyps or adenoma, but some develop into cancer over time (Falterman et al., 1974). Bowel cancers can be adenocarcinomas, lymphomas or squamous cell carcinomas. Approximately 5% of individuals affected with CRC have inherited one of two hereditary forms to include familial adenomatous polyposis (FAP) or hereditary non-polyposis CRC (HNPCC). Those diagnosed with FAP have a nearly100% chance of developing colon cancer at an onset before the age of 40 (National Human Genome Research Institute, 2012). Those diagnosed with HNPCC have a nearly 80% risk probability of developing CRC throughout their lifetime (National Human Genome Research Institute, 2012).

2.2.2 Risk factors of CRC

Several risk factors are associated with the incidence of CRC which can be categorized into non-modifiable and modifiable factors. Those that an individual cannot control such as age and heredity factors consider as non-modifiable factors while environmental and lifestyle risk factors count as modifiable risk factors. More than 90% of CRC cases are found to be in people aged 50 or older and the likelihood of CRC diagnosis increase after age of 40 (**Fig. 2.1**) (National institute for health, 2006, Ries et al., 2006 Thrumurthy et al., 2016). However, CRC appears to be increasing among

young generation by being one of the 10 most commonly diagnosed cancer among both men and women aged 20-49 years (Fairley et al., 2006, O'Connell et al., 2003, O'Connell et al., 2004). Around 95% of the sporadic CRC develop from neoplastic polyps or colorectal adenoma; the precursor lesions of the CRC. An individual with a history of adenomas has an increased risk of developing CRC than an individual with no previous history of adenomas (De Jong et a., 2005). In addition, people with IBD has 4-20% of overall risk of developing CRC than the normal counterpart with no IBD history. The majority of CRC occurs randomly among people with no family history of CRC or predisposing illness. Nevertheless, 20% of people who develop CRC have one or more first-degree relatives with history of CRC or adenomatous polyps (World cancer research fund., 2007). Even though, the reason for increased risk is not clear, it may be due to inherited genes and/or shared environmental factors.

Bowel cancer is considered as an environmental disease due to its solid relation with wide range of ill-defined cultural, social and lifestyle factors. In addition, it is one of the readily identified modifiable causes of the cancers and according to the American Cancer Society, over 47% of cases are theoretically preventable through modifying these environmental factors such as diet and food habits, physical inactivity and life style, high alcohol consumption, and tobacco smoking (Boyle and Langman, 2000, Willett, 2005). Among the environmental factors, diet is strongly associated with CRC and changes in dietary habits might reduce up to 70% of cancer burden (Larsson et al., 2006). Diets high in animal fat, linked to the concept of typical western diet are a major risk factor for CRC (Boyle and Langman, 2000, Janout and Kollárová, 2001) due to their involvement in increasing the bacterial flora capable of degrading bile salts to potentially carcinogenic *N*-nitroso compounds. High red meat consumption has also

been identified as one of the major risk factors of CRC (Larsson and Wolk, 2006, Santarelli et al., 2008) in which the positive association is stronger for colon cancer than rectal cancer. Specially, the presence of heme iron in red meat and the heterocyclic amines and polycyclic aromatic hydrocarbons produced when subjected to high temperature, are believed to possess carcinogenic properties (Santarelli et al., 2008). In addition, some studies claim people who consume comparatively low fibrous foods including vegetables and fruits are at high risk of developing CRC which might describe the cultural and geographical differences in the rates of colorectal incidence (Janout and Kollárová, 2001). For instance, dietary fiber has been proposed as accounting for the difference in the CRC incidence rate between Africa and Westernized countries, suggesting adequate amount of dietary fiber may dilute fecal content, increase fecal bulk, and reduce transit time thus, lower the CRC risk (World cancer research fund., 2007).

Physical activity and excess body weight are interrelated risk factors, that are reported to account for about a fourth to a third of CRC. Regular physical activity and a healthy diet can help to lower the risk of CRC although the evidence is stronger for colonic than for rectal disease (World cancer research fund., 2007). The *in vivo* and cohort evidences suggest that increase in metabolic rate and maximal oxygen uptake (De Jong et al., 2005), elevation of metabolic efficiency and capacity, reduction of blood pressure, and insulin resistance (Lee et al., 2007) are associated with regular physical activity which may be the reason for low CRC risk. Moreover, lack of physical activity is closely associated with increased rate of obesity in both men and women, another risk factor of CRC (Bazensky et al., 2007, De Jong et al., 2005). Several biologic correlates of being overweight and obese, notably increased circulating estrogens and decreased insulin

sensitivity are believed to influence cancer risk (De Jong et al., 2005). However, studies suggest that individuals who use energy more efficiently may be at a lower risk of CRC despite high energy intake (Boyle and Langman, 2000).Though the association between tobacco smoking and lung cancer is very well established, recent evidence shows that 12% of CRC deaths are also due to smoking (Zisman et al., 2006). The carcinogens found in tobacco are known to contribute to formation, size and growth rate of adenomatous polyps (Botteri et al., 2008). Alcohol consumption is a factor in the onset CRC at a younger age similar to tobacco smoking (Tsong et al., 2007, Zisman et al., 2006). Not only, reactive metabolites of alcohol such as acetaldehyde, a well known carcinogen but also alcohol, function as a solvent enhancing penetration of other carcinogens into mucosal cells (Pöschl and Seitz, 2004).

2.2.3 Colon carcinogenesis

The development of CRC is a multistep process, accompanied by multiple genetic and epigenetic alterations and associated with the progressive inhibition of cancer cell apoptosis as well as an increase of cell proliferation. Morphologically, it is characterized by the progression from normal colon tissue to cancer via the earliest neoplastic lesions or ACF to precancerous adenomas (mostly polyps) to adenoma-containing carcinomas and adenocarcinomas followed by malignant transformation (**Fig. 2.2**) (Kinzler and Vogelstein, 1996, Thrumurthy et al., 2016). ACF are clusters of mucosal cells characterized by their prominent structure compared to normal mucosa, altered luminal openings, thickened epithelia, and increased size (larger than normal counterparts). Histologically, they can be divided in to hyperplasia and dysplasia which are known as micoadenomas due to their histologic resemblance to adenomatous polyps

with enlarged, elongated and depolarized nuclei which are believed to be the actual precursors of CRC.

Aberrant crypt foci, were first reported in both experimental animals and human as a potential biomarker of human CRC (Pretlow et al.,199, Roncucci et al., 1991, Tudek et al., 1989). Gupta et al., reported that ACF plays an important role in the adenoma carcinoma sequence in epidemiologic studies supporting ACF as a biomarker for colorectal carcinoma (Gupta et al., 2007). Further confirming above findings, Stevens et al., also concluded that ACF can be used as an intermediate endpoint due to its strong relationship to the known risk factors for CRC (Stevens et al., 2007). In the same article the authors discuss the possibility of using ACF in rodent models as a biomarker even in a short term experimental period of 8 weeks. However, incidence of ACF depends on carcinogen dosage, rodent strain, administration method, intervention time at the carcinogenic state, the size and the crypt multiplicity increase with time as the disease develop (Bird, 1987).

Development of earliest neoplastic lesions into malignant cancer involve in accumulation and alterations of multiple genes which cause genomic instability. These alterations include mutational activation of oncogenes and mutational inactivation of tumor suppressor gene which include at least four muted genes (Fearon and Vogelstein, 1990). Frequent mutations or deletion involve the oncogene KRAS and the three tumor suppressor genes: APC, deleted in colon cancer (DCC) and p53 (Takahashi et al., 2004). Mutations in the APC gene, usually appear in the early stage of tumorigenesis. The mutation of APC leads to the loss of APC function as part of a protein complex targeting β-catenin for its degradation and thus, activates β-catenin/Tcf-4 to induce proliferation

in intestinal crypt epithelial cells (Bright-Thomas and Hargest, 2003). β-catenin normally plays a role in the cadherin-mediated cell-cell adhesion system (Takahashi et al., 2004). However, this accumulation promotes the expression of c-myc, cyclin D1 and c-jun which are critical for cancer development. The mutation of APC is followed by KRAS, and KRAS oncogene mutation activate its downstream signaling pathways, the raf/MEK/MAPK and P13K/Akt/PKB which signals extracellular growth in epithelial cell line facilitating ACF and adenoma development (Smith et al., 2002). Alteration of p53 and DCC suppressor genes are involved in later stages. The tumor suppressor p53 protein is the most commonly mutated gene in human cancer occurring in the late development of CRC and the mutation of p53 gene could be identified in up to 75% CRCs but rarely found in adenomas and aberrant crypt foci (Manne et al., 1997). In addition, this protein is also involved in cell cycle and apoptosis regulation (Takahashi et al., 2004). However, the relationship between dietary risk factors and genetic alterations is as yet incompletely understood.

Besides, epigenetic alterations that are central to the process of genomic imprinting, such as histone modification which modulate the gene expression pattern during cell differentiation activate oncogenes and inactivate tumor suppressor genes in CRC. DNA methylation which may turn off gene expression and can lead to inactivation of tumor-suppressor genes in the process of carcinogenesis, (Curtin et al., 2011) and changes in non-coding RNA which regulate protein expression by inhibiting mRNA translation known to affect the gene expression patterns independent of or in combination with inherited or somatic changes in the DNA sequence. These epigenetic alterations are known to strongly associate with dietary food components and secondary metabolites (Tan et al., 2009, Yang et al., 2008, Zeng et al., 2011). Other mechanisms suggested to

be involved in CRC carcinogenesis include cytotoxic and mutagenic effects of food metabolites and oxidative stress, immune regulation, and alterations in the mucosal inflammatory milieu caused by food metabolites or their by-products (Barone et al., 2012, Bruce et al., 2000, Diggs et al., 2011, Ferguson et al., 2004, Kim and Milner, 2007). The responsiveness to the environment, particularly diet, and the potential reversibility, has made epigenetics a promising target for dietary interventions in the chemoprevention of CRC (Gingras and Béliveau, 2011, Schnekenburger and Diederich, 2012).

2.2.4 Animal models in CRC

Animal models are frequently used in studying the different aspects of human disease, even though they may not precisely represent the complexity of human nature and diseases. However, varieties of animal models mainly mice and rats are in use for studying the underlying mechanism of initiation and progression of CRC. Particularly, genetically modified animals such as $Apc^{Min/+}$mice are used to mimic and study hereditary CRC, whereas chemically induced CRC models are used to imitate non-hereditary CRC. At an earlier time, tumorigenesis in the forestomach and colon were induced by injecting polycyclic aromatic hydrocarbon (Lorenz et al.,1940), methylcholanthrene, radioactive yttrium (Lisco et al., 1947) and 4-aminodiphenyl and 3,2-dimethyl-4-aminodiphenyl. However, the most commonly used models for sporadic CRC takes advantage of the organotropism of the colon carcinogens, DMH and AOM (Rosenberg et al., 2009). DMH, a metabolic precursor of methylazoxymethanol (MAM) was reported to produce colon tumors in rodents that exhibits many of the pathological features associated with the human disease (Haase et al., 1973, Martin et al., 1973, Shamsuddin et al., 1981, Ward, 1974). Thus, this

methylating agent has provided reproducible experimental system to study sporadic forms of CRC (LaMont, and O'Gorman, 1978). However, AOM, a downstream metabolite of DMH offers advantages over DMH including enhanced potency and greater stability in dosing solution (Neufert et al., 2007, Papanikolaou et al., 1998). Nevertheless, both are in use of CRC studies for many years (Rosenberg et al., 2009). DMH has the advantage of low cost, simple application, and high potency and reproducibility. Even though DMH has many advantages, DMH-induced tumors are not effective on studying mucosal invasiveness and metastasis which are common in human CRC (Boivin et al., 2003, Nambiar et al.,2003, Shamsuddin, 1984). Conversely, DMH-induced rodents as a biomarker of pre-cancerous lesion development or ACF formation are widely in use. ACFs are specific to colon carcinogenesis and are induced by colon specific carcinogens such as AOM and DMH in a dose dependent manner and strongly associate with tumor occurrence in rodents (Bird, 1987) which make them ideal for CRC studies.

2.2.5 Prevention of CRC

Cases in colon cancer is projected to reach 2.2 million from 1.2 million over the next two decades, with over half of the incidents (62%) in the developing countries (Karsa et al., 2010). This may be probably due to the increase in population and increased life expectancy in these regions. Further, the incidents rates could outgrow the current estimation due to adaptation of westernized lifestyle and food habits (Huxley et al., 2009, Karsa et al., 2010). This upcoming burden of CRC is demanding development in areas of CRC early detection, staging and therapies, such as radiography, surgery and chemotherapy. Surgery is the primary treatment for patients with localized CRC (stage I/II) but the survival percentage is as low as 50%. In addition, those patients have to go

through adjuvant therapies to avoid reoccurrence which comes with side effects. The stage III patients go through wide surgical resection with postoperative chemotherapy such as 5-FU and leucovorin which demands long term observations and treatments. On the other hand, the goal of therapies for metastatic disease are to prolong survival and improve the quality of life which include surgical reception, palliative chemotherapy, biological therapy and radiation therapy (Labianca et al., 2010). However, these therapies might not be effective as expected and the available resources might not be enough to meet the demand which make preventive strategies are more important to control CRC incidence rates.

In addition, prevention by early detection through stool-based testing at early stage of CRCs and endoscopic-based testing, such as flexible sigmoidoscopy and colonoscopy and removal of polyps are currently available methods which help to lower the disease mortality (Levin et al., 2008). Even though the council of European Union and the *ad-hoc* United States task force recommend starting population screening at age 50 (Segnan et al., 2010, US Preventive Service Task Force, 2008), this cannot be generalized from one population to another and the age of screening initiation needs to be determined based on local epidemiological data and on the life expectancy of the population. However, these screening methods depends on availability of the necessary resources, sufficient funding or economical background, stratification of risk and the choice of most suitable screening tests which might be limited in many developing countries.

2.2.5.1 Drugs for chemoprevention

Since the progression from a precursor lesion to CRC takes approximately around 10 to 20 years, allowing time for effective intervention and prevention, ideal chemopreventive agent/s are in high demand. The ideal chemopreventive agents are supposed to be non-toxic, highly effective in multiple sites, orally consumable, inexpensive, accepted by the population, and have well known mechanism of action (Rajamanickam and Agrawal, 2008). Currently, only celecoxib is proven for chemoprevention of CRC but it can be used only for patients with FAP due to cardiovascular toxicity. Indeed, recent studies proved aspirin, statin, sulindac and metformin as effective agents on reducing CRC incidences and risk of distant metastasis, however, are associated with side effects such as gastrointestinal toxicity (Half and Arber, 2009).

In addition, currently available drugs are monotargeted which make them less efficient in suppression of CRC since growth and progression of cancer is a multiple-step process which include dysregulation of multiple cell signaling pathways. Extensive research has revealed that most naturally occurring compounds could modulate multiple cell signaling pathways and thus are multitargeted naturally (Xu et al., 2010).

2.2.5.2 Nutritional chemoprevention

Diet play a vital role in the etiology of CRC, which make nontoxic dietary phytochemicals and secondary metabolites the most natural option in chemoprevention. Many studies reported convincing evidence for decreased risk of CRC by reduced red and processed meat consumption and increased consumption of fiber rich foods such as unrefined grains, legumes, mushrooms, fruits and vegetables, poultry, and fish (Chan

and Giovannucci, 2010). Therefore, promoting a healthier diet, characterized by consumption of less animal fat, red meat, and more dietary fiber with increased physical activity could be a good strategy for primary CRC prevention (Gingras and Béliveau, 2011). In addition, many natural dietary compounds in fruits and vegetables are known to have pleiotropic anticancer effect and are able to modulate various cell signaling pathways to prevent and suppress CRC (Rajamanickam and Agrawal, 2008). These phytochemicals and secondary metabolites include phenolic compounds, flavonoids, carotenoids, polysaccharides, saponins, reseratols, spingolipids, folic acid, vitamin D, butyrate, calcium, nitrogen containing compounds, and organosulfur compounds. These compounds exhibit many biological activities such as immunomodulatory, anti-inflammatory, antioxidant, anti-mutagenic, anti-bacterial, anti-viral and anti-hepatoxic (Rajamanickam and Agrawal, 2008). Thus, dietary compounds are able to control various molecular pathways involved in CRC initiation and progression including cell cycle progression, apoptosis, cell proliferation, angiogenesis, tumor cell invasion, metastasis and tumor promoting signal transduction pathways such as cyclooxygenase 2 (COX-2) induced PGE2 levels, Wnt/β-catenin pathway, ERK pathway and PI3K/Akt signaling pathway (Rajamanickam and Agrawal, 2008).

Combination of two natural compounds may produce improved (synergistic), reduced (antagonistic) or identical (additive) effects compared to their effect when acting separately (Redondo-Blanco, 2017). Several studies have shown that synergistic effect of food components such as curcumin and catechins, resveratrol and grape seed extract (Reddivari et al., 2016), curcumin and quercetin (Temraz et al., 2013) on prevention of precancerous lesion development and inhibition of colon carcinogenesis were not only effective but also were associated with multi-signaling pathways. Therefore, it is

expected that the combination of natural compounds could be more efficacious than anticancer drug alone since the natural compounds target several molecular pathways that are not inhibited by drugs (Temraz et al., 2013).

2.2.6 Potential CRC preventive effect of edible mushrooms

Mushrooms have been regarded as gourmet cuisine across the world since antiquity for their unique taste and subtle flavor. Recently mushroom have emerged as a source of nutraceuticals, anti-oxidants, anti-cancer, prebiotic, immunomodulation, anti-inflammatory, cardiovascular, anti-microbial, and anti-diabetic (Barros et al., 2007, Kim et al., 2007, Sarikurkcu et al., 2008, Synytsya et al., 2009, Wang et al., 2004) and widely in use as a bio therapeutic agent. Numerous clinical trials have been conducted to assess the benefits of using edible mushrooms and their extract in cancer therapy. Evidences support that bioactive components in edible mushroom can be used individually and as adjuncts to suppress cancer development (Patel and Goyal, 2012). Findings suggest that the synergistic effect of some mushrooms in combination with commercial anti-cancer drugs might be an effective tool for treating CRC.

Further, mushrooms are known to complement chemotherapy and radiation therapy by diminishing the side-effects of cancer, such as nausea, anemia and bone marrow suppression. The bioactive compounds responsible for conferring anti-cancer potential include polysaccharides: specially β-glucan and proteins, fats, ash, glycosides, vitamin D and precursors, alkaloids, glucosylceramides, volatile oils, tocopherols, phenolic compounds, flavonoids, carotenoids, folates, ascorbic acid enzymes, and organic acids. Among, the best known and most potent mushroom-derived substances with anti-tumor and immunomodulating properties are polysacchraides. The polysaccharide β-glucan,

which consist of a backbone of glucose residues linked by β-(1 → 3)-glycosidic bonds, often with attached side-chain glucose residues joined by β-(1 → 6) linkages (Chen and Seviour, 2007) is the most versatile metabolite.

Fruiting bodies, water extracts and ethanol extracts of over 20 types of edible mushrooms around the world are proven to be involved in different cell signaling pathways such as cell differentiation, apoptosis, immune surveillance, angiogenesis, metastasis, cell cycle and signal transduction control in order to prevent initiation and development of CRC. Further, multi-targeting ability together with the inherently vast structural diversity of natural compounds found in mushrooms make them the foremost food components in discovering new drug that rationally target the abnormal molecular and biochemical signals leading to CRC.

2.2.6.1 *Grifola frondosa* as an anticancer agent

Grifola frondosa is one of the most valued traditional medicines and has been used as a health food for long time in east Asian countries. Fruiting bodies are rich in a variety of nutrients similar to but higher than other edible mushrooms (Aoki et al., 2018, He et al., 2017). The polysaccharide fraction of maitake contains glucan, xylan, mannans, galactans and chitin with the higher proportion of β-glucan. Particularly, β-glucan in maitake are comprised of unique and complex structure containing both 1, 6 main chain with 1, 3 branches and 1, 3 main chain with 1, 6 branches which might be the responsible ingredient of higher bioactivity of maitake (Mayell, 2001). Water soluble polysaccharides fraction from maitake fruiting bodies has been proven to possess antitumor and anti-carcinogenic activities against liver, prostatic, gastric and breast cancers. D-fraction, which contains β-glucan and protein with molecular weight around

1000 kDa and the more purified version of D fraction with better bioactivity: MD fraction, were demonstrated to have anti-cancer effect in *in vivo*, *in vitro* and clinical trials (Aoki et al., 2018). In addition, β-1,3 glucan from cultured maitake mycelium showed anti-tumor activities in mice bearing brain tumors (Aoki et al., 2018). Further, triterpene components, low molecular weight substances, and ergosterol (vitamin D2) in maitake are also known to have anti-cancer effect against several cancer types (Aoki et al., 2018,).

Maitake and its polysaccharide fraction have also shown synergistic anti-cancer effect in conjunction with other nutritional therapies and conventional cancer therapy (Aoki et al., 2018,). The effect of chemically sulfated polysaccharide derived from water-insoluble polysaccharide of *maitake against human gastric carcinoma cells showed anti-cancer activity through apoptotic induction and the effect was accelerated in combination with* 5-fluorouracil (Shi et al. 2007). The apoptotic mechanism was associated with drop in mitochondrial trans-membrane potential, up-regulation of Bax, downregulation of Bcl-2 and activation of caspase-3. In addition, water-soluble polysaccharides from *maitake* fruiting body showed a significantly reduced levels of Wnt2, p-β-catenin, p-β-catenin(Ser675), p-c-Myc(Ser62), c-myc, TCF4, and p-GSK3β (SER9), resulting in a gradual decrease in the ratio of p-c-Myc(Ser62)/c-Myc and p-GSK3β(SER9)/GSK3β in C57BL/6 mouse colon tissue (Meng et al., 2019). However, the molecular targets and mechanisms of the effects of whole maitake fruiting bodies on CRC have not yet been fully elucidated.

2.2.7 Potential CRC preventive effect of bovine milk

Milk and milk products are recognized as functional foods, suggesting the direct and significant effect on health and wellbeing of humans. Further, milk is considered to be the only foodstuff that contains approximately all different substances known to be essential for human nutrition (Goodman et al., 2002, Laakkonen and Pukkala 2008). Milk contains carbohydrates in the form of lactose, protein, fat, calcium, the B-group vitamins (thiamin, riboflavin, niacin, vitamin B_6, and folate), vitamin A, vitamin C, magnesium, and zinc (Jelen 2005, Miller et al., 2007). In addition, milk is one of the major sources of conjugated linoleic acid (CLA) in the diet, although it is a minor component of milk fat (Jelen 2005). The consumption of milk and milk products are known to correlate with a reduced risk of numerous cancers (Keri Marshall 2004). Specially, milk constituents such as vitamin D, proteins, calcium, sphingolipids, CLA and butyrate might be responsible for prospective association between dairy products and low risk of cancers (Bingham and Day 2006, Cui and Rohan 2006, Laakkonen and Pukkala 2008, McCann et al., 2004, Parodi 2005).

A variety of studies (epidemiological, animal, laboratory, and clinical trials) have shown inverse association between CRC and dairy milk components. The higher ingestion of milk calcium, particularly, 1200 to 1500 mg/d, or 4 servings of dairy products per day was known to significantly reduce the risk of colon cancer (Cho et al., 2004a, Chan and Giovannucci 2010, Holick 2008). In addition, epidemiologic intake and intervention studies have shown that calcium administration lowers colorectal adenomatous polyps, cancer cell proliferation and upregulate cancer cell apoptosis (Fedirko et al., 2009, Ahearn and others 2011). However, many evidence suggests that the vitamin D is essential to modulate the effect of calcium on colorectal

carcinogenesis (Mizoue et al., 2008) thus, the simultaneous intake of calcium and vitamin D is important for effective suppression of CRC. Fat components of dairy products including CLA, sphingolipids and butyric acid have also been proven to be protective against CRC in experimental studies (Liew et al., 1995), Hague and Paraskeva 1995, Parodi 1997). Casein is known to protect against colon cancer by inhibition of enzymes that are produced by intestinal bacteria and are responsible for deconjugation of procarcinogenic glucuronides to carcinogens (Parodi 1998). Although, milk components are known to have protective effect against colon cancer, the effect of whole bovine milk is not very well known (Davoodi et al., 2013)

2.3 Probiotics, prebiotics and intestinal inflammation

2.3.1 Probiotics

Probiotic bacteria, by definition, are living microbial food ingredients which have a beneficial effect on human health. However, according to the WHO, FAO and EFSA (the European Food Safety Authority), probiotic strains must meet both safety, functionality and technological usability criteria (**Table 2.1**) (FAO/WHO 2002, Simon et al., 2005).The safety is defined by the origin, the absence of association with pathogenic cultures, and the antibiotic resistant profile while functional aspects are defined by the ability to survive in the gastrointestinal track and the immunomodulatory effect (Markowiak and Śliżewska, 2017). In addition, probiotics should have the ability to survive and maintain the probiotic properties throughout the storage and distribution process (Lee and Salminen, 2002). Probiotic strains are not associated with the genus or species of a microorganism, however human probiotic microorganism mostly belong to the bacterial genera *Lactobacillus, Bifidobacterium, Lactococus, Streptococcus, Enterococcus* and fungal genus *Saccharomyces* (Simon et al., 2005).

Humans have numerous health benefits from probiotics for the maintenance of health and wellbeing. The main advantage is maintaining the proper balance between beneficial and pathogenic bacteria, through the production of anti-microbial substances to kill the pathogens, competition with pathogens for adhesion to the epithelium, immunomodulation of the host, inhibition of bacterial toxin production and nutrition (Guillot, 2003, Isolauri et al., 2001, Vandenbergh, 1993). The ability to co-aggregate, leads to the form action of the protective barrier, preventing colonization of pathogenic bacteria (Schachtsiek et al., 2004) and also triggers the signaling cascade for immunomodulation which includes increased production of immunoglobulins, enhanced activity of macrophages and lymphocytes, and stimulation of γ-interferon production (Oelschlaeger, 2010). Thus, co-aggregation of probiotics is important in the prevention and treatment of contagious diseases, chronic inflammation, and elimination of cancer cells (Borchers et al., 2009, EFSA, 2013).

Moreover, secondary metabolites in particular, low-molecular-weight substances produced by probiotic microorganisms such as SCFA, hydroperoxide, and bacteriocins are proven to have the ability to suppress the colonic inflammation and related diseases including IBD, peptic ulcers, and CRC (Geier et al., 2006, Geier et al., 2007, Lesbros-Pantoflickova et al., 2007, Levri et al., 2005). Several studies have demonstrated that intracellular butyrate and propionate inhibit the activity of HDACs in colonocytes and immune cells to promote hyperacetylation of histones, and downregulate pro-inflammatory cytokines such as IL-6 and IL-12 and upregulate anti-inflammatory cytokines to control intestinal inflammation and development of CRC (Chang et al., 2004, Fung et al., 2008, Hammer et al., 2008, Smith et al., 2013, Wilson et al., 2010,).

2.3.2 Prebiotics

Prebiotics are mostly fibers that are non-digestible food ingredients and beneficially affect the host's health by selectively stimulating the growth and/or activity of some genera of microorganisms in the colon, generally lactobacilli and bifidobacteria (DeVrese and Schrezenmeir 2008). FAO/WHO defines prebiotics as a non-viable food component that confer health benefit(s) to the host and associated with modulation of the beneficial microbiota. An ideal prebiotic should be 1) resistant to digestion in the upper segment of the alimentary tract, 2) easy to ferment by the beneficial intestinal microflora, 3) beneficial for the host 4) selectively stimulate the growth and/or activity of beneficial bacteria 5) stable in various food processing conditions and remain unchanged, non-degraded or chemically un- altered (Wang 2009).

The majority of the identified prebiotics can be categorized based on the origin, fermentation profile, and dosage required for health effects. Some of the sources of prebiotics include: breast milk, soy beans, inulin sources, raw oats, unrefined wheat, unrefined barley, yacon, non-digestible carbohydrates in particular non digestible oligosaccharides (Pandey 2015). In addition, dietary fiber such as cellulose, hemi cellulose, β-glucan, and pectin are considered as potential prebiotics which are fermented by not only probiotic microorganisms but also by other majority of the colonic microorganisms (Ouwehand et al., 2005). Prebiotics are either present naturally in some food products or added to food as an additional support to intestinal probiotic microorganism. Consumption of prebiotics largely modulates the composition of the intestinal microbiota and their metabolic activities, especially through modulation of lipid metabolism, enhanced absorbability of calcium, modulation of immunological system, and modification of bowel function (Van-Loo et al., 2005). Providing an energy

source that only specific microorganism species can utilize has a greater impact on microbial composition and metabolic activities than any other factors. However, the molecular structure of prebiotics determines the physiological effects and type of microorganisms that are able to use as the substrates (Crittenden and Playne, 2009). Despite of the prebiotic type, all the prebiotics are involved in the production of SCFA, regulation of hepatic lipogenic enzymes as a result of increased SCFA production, modulation of mucin production, increasing the number of lymphocytes and /or leukocytes in gut-associated lymphoid tissues (GALTs) and in peripheral blood, and increased secretion of IgA (Schley and Field, 2002). As a result of these functions, bioavailability and activity of probiotic microorganism increase improving host health.

Regular intake of prebiotics may lead to numerous health benefits. Studies have shown that prebiotics: inulin and oligofructose has the ability to reduce blood low density lipoprotein (LDL) level in rats after 5 weeks of oral administration and the authors suggest this may due to acceleration of lipid catabolism by prebiotics (Mojka, 2014, Socha et al, 2002). In addition, studies on colorectal carcinoma demonstrated that the disease occurs less commonly in people who intake fiber rich diets (Mojka, 2014, Pena, 2007). Results of rat studies proved that a prebiotic-enriched diet lead to significantly reduced indexes of carcinogenesis and further they demonstrated that butyric acid may be a chemopreventive factor in carcinogenesis or act as agent to suppress development of carcinoma through the promotion of cell differentiation (Kim et al., 1982, Scheppach and Weiler, 2004). *In vitro* studies on human L97 and HT29 cell lines demonstrated that inulin caused a significant inhibition of growth and induction of apoptosis in human colorectal carcinoma (Munjat et al., 2009). In addition, rats induced with azoxymethane

colorectal carcinoma showed an inhibition of cancer growth after administration with inulin and oligofructose (Verghese et al., 2002).

2.3.3 Synbiotics

Synbiotic are generally known as synergistic combinations of pro- and prebiotics which are developed to overcome the possible survival difficulties of probiotics and play a vital role in maintenance of intestinal homeostasis (Cencic and Chingwaru 2010). A synbiotic product improves host health and wellbeing by selectively stimulating the growth and/or activating the metabolism of one or a limited number of health promoting bacteria: particularly probiotic species through supplementation of species specific substrate. Several studies have shown that application of synbiotics for the modulation of selected intestinal probiotics seems promising (Markowiak, and Śliżewska, 2017). Among available synbiotics, a combination of *Bifidbacterium* or *Lactobacillus* genus with FOS products seems to be the most popular. Other than that, inulin or oligofructose combine with *Bifidbacterium* or *Lactobacillus* are also in use (Markowiak, and Śliżewska, 2017). Those synbiotics are known to reduce concentration of undesirable metabolites, inactivate the nitrosamines and cancerogenic substances, increase levels of SCFAs, ketones, carbon disulphides and methyl acetates which potentially result in positive effect on the host's health (Manigandan et al., 2012). The health benefits of synbiotics include, 1) increased levels of *Bifidobacterium* and *Lactobacillus* genus and maintenance of the balance of gut microbiota, 2) improvement of hepatic function in cirrhotic patients, 3) improvement of immunomodulative abilities, 4) prevention of bacterial translocation and reduced incidences of nosocomial infections in surgical patients. (Zhang et al., 2014).

Synbiotics are well known to possess anti-inflammatory, anti-bacterial, anti-cancerogenic, and anti-allergic effects. A randomized trial that use synbiotics containing five probiotics (*Lactobacillus plantarum*, *Lactobacillus delbrueckii* spp. *bulgaricus*. *Lactobacillus acidophilus, Lactobacillus rhamnosus,* and *Bifidobacterium bifidum*) and inulin as prebiotic in adult subjects with non-alcholic steatohepatisis (NASH) showed a significant reduction of intrahepatic triglycerol (IHTG) within a six-month period (Wai-Sung et al., 2015). One study wherein hypercholesterolromic male rats were fed with rice bran fermented with *Lactobacillus acidophilus* showed controled lipid profile (Oberreuther-Moschner et al., 2004). Synbiotics seems to be involved in immune functions and immunomodulation. In another study, 52 adults fed with a synbiotic product containing a blend of probiotics (*Lactobacillus casei, Lactobacillus rhamnosus, Streptococcus thermophiles, Bifidobacterium breve, Lactobacillus acidophilus, Bifidobacterium longum, Lactobacillus bulgaricus*) with FOS for 28 weeks showed the inhibition of Nuclear factor $_{K}$B (NF-$_{K}$B) and reduced production of TNF-α and colonic inflammation (Eslamparast et al., 2014). In another experimental research synbiotic supplementation of *Lactobacillus, Bifidobacterium* and 10% FOS in rats fed with high-fat, low-fiber diet suppressed intestinal and systemic inflammation (Delcenserie et al., 2008). Anti-carcinogenic effect of synbiotics emerge due to their ability to modulate various cell signaling pathways at the same time. The proposed mechanisms include 1) altering the immune functions associated with immune response 2) anti-proliferative effects via regulation of cell apoptosis and cell differentiation, 3) suppressing the production of enzymes like β-glucuronidase, urease, choloylglycine hydrolase, azedoreductase and nitro-reductase. Bifidobacteria probiotics when used with FOS reduced colon carcinogenesis induced by DMH in mice and also showed tumor inhibition in liver (Fotiadis et al., 2008). Dietary administration of

Bifidobacterium longum and oligofructose and inulin inhibits the formation of pre-neoplastic lesion development in DMH colon carcinogenesis induced rats (Kaur and Gupta 2002). Other than that, synbiotics may be highly efficient in the prevention of obesity, T2D, and osteoporosis, reduction of serum cholesterol and glucose levels, treatment of brain disorders associated with abnormal hepatic function (Pandey et al., 2015) and prevention of constipation and diarrhea.

2.3.4 Potential prebiotic activity of mushrooms

Mushroom has an emerging identity as a potential prebiotic. The major components rendering prebiotic action in mushroom are non-digestible polysaccharides such as glucan, chitin, and heteropolysaccharides. Similar to other prebiotics, mushroom also exert several beneficial effects for the host through enhancing the growth and activity of probiotics. Particularly, several mushroom polysaccharides such as pleuran, lentinan, schizophyllan, α and β-glucans, mannans, xylans, galactans, chitin, inulin and hemi celluloses can be considered as well studied, promising prebiotics agents (Singdevsachan et al., 2016). Pleuran from oyster mushroom and lentinan from shiitake mushroom are shown to suppress intestinal inflammation and development of intestinal ulcers in rats (Nosalova et al., 2001, Zeman et a., 2001,). Besides, β-glucans from mushrooms *Pleurotus tuber-regium*, *Polyporous rhinoceros* and *Wolfiporia cocos* showed a potential prebiotic effect through modulation of the growth of both bifidobacteria and lactic acid bacteria (Wong et al., 2005). Synytsya et al., showed that water extract mushroom glucans from *Pleurotus ostreatus* and *Pleurotus eryngii* were able to stimulate the growth of probiotics: 3 strains of *Bifidobacterium* spp, 4 strains of *Lactobacillus* spp and *Enterococcus faecium* (Synytsya et al., 2009). Additionally, the prebiotic effects of three crude polysaccharides (PS I, PS II, and PS III) from *Agaricus*

blazei on the growth of lactic acid bacteria was studied by Yan and Han, and found PS I and PS II have better growth promoting effect on lactic acid bacteria (Yan and Han 2008). In another study the crude polysaccharide extract from *Ganoderma lucidum* fermented in batch culture fermentation of human feces culture unit showed a growth promotion of *Bifidobacterium* sp, *Lactobacillus* sp and growth inhibition of *Salmonella* sp (Yamin et al., 2012). Another recent study also showed the prebiotic activity of crude polysaccharides from *Lentinula edodes*, *Pleurotus eryngii* and *Flammulina velutipes,* and also showed that prebiotic tested can enhance the survival rate of probiotics during cold storage (Chou et al., 2013).

Among all the mushroom polysaccharides, β-glucans are important due to their well-known therapeutic properties. Particularly, β-1-3,1-6-glucans from fungi are known as "biologic response modifiers" due to their immunomodulation ability against infectious diseases and possible tumoricidal activity (Firenzuoli et al., 2008). Wasser, stated that anti-tumor activity of mushroom mainly depends on the presence of β-1-3 linkage in the backbone of the glucan and presence of β-1-6 branch points can enhance the bioactivity (Wasser et al., 2002). Further, β-1-3-glucan from fungi known to have 99-100% of tumor inhibition while other polysaccharides exhibit only 10-40% inhibition (Blaschek et al., 1992). In addition, orally administrated soluble barley β-1-3,1-4 glucan or *in vitro* anti-tumor monoclonal antibodies were ineffective as single agents, but when combined, showed a significant anti-tumor effect (Cheung et al., 2002) showing that β-glucan potentially can be used to generate a novel cell-mediated effector mechanism for tumor vaccines and antibodies against tumors. Other than that, β-glucan from different mushrooms: *Agaricus brasiliensis, Grifola frondosa, Pleurotus florida, Lentinus squarrosulus, Calocybe indica, Termitomyces roustus, Russula albonigra,*

Ramaria botrytis and *Tricholoma crassum* showed immunomodulatory and anti-tumor activities (Singdevsachan et al., 2016). Unlike most of the natural components, purified β-1-3-glucans retain their bioactivity and can work at cellular levels to maintain the intestinal homeostasis through modulation of the growth of colonic probiotics (Brown and Gordon, 2001).

2.3.5 *Grifola frondosa* as a potential prebiotic agent

Grifola frondosa contains natural oligofructoses, FOS, lactulose, galactomannan, and indigestible polydextrose, mannan, xylose, indigestible dextrin and β-glucan as polysaccharides (Jayachandran et al., 2017). The D fraction of maitake which is rich in proteoglucan is well known for its immune-stimulatory capacity. In particular, decreased cell viability, increased cell adhesion and reduction in migration and invasion of mammary tumor cells were observed in murine model of breast cancer, fed with maitake D-fraction (Alonso et al., 2017). The β-glucan fraction of maitake, known as grifolan has triple helix structure and studies have shown that grifolan can stimulate the growth of cultured *Bifidobacterium* and *Lactobacillus* while suppressing the growth of *Salmonella*. This further suggest that grifolan may have the prebiotic effect on intestinal microbiota. In addition, one study showed that maitake has the ability to inhibit hepatocellular carcinoma in Hep3B hepatoma cells by inhibiting proliferation, inducing cell cycle arrest, and inducing apoptosis (Lin et al., 2016). Further, maitake is known to be involved in lipid metabolism, and helps to prevent viral infections.

Table 2.1: Selected criteria of probiotic strains (adapted from Food and Agriculture Organization)

Criterion	Required Properties
Safety	• Human or animal origin. • Isolated from the gastrointestinal tract of healthy individuals. • History of safe use. • Precise diagnostic identification (phenotype and genotype traits). • Absence of data regarding an association with infective disease. • Absence of the ability to cleave bile acid salts. • No adverse effects. • Absence of genes responsible for antibiotic resistance localized in non-stable elements.
Functionality	• Competitiveness with respect to the microbiota inhabiting the intestinal ecosystem. • Ability to survive and maintain the metabolic activity, and to grow in the target site. • Resistance to bile salts and enzymes. • Resistance to low pH in the stomach. • Competitiveness with respect to microbial species inhabiting the intestinal ecosystem (including closely related species). • Antagonistic activity towards pathogens (e.g., H. pylori, Salmonella sp., Listeria monocytogenes, Clostridium difficile). • Resistance to bacteriocins and acids produced by the endogenic intestinal microbiota. • Adherence and ability to colonies some particular sites within the host organism, and an appropriate survival rate in the gastrointestinal system.
Technological usability	• Easy production of high biomass amounts and high productivity of cultures. • Viability and stability of the desired properties of probiotic bacteria during the fixing process (freezing, freeze-drying), preparation, and distribution of probiotic products. • High storage survival rate in finished products (in aerobic and micro-aerophilic conditions). • Guarantee of desired sensory properties of finished products (in the case of the food industry). • Genetic stability. • Resistance to bacteriophages.

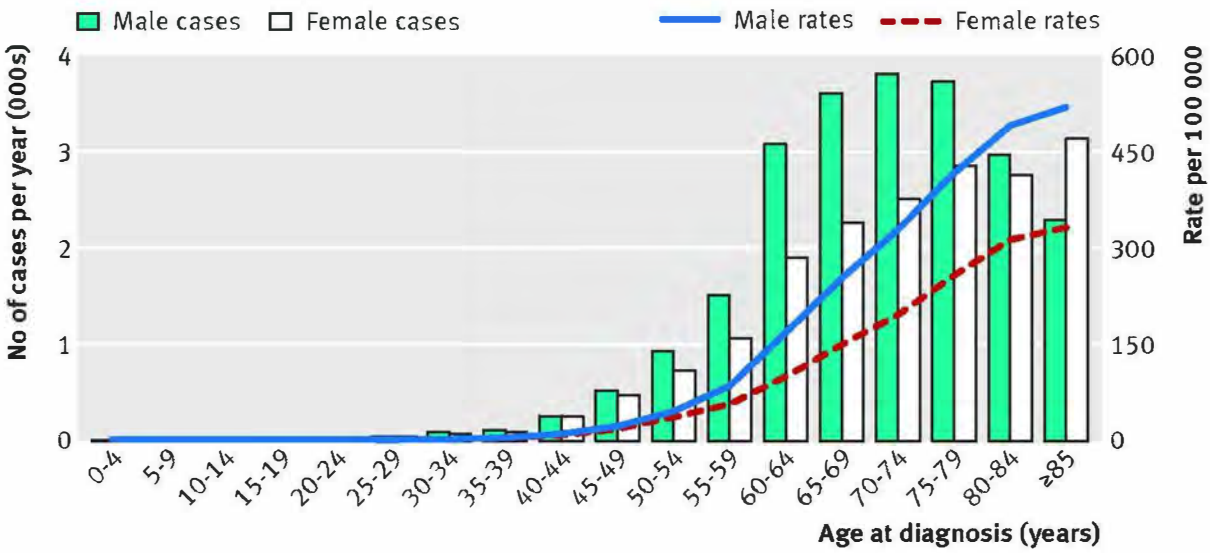

Figure 2.1: Age specific incidence rates of colorectal cancer per 100 000 global populations (adapted from Cancer Research institute, UK)

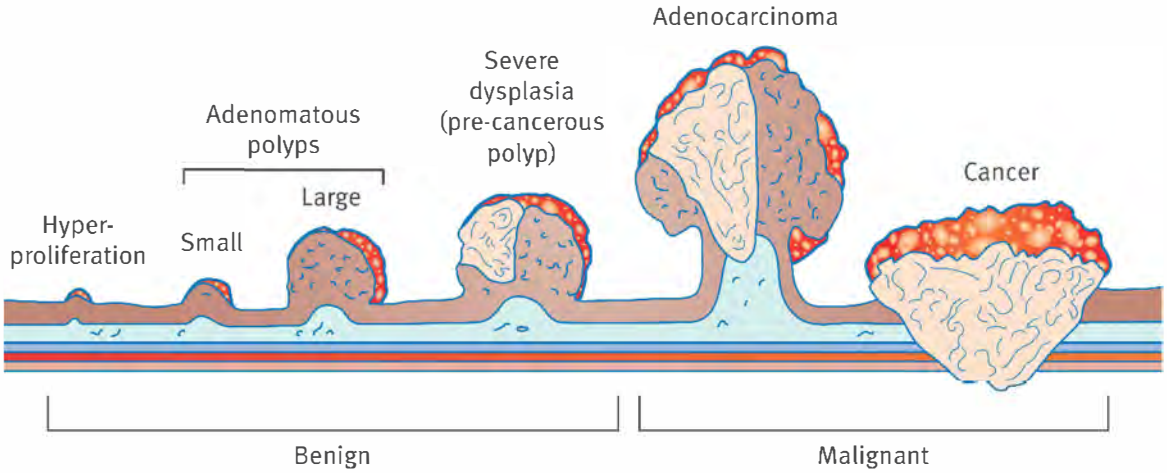

Figure 2.2: Progression from colorectal polyp to cancer (adapted from Thrumurthy et al., 2016)

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3. Effect of oral intake of maitake and bovine milk on mice treated with 1,2-dimethylhydrazine-colon carcinogenesis

3.1 Introduction

Cancer is one of the major non communicable diseases and cause considerable suffering and economic loss worldwide. CRC is a malignant tumor with high morbidity and mortality rate which cause around 600,000 annual deaths making it the fourth most common form of cancer and the third leading cause of cancer-related death worldwide (Center et al., 2009). Diet and lifestyle have been thought to have a role in the etiology of CRC. Diets rich in animal fat are particularly known to promote tumor growth while plant-derived fibrous diet are known to suppress pre-cancerous lesions and colon cancer developments (Triantafillidis et al., 2009). In an attempt to clarify the relationship between diet and CRC, researchers are examining individual dietary components, such as red meat, calcium, fiber, folic acid, and vitamin D on adenomas which are precursors to CRC, or later stage of CRC development or both.

Due to the limited efficacy and the cytotoxicity of chemotherapeutic agents used for treatments in advanced CRC, prevention has gained in importance, based on the multistep sequence and the long latency of CRC. Even though CRC prevention by screening fecal occult blood testing or colonoscopy is popular and efficient, compliance of the population remains low due to low availability of facilities, social levels and education. Therefore, nutritional chemoprevention which is a low cost, highly available,

efficient with minimum side effects is on demand. Non-toxic phytochemicals and secondary metabolites such as curcumin, polyphenols, flavonoids, polysaccharides (mushroom glucans), saponins, resveratrol, sphingolipids, calcium, butyrate and vitamin D are in high demand for chemoprevention due to their ability to trigger several pathways related to carcinogenesis like apoptosis, inflammation and angiogenesis (Gupta et al., 2010, Kelloff et al., 2000, Naitani et al., 2008).

Many studies in a variety of *in vivo* and *in vitro* models have shown that different species of edible mushrooms and their fractions (e.g., GlcCer, vitamin D and precursors and β-glucan) have anti-oxidative, anti-inflammatory and anti-tumor effects which can inhibit proliferation and metastasis and induce apoptosis in various malignant tumors, including colorectal cancer (Shomori et al., 2008). Maitake is an edible mushroom, rich in dietary fiber which includes both soluble and insoluble fibers, vitamin D and pro-vitamin D2 (Phillips et al., 2011). The β-glucan fraction of maitake mushroom has been reported to possess anti-tumor activity both *in vivo* and *in* vitro (Aggarwal, 2003, Akazawa et al., 2002, Olsen et al., 2016, Pullman et al., 1982, Rabb et al., 1993). Besides, Shomori et al., showed the *in vitro* antitumor effects of water soluble fraction of maitake on gastric cell line (Shomori et al., 2009). In contrast to the above, only a few studies have examined the effect of maitake fruiting bodies on the prevention of bowel cancer (Shomori et al., 2009).

Milk is one of the most popular beverages in the world and in 2016 alone, the worldwide milk consumption was 223 billion liters (Pulina et al., 2018). Cow's milk is the most frequently encountered, although there is considerable geographical variation, with goat, sheep and camel milk consumed in the Middle East and the water buffalo as a

traditional source in South Asia (Tsuda and Sekine, 2000). Milk and milk components have been hypothesized to have possible anti-carcinogenic properties due to higher bioavailability of nutrients such as calcium, micellar calcium phosphate (MCP), casein, substances that accelerate calcium absorption (i.e., casein-phosphopeptide (CPP) and lactose), butyric acid, ether lipids, sphingomyelin, conjugated linoleic acid, vitamin A, carotene and vitamin D (Jenness, 1979, Pulina et al., 2018, Tsuda and Sekine, 2000).

Growth and progression of cancer is a multiple-step process accompanied by multiple genetic and epigenetic alterations which result in the dysregulation of multiple cell signaling pathways, thus, suggesting the importance of multi-targeted drug more than mono-targeted drug on treating CRC. Wide ranging researches have revealed that the naturally occurring compounds in fruits, vegetables, herbs, and other foods could modulate multiple signaling pathways and thus are multi-targeted naturally (Rajamanickam and Agrawal, 2008). Meanwhile, combinatorial trials have shown the synergistic inhibitory effect of some naturally available compounds against cancer cells. In one study, curcumin and celecoxib, a specific COX-2 inhibitor showed effective inhibitory effect against ACF formation in DMH treated mice when treated together than independent treatments (Shpitz et al., 2006). Further, Ohishi et al., concluded that sulindac, a well-known, promising anticancer agent with adverse side effects, can suppress ACF formation in DMH treated animal without any notable side effects when combined with epigallocatechin gallate (EGCG) (Ohishi et al.,2002). Multifactorial nature of colon carcinogenesis and multi-target effect of mushroom and milk lead us to assume more beneficial effect in combining these two agents with different mode of actions, rather than a single agent on preventing colon carcinogenesis.

Therefore, in this chapter we aimed to evaluate the chemopreventive properties of maitake, dietary bovine milk and their combinations in mice treated with 1,2-dimethylhydrazine colon carcinogenesis. The possible mechanism by which maitake and milk may exert their action together or individually were also studied.

3.2 Experimental section

3.2.1 Diet preparation

Commercially available milk powder (Yotsuba Milk Products Co., Ltd) and fresh maitake fruiting bodies (Hokuto Corporation) were purchased from a local market in Hokkaido, Japan. Fresh maitake fruiting bodies were cut into small pieces prior to boiling for 10 min (Dikeman et al., 2005) with continuous agitation. The excess water was drained and the mushroom cooled down to room temperature on the metallic mesh. Remaining excess water was further removed by pat drying and the dried fruiting bodies were lyophilized, milled and stored at -30 °C until used for diet preparation.

3.2.2 Animals and Diet

Experimental diets were based on sphingolipid free AIN-76 standard for purified diets for rats and mice and contained either milk, maitake or both as follows; 10% milk (10MK), 10% maitake (10MM), 5% milk + 5% maitake (5Mix), 10% milk + 10% maitake (10Mix) (**Table 1, Appendix**) and normal AIN-76 diet was used as the control. Each group of mice was fed one of the five diets (**Table 1, Appendix**). and had free access to food and water.

Four-week old male BALB/c mice (n=72) were purchased from Japan SLC, Inc (Shizuoka, Japan) and housed in micro-isolator cages (four mice per cage) under 12-h light/dark condition with a relative humidity of 50-60% and room temperature of 22 °C. Mice were given tap water and AIN-76 standard diets *ad libitum*. After acclimation for one week, mice were randomly divided into six groups (n = 12) namely: blank, control, 10 MM, 10MK, 5Mix and 10Mix and fed with experimental or normal diets. After one week of acclimatization for the experimental diets all of the mice (n = 60) were injected subcutaneously with DMH-HCl (Tokyo Chemical Industry Co., Ltd., Japan) at a dosage of 15 mg/kg body weight once a week for 10 weeks except the animals in the blank group to induce pre-cancerous lesion development and colorectal cancer. Precisely, 30 mg of DMH was dissolved using autoclaved saline (0.9% NaCl in 1mM EDTA-2Na) in 15 mL sterile conical centrifuge tubes and pH was adjusted to 7.0 using 0.5 N NaOH.

The animals in the negative control group (Blank) fed with normal AIN-76 chow diet received 0.9% saline with the same volume as DMH given to the other groups. The maitake and milk were administrated in a modified dose based on the previous work in rats (Sliva et al., 2012). Daily food consumption and weekly changes in weight were recorded. At the end of the 10th week, the animals were sacrificed under anesthesia.

3.2.3 Serum and organs collection

Serum was collected via cardiac puncture and placed in tubes containing anticoagulant (heparine) and stored at -80 °C for hematological examinations. Liver and spleen were removed, washed with cold saline, and the weights were recorded. Liver was snap frozen and stored at -80 °C. The cecum was excised, weighed and the digesta was scraped off from the cell wall and a known weight placed in three volumes of saline for

pH measurement and the rest of the digesta was stored at −20°C for later analysis of SCFA.

3.2.4 Identification of aberrant crypt foci

ACF was identified as described previously (Wijnands et al., 2004). Five colons per group were removed, rinsed with 0.9% cold saline to remove residual bowel contents, cut opened longitudinally and fixed flat in 10% buffered formalin for 24 h at room temperature before they were made into specimems. The mucosal surface of the colons was stained with 0.3% methylene blue in saline for 30 min at room temperature to view ACF. The number of aberrant crypt foci were examined under 10× magnification using a light microscope in a blinded manner and distinguished from the normal crypt by their larger size (larger than adjacent normal crypts), irregular and dilated luminal opening and thicker epithelial lining. Observed aberrant crypt foci were categorized in three groups (AC1, AC2, AC3≥) based on the number of aberrant crypt observed in each focus.

3.2.5 Dietary lipid analysis

To extract the total lipid from diet and dietary components (maitake, milk and corn oil), 500 mg of samples were extracted with 3 ml of chloroform: methanol (2:1 v/v) in 0.002% butylated hydroxytoulene (BHT). After repeating the extraction procedure two times, extracts were combined, washed with 0.9% KCl and centrifuged at 3000 rpm for 10 min after vigorous vortex. The organic layer was collected, evaporated at RT under nitrogen steam and dry residue was re-dissolved in 2 mL of chloroform: methanol (2:1 v/v) and stored at -20 °C for further use.

3.2.5.1 Total lipid analysis

Total lipid profile in diet was analyzed as described by Reis et al., (Reis et al., 2012) Briefly, two sets of 100 µL of lipid extracts were placed in sealed vials, dissolved in 2 mL of methanol-acetyl chloride mixture (9:1 v/v) and one set was treated with 100 µL of internal standard (17:0, 0.2mg/mL) and incubated at 100°C for 2h. Fatty acid methyl esters (FAME) and dimethyl acetals (DMA) were extracted with 3.5 mL of hexane and distilled water (v/v: 6:1). The mixture was vortexed vigorously and centrifuged at 3000 rpm for 10 min at RT and the upper layer was extracted with 2% $KHCO_3$ and vigorous vortexing for 1 min. After centrifugation at 3000 rpm for 10 min at RT, the organic layer was collected, evaporated at RT under nitrogen steam and dry residue was re-dissolved in 0.5 mL of hexane and prepared for gas chromatography (GC) by sealing the hexane extracts under nitrogen. The analysis was carried out with a GC-2010 instrument (Shimadzu, Japan) equipped with a split injector, a flame ionization detector (GC-FID at 240 °C) and a CP-SIL 88 column (50 m × 0.25 mm ID × 0.20 µm) (Vairan USA). The oven temperature program was as follows: the initial temperature of the column was 80 °C, held for 2 min, then a 10 °C/min ramp to 160 °C for 5 min, followed by 2 °C/min ramp to 220 °C for 30 min, and a final column temperature 220 °C held for 10 min and detection temperature was 240 °C. The column flow rate was 0.7 ml/min. Sample injection (2 µL) was carried out at 230 °C. Fatty acid identification was made by comparing the relative retention times of FAME and DMA peaks from samples with standards (bovine heart lipid extract) and quantification was done against internal standard (C17:0). Results were expressed as µmol/ mL and mol%.

3.2.5.2 Sphingolipid analysis

Total lipid was extracted using the protocol in 3.2.5 with some modifications (Takakuwa et al., 2002). Five grams of samples were used and after subsequent extracts the organic layer was dried using rotary evaporator at 100 rpm at 30 °C and further extracted using the method described by Wang and Benning, with some modifications. The collected organic layer reconstituted with 50 mL of 0.4M KOH sonicated for 5 min in a sonic water bath at 20 °C and mixed in a water bath at 38 °C for 2 h. The solvent mixture was mixed with chloroform: methanol: water (1:4:0.8, v/v) in a separating funnel with stirring for phase separation and the lower phase was collected. The collected organic layer further washed overnight, with Chloroform: Methanol: Water (3:48:47, v/v) to remove non-lipid compounds, dried using rotary evaporator and re-dissolved in 200 µL of samples. Thin layer chromatography was used to separate the lipid with standards and chloroform: methanol (95:12 v/v) was used as the mobile phase. Separated lipids were reversibly stained with iodine and silica from identified lipids were scraped out and collected in a screw cap vial. Silica was removed, the lower layer after reconstituted with water, was collected, dried under nitrogen steam and re-dissolved with 5 mL of chloroform: methanol (2:1 v/v). Two sets were prepared using 2 mL in each vial, one set was treated with 5 µL of internal standard (DL erythro/thero sphinganine d18:0, Cayman) 1 N hydrochloric acid (HCl) in anhydrous methanol was added to the mixture and incubated at 70 °C for 18 h. FAME were separated using hexane, dried under nitrogen steam and reconstitute with 10 µL of hexane prior to quantify with the gas chromatography mass spectrometry (GC-MS). The GC-2030 GC-MS (Shimadzu, Kyoto, Japan) equipped with a CP-SIL-88 column (2 µm, i.d. 50 m × 0.25 mm, Varian, USA) and a flame ionization detector with oven cycle program of 80 °C to 160 °C, at 10°C /min for 5 min, followed by 160°C to 220°C at 2°C /min

for 30 min, and a final column temperature 210 °C for 5 min. Injection and detection temperature were 203°C and 240 °C respectively, and column flow rate was 0.7 mL/min. 2 µL sample was used for sphingolipid evaluation. Sphingolipid detection was done by comparing the relative retention times of peaks from samples with standards and quantification was done against internal standard (d18:0). Results were expressed as µmol/ mL and mol%.

3.2.6 Analysis of cecal content

Cecal contents were collected during the autopsy at the end of the study for pH and SCFA measurements.

3.2.6.1 Cecal pH

The cecal contents were vortexed, centrifuged at 5000 rpm for 3 min and the supernatant was used to measure the pH (portable pH meter, AS ONE).

3.2.6.2 Cecal short chain fatty acid analysis

The SCFA concentrations including acetate, butyrate, propionate and lactate in the cecum were analyzed by High Performance Liquid Chromatography (HPLC) (Shimadzu, Kyoto, Japan) equipped with a Shim-pak SCR-102H column (7 µm, i.d. 8.0 mm × 300 mm, Shimadzu) and a conductivity detector. Briefly, known amount of cecum (0.2- 0.3 g) were dissolved in three-fold volume of distilled water, homogenized, centrifuged at 16,000 × g for 10 min and the supernatant was filtered in a 0.45 µm filter for HPLC analysis. The mobile phase was pH buffer for organic acid analysis (Shimadzu) at a flow rate of 0.8 mL/min with a column temperature of 40 °C. A standard SCFAs mixture containing acetate, propionate and butyrate (Sigma-Aldrich,

St. Louis, MO) was used for calculation: acetate: y=0.0508x (R_2=1), propionate: y=0.0447x (R_2=1), butyrate: y=0.0371 (R_2=1) respectively. The concentration of SCFAs was expressed as concentration (μmol/g).

3.2.7 Liver analysis

Frozen liver tissue samples were homogenized with three volumes of cold phosphate buffer solution (PBS) containing 30% of 1mM EDTA using a digital homogenizer (AS one corporation, Osaka, Japan) at 1000 rpm under ice bath condition. Aliquots were stored at -80 °C for total lipid, protein and thiobarbituric acid reactive substance (TBARS) measurements.

3.2.7.1 Analysis of hepatic lipid and fatty acid profile

Direct total lipid analysis was conducted as described by Neves et al., (Neves et al., 2019) Briefly, two sets of 200 μL of homogenized liver samples were dissolved in 2 mL of methanol-acetyl chloride mixture (9:1, v/v) and one set was treated with 100 μL of internal standard (17:0, 0.2mg/mL) and incubated at 100°C for 2h. Fatty acid methyl esters were extracted as described in 3.2.5.1

3.2.7.2 TBRAS analysis

Levels of thiobarbituric acid reactive substances (TBARS) in liver homogenates were estimated by the method of Okhawa et al., (1979) [18]. Concisely, 1.5 mL of 20% acetic buffer solution (pH 3.5), 200 μL of 8.1% SDS solution, 50 μL of butylated hydroxytoluene (BHT) solution and 1.5 mL of 0.8% thiobarbituric acid (TBA) were added to the 100 μL of liver homogenates. The mixture was made up to 4.0 mL with distilled water and let stand at 5°C for one hour followed by heating in boiling water

bath (< 95°C) for 60 min. After cooling down to room temperature, 1.0 mL of distilled water and 5.0 mL of n-butanol/pyridine (15:1 v/v) were added and mixed vigorously. After centrifugation at 3000 rpm for 10 min, the absorbance of the organic layer was measured at 532 nm. As standard 1,1,3,3-tetramethoxypropane (TEP) was used and the levels of TBARS were expressed as mmol/mg tissue.

3.2.8 Analysis of colon mucosa

The colon mucosa from five animals per group were scraped, homogenized in PBS containing proteinase inhibitors (Protease inhibitor cocktail set III, Fujifilm Wako Pure Chemical Corp., Japan), and after addition of Triton X-100 aliquots were snap frozen and stored at -80 °C for further use.

3.2.8.1 Cytokine assay

Relative expression levels of 40 mouse cytokines (cytokines and chemokines) were evaluated using a mouse cytokine array kit, according to the manufacturer's instructions (Mouse Cytokine Array Panel A, R&D systems). Concisely, the nitrocellulose membrane containing immobilized capture antibodies was blocked with Array buffer 1 for 1 h on a rocking platform shaker at room temperature. The membrane was then incubated with lysates treated with array buffer 4 along with Detection Antibody Cocktail overnight at 2 °C to 8 °C. The membrane was incubated with streptavidin horseradish peroxidase conjugate followed by chemiluminescent detection reagent. The membrane was scanned using autoradiography cassette (Amersham Pharmacia Biotech). The pixel density in each spot volume was determined (Schneider et al., 2012) corrected for background and expressed as fold change using ImageJ version 1.46 software (http://imagej.nih.gov/ij). The analyzed cytokines were as follows: B

lymphocyte chemoattractant (BLC); chemokine (C-C motif) ligand 1 (I-309); complement component 5a (C5/C5a); eotaxin; granulocyte macrophage colony-stimulating factor (GM-CSF) complex; interferon-γ (IFN-γ); interferon-γ-induced protein 10 (IP-10); interleukin (IL)-1ra, -2, -3, -4, -5, -6, -7, -10, -12, -13, -16, -17, -23, and -27; metallopeptidase inhibitor 1 (TIMP-1); monocyte chemoattractant protein 2 (MCP-2); macrophage inflammatory protein-1β (MIP-1β); macrophage inflammatory protein-1α (MIP-1α); monokine induced by gamma interferon (MIG); monocyte chemotactic protein 5 (MCP-5); monocyte-specific cytokine MCP-1 (JE); macrophage colony-stimulating factor (M-CSF); neutrophil-activating protein 3 (KC); interferon-inducible T cell alpha chemoattractant (I-TAC); regulated on activation, normal T cell expressed and secreted (RANTES); soluble intercellular cell adhesion molecule-1(sICAM-1); stromal cell-derived factor 1 (SDF-1); thymus and activation regulated chemokine (TARC); triggering receptor expressed on myeloid cells 1 (TREM-1); and tumor necrosis factor-α (TNF-α).

3.2.8.2 TNF-α assay

TNF-α levels in colon mucosa was measured by using an ELISA kit specific to TNF-α (FUJIFILM Wako Shibayagi Corp., Gunma, Japan) in accordance with the supplier's protocol. In brief, the microplate pre-coated with mouse TNF-α antibody was diluted and samples were then incubated on the ELISA plate for 2 h followed by incubation with biotin-labelled detection antibody. Samples were then incubated with streptavidin-HRP prior to detection with TMB substrate. The reaction was stopped with stop solution, and the quantification was performed by determination of the optical density within 30 min, using a microplate reader set to 450 nm (Thermo Scientific Multiscan FC, Finland). Each assay was repeated for a minimum of three times.

3.2.8.3 Apoptosis assay

Relative levels of 21 mouse apoptosis-related proteins were detected and analyzed using a mouse apoptosis array kit, according to the manufacturer's instructions (R&D systems). Briefly, the membrane containing immobilized apoptosis-related antibodies was blocked with the provided blocking buffer for 1 h followed by incubation with Detection Antibody Cocktail for 1 h at room temperature. After treatment with streptavidin horseradish peroxidase conjugate and chemiluminescent detection reagent respectively, the spots were detected. Spots intensities were quantified and expressed as fold change (ImageJ version 1.46 software). The analyzed apoptosis related proteins are: B-cell lymphoma 2 (Bcl-2); Bcl/leukemia x (Bcl-x); catalase; claspin; (MCL-1);.p27 cyclin dependent kinase 4 inhibitor 1B (p27/Kip1); X-linked inhibitor of apoptosis (XIAP); Bcl-xL/Bcl-2 associated death promoter (Bad); cytochrome c; second mitochondria-derived activator of caspase/direct inhibitor of apoptosis-binding protein with low pI (SMAC/Diablo); fibroblast-associated (Fas); TNF receptor 1 (TNF R1); TNF-related apoptosis-inducing ligand receptor 2 (TRAIL R2); cleaved caspase-3; p53; hypoxia-inducible transcription factor (HIF)-1α; heme oxygenase (HO)-1; HO-2; heat shock protein (HSP)27; HSP60; HSP70.

3.2.9 Statistical analyses

Differences among groups were assessed using one-way ANOVA with Tukey's post hoc test. P values of <0.05 were considered to be statistically significant. All data were analyzed using either BellCurve for Excel (Social Survey Research Information Co., Ltd., Tokyo, Japan) or R 3.5.2 with the RStudio 1.1.453 package (R-foundation) and expressed as the mean ± standard error of the mean (SEM) unless otherwise indicated.

3.3 Results

3.3.1 Effect of maitake and milk on food consumption, body weight, organs weight and colon length

Body weight gains and food intake did not differ among mice fed with different experimental diets over the period of 10 weeks. Even though, all the animals gained weight throughout the experimental period, the body weight gained of the DMH un-treated group was slightly higher than that of DMH treated groups **(Fig. 3.1)**. On the other hand, food disappearance of mice fed experimental diets was not different from the blank group, nevertheless, 5Mix group showed slightly higher food consumption after the 5^{th} week till the end of the experiment **(Fig. 3.2)**. Likewise, no significant effect of diets or DMH treatment were observed on liver and spleen weight at the end of the 10^{th} week. Cecum weight of 10Mix mice, however, was significantly higher than the control (0.30 ± 0.02 vs 0.24 ± 0.01; $p < 0.05$) which was comparable with maitake treated group **(Table 3.3)**. With regard to the colon length, the lowest value was observed in 10Mix followed by 10MM, 10MK, and 5Mix and were significantly shorter than that of the control (**Table 3.3**).

3.3.2 Effect of maitake and milk on ACF formation

After 10 weeks of dietary treatments with milk, maitake and the combinations, colonic ACF were identified in all the six groups. Animals treated with DMH experienced remarkable development of ACF, compared to the untreated group (blank). The development of ACF was evidenced by the occurrence of single crypt or multicrypt with increased sizes, reformed luminal openings and thickened epithelia (**Fig. 3.3**). ACF were present predominantly in the distal and middle colon but were also observed at low levels in the proximal colon. The total number of ACF, AC and multicrypt of

ACF per cm^2 of the colon were significantly higher ($p < 0.05$) in the control compared to the blank and treatment groups (**Fig. 3.4 pic** and **Fig. 3.5**). Interestingly, treatment groups exhibited over 40% total reduction of ACF formation at the end of the 10^{th} week showing the highest suppressing ability in the 10Mix group. Even though 10Mix group was effective on suppressing overall ACF, the no of foci containing more than 2 crypts were not significant among milk, maitake or combination treated groups **(Fig. 3.5)**.

3.3.3 Dietary lipid composition

Fatty acid content of main dietary ingredients (corn, maitake and milk) were expressed as mol% of diets (**Table 3.1**). Highest amounts of saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA) were observed in 10Mix combination diet while the highest amount of polyunsaturated fatty acids (PUFA) were observed in maitake only treated 10MM diet. Further, omega 3 PUFA were significantly higher in maitake treated groups. Further n3/n6 ratio which is an indicator of CRC suppression was highest in milk only treated group. Sphingolipid analysis revealed the presences of ceramide, GlcCer in both maitake and milk but the presence of sphingomyelin was observed only in milk. Total amount of both ceramide, GlcCer were higher in maitake (26326 nmol/ 100 g sample, 489 nmol/ 100g sample respectively) than that of milk (939 nmol/ 100 g sample, 221 nmol/ 100g sample respectively). The amount of sphingomyelin observed in milk was 2442 nmol/ 100g (**Table 3.2**).

3.3.4 Effect of maitake and milk on cecal pH and SCFA

Cecal pH and Cecal SCFA were assessed in order to determine the colon condition. Significantly lower cecal pH values were observed in 10Mix and 10MM compared to control and those values were comparable with the blank (**Table 3.3**). Total SCFA

concentration were significantly ($p < 0.05$) higher in maitake and combination treated groups compared to DMH treated control group and these values were comparable with the blank **(Table 3.4)**. Conversely, total SCFA in 10MK group, was lowest among dietary treatments groups and was equivalent to control. In all groups, acetate was the most abundant SCFA followed by propionate and butyrate. Animals treated with 10Mix diet showed moderately higher amount of acetate, propionate and total SCFA than that of the blank while the butyrate was equal.

3.3.5 Effect of maitake and milk on liver lipid profile and fatty acid composition

Total saturated fatty acids levels were similar among groups and the least SCFA levels were observed in 10MM treated animals while highest were in combination treated groups (**Table 3.4**). Higher palmitic (C16:0) content were detected in 10Mix treated mice while higher stearic (C 18:0) contents were detected in mice fed with standard diet. Administration of milk or maitake increased MUFA content in liver compared to standard diet fed animals while maitake and milk administration showed a negative effect on liver PUFA levels. Total amount of n-3 PUFA was not differ among groups however, not only milk administration was able to decrease n-6 levels in liver but also increase n-3 to n-6 ratio which are known to have suppressive effect against colon inflammation and CRC development.

3.3.6 Effect of maitake and milk on liver lipid peroxidation

Liver lipid peroxidation was determined by measuring thiobarbituric acid reactive substances (TBARS) in proximal and distal colon tissues of blank and experimental mice. Liver TBARS were significantly higher in the DMH treated mice (control group)

at the end of 10 weeks as compared to the DMH untreated mice (blank group). Maitake, milk and combination treatments significantly lowered ($p < 0.05$) TBARS as near to normal compared to mice treated with DMH alone and the effect was more pronounced in 10Mix supplemented group (**Fig. 3.6**).

3.3.7 Effect of maitake and milk on the expression of inflammatory-related cytokines

The effect of the different treatments on release of the pro-inflammatory cytokine TNF-α, a representative inflammatory-related cytokine in colon mucosa was analyzed using ELISA kit. In the control group the release of the cytokine by the colonic mucosa was over threefold higher than in blank mice. In all maitake and/or milk treated groups, DMH treatment failed to up-regulate the amount of TNF-α released and the difference from the control group was significant. TNF-α levels in the 10MK and 10Mix were similar to that in the DMH untreated blank group **(Fig. 3.7)**. Other than that, significantly higher levels ($p < 0.05$) of IL-1ra, an anti-inflammatory cytokine, lower levels of IL-16, a pro-inflammatory cytokine, and IP-10, a chemokine, were observed in groups treated with 10MK, 10MM, and 10Mix. In addition, moderately higher levels of BLC, RANTES and sICAM-1 were observed in treatment groups compared to control group (**Fig. 3.8**). Even though 40 different cytokines and chemokines were tested we could not detect all of them.

3.3.8 Effect of maitake and milk on the expression of apoptosis related proteins

To further understand the mechanism of apoptosis in DMH treated mice, the expressions of apoptosis-related proteins were observed (**Fig. 3.9**). All the anti-apoptotic protein tested, were lower in dietary treatment groups compared to that of the

control showing significant reduction of Bcl-2, Bcl-x, MCL1, XIAP and p27/Kip (**Fig. 3.9**). Further, the highest effect was observed in 10Mix group. In addition, tested apoptosis related proteins were moderately higher in maitake and/or milk treated groups except pro-apoptotic protein: SMAC/Diablo. The levels of cleaved caspase-3 the effector caspase, in 10 MM or 10 Mix were significantly high compared to control. Besides, higher amount of p53, cytochrome c, Bad, TRAIL R2, TNF R1 and Fas were observed in maitake treated groups.

3.4 Discussion

Growth and progression of cancer is a multiple-step process which is the result of the dysregulation of multiple cell signaling pathways, which in turn suggests that a drug, which can interact with multiple target molecules, will be more efficacious than the current mono-targeted anticancer drugs. Extensive research has revealed that most naturally occurring food components and compounds, could modulate multiple cell signaling pathways and thus are multi-targeted naturally (Xu et al., 2010). Maitake is rich in bioactive compounds including polysaccharides, phenolic compounds, essential lipids and vitamin D that are known to have suppressive effect against CRC development (Patel and Goyal, 2012) was used in the present study with or without combination with milk. Milk is rich in complex sphingolipids, casein, calcium and vitamin D (Pulina et al., 2018, Tsuda and Sekine, 2000) which possesses anti-cancer effects against different types of cancers such as breast cancer, gastric cancer and rectal cancer and may have the potential to inhibit the pathogenesis of colorectal cancers. In addition, some of these agents when used in combination exert more efficient inhibitory effect on cancers than independent intake (Ohishi et al., 2002, Shpitz et al., 2006). In

particular, previous studies have suggested that expression of calcium-sensing receptors (CaSR) which is known to suppress TNF-α induced inflammation and upregulate apoptosis in muted cells depend on bioavailability of extracellular calcium ion and vitamin D (AGGarwal et al., 2016). Multifactorial nature of colon carcinogenesis, multitarget ability of maitake and milk and effect of calcium and vitamin D against colon inflammation and tumor prevention lead us to assume a more benefit in combining these two food components to prevent colon carcinogenesis. Therefore, in the present study, DMH-induced mice colon carcinogenesis model was used to evaluate the effects of maitake and the synergistic effect of maitake when combined with milk on ACF formation and tumor incidences.

In the present study animals did not show any significant difference in weight gain or food intake throughout the experimental period. However, in one study, rats treated with DMH showed reduced body weight and lower growth rate during the 15 weeks of experimental time indicating the increased tumor burden, loss of appetite accompanied by increase in polyps driven-cachexia and anorexia (Thangaraj et al., 2018). In that experiment, daily dose was 10 mg/kg and 20 mg/kg body weight, while in our study it was 15 mg/kg body weight and also the experimental time was different by 5 weeks. This may explain why the animals in the present study was at the initial stage of the CRC development. Confirming the present observations, Yamashita et al., also stated that DMH administered balb/c mice fed with GlcCer from four difference types of flour did not show any different in weight gain or food intake during the 7 weeks of experimental time (Yamashita et al., 2017).

Formation of ACF, which is regarded as an ideal biological indicator for evaluating the development of CRC (Furukawa et al., 2002, Katyama et al., 2002, Luebeck and Moolgavkar, 2002, Tanaka et al., 2000). was observed in all the experimental groups (**Figs. 3.3 and 3.4**) except in the DMH negative control. Although the ACF suppressive components in maitake and milk are different, both food components significantly suppressed ACF formation and damages in colon tissues induced by DMH treatments. Furthermore, combination treated 10Mix group was more effective in suppressing ACF formation than maitake or milk alone (10MM and 10MK, **Fig. 3.5**). Our result was comparable with previous studies conducted in combination trials and these results also suggest that synergistic inhibitory effect in suppressing neoplastic lesion development is more effective than independent intake. (Xu et al., 2010). However, it should be noted that the effect of ACF suppression by the simultaneous intake of milk and maitake is likely to be dose-dependent. The ACF multiplicity (crypts >=2) has been suggested to be a better predictor of tumor incidence since, large ACFs have much greater potential to progress into cancer (Half and Arber, 2009). In our study the numbers of large ACF (crypts >=2) in different diet groups reflected the same pattern as was observed with the total ACF number (**Fig. 3.5**).

SCFAs are thought to play an important role in the process of inhibiting colon carcinogenesis, and ACF development due to its involvement in colon inflammation, cell proliferation and mutant cell apoptosis (Byndloss and Bäumler, 2018, Havenaar, 2011, Maslowski et al., 2009, Zhu et al., 2014). SCFAs including acetic, propionic, and butyric are known to be the major end products of microbial fermentation of dietary polysaccharides, milk and some food components (Zhao et al., 2011). In the present study, we observed significantly decreased levels of SCFA in DMH-induced control

groups compared to DMH negative control. However, consumption of milk or maitake was able to increase the SCFA production. Confirming our data, Zhao et al., showed increased levels of SCFA in AOM treated rats fed high amylose corn starch suggesting the involvement of SCFA in suppressing ACF occurrence (Zhao et al., 2011). Butyrate, is the most important among all SCFA which is involved in maintain the mucosal hemostatic (Blottiere et al., 2003, Bauer-Marinovic et al., 2006). It is known to inhibit proliferation of neoplastic colonocytes and stimulate proliferation in normal colon epithelial cells (O'Keefe et al., 2009, Roy et al., 2009). Meanwhile, increased butyrate production is associated with induction of differentiation and enhanced apoptosis in both in vivo and in vitro (Le Leu et al., 2003, Scheppach et al., 1992, Zhang et al., 2010). In human colonic mucosa, butyrate was found to regulate several pathways such as fatty acid metabolism, electron transport, TNF-α signaling and oxidative stress pathways to suppress colon carcinogenesis (Vanhoutvin et al., 2009). In addition, butyrate applied colon carcinoma and adenocarcinoma cell lines showed upregulation of genes involved in apoptosis, DNA synthesis, repair and recombination while downregulation of numerous oncogenes, cell cycle regulators and transcription factors (Dronamraju et al., 2009, Ni et al., 2010, Scott et al., 2008, Schwartz and Hadar, 2014, Vanhoutvin et al., 2009, Zeng et al., 2014). Increased cecal butyrate was observed in all the treatment groups compared to the control, suggesting that distal SCFA concentration might be associated with decreased ACF occurrence and induced apoptosis by milk and maitake (**Table 3.4**). SCFA is a secondary metabolite of probiotics microorganisms and probiotic species mainly use prebiotic substrates to stimulate their growth and activity. Bovine milk contains oligosaccharides that are analogue to human milk oligosaccharides and bioactive peptides (Zivkovic and Barile, 2017). Fermentation of milk oligosaccharides and bioactive peptides by probiotic microorganisms, particularly

Bifidobacterium and *Lactobacillus* decrease colon pH while increasing SCFA and lactate. In addition, oligosaccharides, FOS, lactulose, galactomannan and indigestible polysaccharides such as mannan, xylose and, β-glucan in maitake are considered as promising prebiotics agents (Singdevsachan et al., 2016). Further, mushroom has been known to increase SCFA in order to suppress colon inflammation and related diseases (Singdevsachan et al., 2016, Synytsya et al., 2009). The stimulation of growth and activity of probiotics might be the reason behind increased SCFAs levels and decreased cecal pH in dietary treatments group (**Table 3.3**). However, total SCFA concentration including butyrate was comparatively lower in milk only treated group (**Table 3.4**) than in maitake or combination treated groups. Morita et al., reported that when a highly purified and digestible protein such as casein is the sole protein source or available in excess in the diet with the presence of large amount of fermentable carbohydrates, nitrogen supply may become insufficient to sustain a rapid bacterial proliferation, thus resulting in a decline in fermentation capacity (Morita et al., 1998). This may explain the decline in SCFA production and higher pH level in milk only treated group.

Changes in liver fatty acid profile were found among groups based on given experimental diets (**Table 3.1 and 3.5**). The daily MUFA and PUFA distribution in liver were affected by dietary lipid composition and DMH treatments. The highest amounts of PUFA were observed in maitake added diets and interestingly, highest amount of liver PUFA were also observed in standard fed groups. The PUFA availability depends on permeability and fluency of cell membranes and make them more susceptible for lipid peroxidation (Gutierrez et al., 2004). Nevertheless, many studies have shown the dietary PUFA, particularly n-3 and n-6 play crucial role in colon inflammation and carcinogenesis (Agnihotri et al., 2016). Among essential fatty acids,

n-3 PUFA has shown chemopreventive action against different cancers including colon, lung and breast. On the other hand, n-6 PUFAs are known to possess pro-inflammatory and pro-carcinogenic effect (Benatti et al., 2004) which suggest the importance of ratio of n-3 and n-6 PUFA for the beneficial effect (Simopoulos, 2002). In our study, n-3/n-6 ratio was highest in milk diet followed by standard only diet (**Table 3.1**). However, n-3/n-6 ratio in liver issue were not followed the dietary pattern and instead, showed the highest ratio in milk only treated followed by 10Mix groups suggesting that the bioavailability depend on body tissue and lipid peroxidation.

On the other hand, lipid peroxidation is an indicator of oxidative stress which increased with DMH induced malignant alterations (Das, 2002). DMH is not only colon carcinogen but also a potent necrogenic hepatic tumorigenesis and also in the liver, DMH is converted into ultimate carcinogenic metabolite, dizonium ion which could elicit an oxidative stress and produce tumors in colon (Devesena and Menon, 2007). TBARS is an accurate indicator of liver lipid peroxidation (LPO) (Devesena and Menon, 2007). Enhanced LPO in the liver of DMH treated animals could be attributed to the DMH-induced oxidative stress and production of reactive oxygen metabolites (ROMs). Metabolism of the DMH in the liver produce AOM, leading to produce MAM and carbonium ion known as active carcinogenic electrophiles. Therefore, elevation of hepatic LPO as evidenced by the increased levels of TBARS could be due to the toxic metabolites released during DMH metabolism. Present result also showed, that DMH treatments increase liver lipid peroxidation (**Fig. 3.6**). However, oral ingestion of milk or maitake were able to suppress increased oxidative stress which is indicated with lower TBARS levels in dietary treatment groups. Previous reports suggest, phytochemicals in natural food resources can suppress oxidative stress related colon

carcinogenesis (Sreedharan et al., 2009). In one study, DMH treated animals supplemented with morin significantly decreased hepatic TBARS near to normal levels and they further showed that decreased TBARS is associated with increased activities of detoxification enzymes: superoxide dismutase (SOD), catalase (CAT), glutathione S-transferase (GST), glutathione peroxidase (GPx), glutathione reductase (GR), vitamins C, vitamin E and glutathione (GSH) which underline the antioxidant property exhibited by morin in the liver (Sreedharan et al., 2009). Following same, our study also showed near to normal TBARS levels in 10Mix group which suggest the activity of phytochemicals, particularly vitamin E and D in milk and maitake.

Pro-inflammatory cytokines are known to play an important role in inflammation of the intestinal mucosa (Nakamura et al., 1992). Specifically, increased levels of TNF-α, IL-1β, IL-6 and IL-8 have been reported in CRC patients (Aggarwal, 2003, O'Shea et al., 2002, Pullman et al., 1992, Raab et al., 1993) and TNF-α is known as a critical component in cell death and inflammation (Sedger and McDermott, 2014). Chronic inflammation is suggested to induce cancer via dedifferentiation-associated epigenetic regulations (Shibata et al., 2018). In a previous study the dietary complex sphingolipids were able to suppress the upregulated TNF-α in colon of DMH administrated mice (Yamashita et al., 2017). In the present study, we demonstrated that TNF-α release from colonic tissue samples of the mice in the DMH positive control group was significantly elevated as compared with DMH negative control and with the other dietary treatment groups (**Fig. 3.7**). The tissue levels of TNF-α protein were correlated with the degree of inflammation and macroscopic score, supporting previous, as well as more recent reports (Akazawa et al., 2002, Olsen et al., 2016). In addition, in line with the present results, recently published clinical reports (Lavi et al., 2012, Rutgeerts et al., 2005)

show that treatment with anti-TNF-α, a specific antibody against TNF-α, induces remission in 30–40 % of ulcerative colitis patients. Interestingly, both milk and /or maitake treatments suppressed the increased levels of TNF-α however, the effect of milk was stronger than that of maitake (**Fig. 3.7**). Milk and mushroom contain complex sphingolipids (sphingomyelin and GlcCer respectively) which are known to suppress inflammatory cytokines such as TNF-α. Our results suggest the sphingomyelin in milk is higher than GlcCer in maitake (2442 nmol/100g sample and 489 nmol/ 100g sample respectively) (**Table 3.2**). Confirming our results, previous study showed the presence of higher concentration of sphingomyelin in dried milk (27 mg/100 g) than the GlcCer in dried maitake mushroom (6 mg/100 g). Not only that, same study showed that GlcCer in plants and mushrooms possess lower bioavailability due to the presence of cell wall than sphingomyelin in mammals (Yamashita et al., 2019). In addition, SCFA is known to be involved in the regulation of TNF-α induced inflammation. A SCFA mixture with different proportions of butyrate (5%, 20% and 50%) was used to examine the effect of treatment on barrier function with concomitant addition of pro-inflammatory lipopolysaccharide or TNF-α and authors found out that when the proportion of butyrate was higher in the SCFA mixture (20% or 50%), TER increased significantly despite TNF-α and lipopolysaccharide treatment, suggesting that butyrate incubation can exert protective effect against inflammation (Chen et al., 2017). Calcium is also known to play a crucial role on regulating colon inflammation. Calcium activates a wide range of biological activities including cell differentiation, proliferation in order to suppress colon inflammation, and maintain epithelial homeostasis. In one study DMH treated rats with low calcium diet plus red meat, in which consumption was associated with increased risk of CRC, showed larger number of aberrant crypt and multiplicity than animal fed with low calcium diet (Pierre et al.,2008). Further, low calcium intake was

linked to the pathogenesis of several chronic diseases and is a recognized risk factor for total cancer incidence (Park et al., 2009, Peterlik et al., 2013). In addition, calcium mediated the activity of calcium-sensing receptor (CaSR). The extracellular CaSR is associated with suppressed colonic inflammation and cancer development (Lamartino et al., 2018, Tennakoon et al., 2016) through regulating several cellular processes including secretion, chemotaxis, cell–cell adhesion, and control of proliferation, differentiation, and apoptosis (Brennan et al., 2013, Brown and MacLeod, 2001). On the other hand, Mine and Zhang suggested that CaSR suppress TNF-α-induced inflammation in intestinal epithelial cells to suppress CRC development (Mine and Zhang, 2015). The calcium contents in milk and maitake that were used in this study are 890 mg and trace per 100 g dry weight, respectively as calculated by Japanese Standard Tables of Food Composition. Increased intake of milk calcium might be a reason for suppressed TNF-α in mice fed with milk and milk combined diets. Even though, AIN-76 diet contains 4 g of calcium per kg diet, the absorption and bioavailability is low compared to milk calcium and thus, the effect on suppressing ACF development may be negligible.

The CaSR expression not only depends on the bioavailability of extracellular Ca^{2+} but also on vitamin D (Bennette et al., 2000, Canaff and Hendy, 2002, Chakrabarty et al., 2005). The most active form of vitamin D: 1,25 dihydroxyvitamin D_3 (1,25 D_3) bound to vitamin D receptor (VDR) to induce translation of the CaSR and in contrast the amount and activity of the CaSR affects $1,25D_3$ signaling (Aggarwal et al., 2016). Confirming the above findings, Canaff and Hendy showed that the CaSR expression was significantly reduced in the vitamin D-depleted rats. In another study, dietary vitamin D showed the upregulated CaSR expression in the colon of mice (Aggarwal et

al., 2016) suggesting that intact CaSR expression and function is needed for proper expression of the vitamin D system. The involvement of vitamin D in colorectal tumor development was revealed by Horvat et al., showing significantly higher expression of CYP24A1, vitamin D degrading enzyme in tumor cells compared with the adjacent normal tissue (Horvath et al., 2010). Besides, several other studies showed that vitamin D reduced the risk of CRC by maintaining normal calcium gradient in the colon epithelial crypts, decreasing proliferation of noncancerous but high risk epithelial cells, and stimulating mutual adherence of epithelial cells (Bobek and Galbavy, 2001, Lavi et al., 2012). Maitake fruiting bodies contains around 28.1 μg/100 g of vitamin D while milk contain around 1 μg/100 g of vitamin D (Japanese Standard Tables of Food Composition). The high concentrations of milk calcium and vitamin D in combination diet, 10Mix might be the possible reason for higher bioactivity in suppressing ACF development, pro-inflammatory cytokines TNF-α and upregulating apoptosis. Beta casein in milk is reported to induce colorectal TNF-α compared to whey protein (Rafiee-Tari et al., 2019). In this study we used casein as protein source which were included in all the experimental diets and the casein levels in diets containing milk were adjusted by altering protein; which may suggest the effect of added casein on modulating TNF-α is insignificant. Regarding additional cytokines, the present study did not show any significant difference among groups treated with milk or maitake. However, pro-inflammatory cytokine; IL16, anti-inflammatory cytokine; IL-1ra, and chemokine; IP 10 were significantly different between control and maitake and/or milk treatment groups (**Fig. 3.8**). Vitamin D and calcium are also proven to increase IL-1ra, which inhibits the expression of IL-1α through the blockage of the IL-1/PI3K/NF-κB pathway (Ma et al., 2017).

Apoptosis of individual cells may be recognized as a protective mechanism against neoplastic development in the organism by eliminating genetically damaged cells or excess cells that have improperly been induced to divide by a mitotic stimulus (Elmore 2007). Colonic inflammation eventually regulates apoptosis and anti-apoptosis by multiple pathways through binding to TNF R1 which is considered as an effective method for a wide variety of cancer treatments (Fesik, 2005, Sedger and McDermott, 2014). Previous studies showed different food components and food bioactive compounds work differently in normal cells and mutant cells. For instance, Chinese yam and the purified protein are reported to suppress ACF formation through upregulating apoptosis in mutant cells (Arai et al., 2013). Further several other studies have shown significant apoptosis in colonic epithelial cells suffering mild acute inflammation induced by DSS (Elmore 2007). In another study, sphingoid which was digested from complex sphingolipids markedly induced apoptosis in colorectal cancer cells but with no change in normal cells (Yamashita et al., 2019).

Bcl-2 family proteins play a vital role in apoptosis which includes anti-apoptotic members such as Bcl-2 and Bcl-x, pro-apoptotic members, such as BAK and Bad and Bax and apoptotic members, such as Bid and Bim (Wei et al., 2001). Proteins of these three classes are capable of forming either homo-oligomers or heterodimers with one another and appear to play distinct roles in governing mitochondrial membrane permeabilization and apoptosis (Zhu et al., 2007, Zong et al., 2001). In our present study, significantly decreased expression levels of the anti-apoptotic proteins Bcl-2 and Bcl-x were evident during the induction of apoptosis by milk and maitake, in contrast to the increased expression level of the pro-apoptotic protein Bad (**Fig. 3.9**). It is well-established that Bad is positively regulated by p53 protein and negatively controls Bcl2

expression (Kumar et al., 2017). Butyrate also has been reported to induce colorectal carcinoma cell apoptosis through activation of caspases cleavage of the cyclin-dependent kinase inhibitor p21, Bcl-2 protection and DNA fragmentation (Jan et al., 2002). Indeed, high fiber diet which favor butyrate production reduce BCL-2 expression, suppress ACF formation and reduce the incidences of CRC (Avivi Green et al., 2001, Bingham, 1990, Perrin et al., 2001). A study conducted using fruiting body and mycelia extracts of the edible mushroom *Pleurotus pulmonarius showed decrease proliferative potential in colon cancer cell lines and suggested that* the downregulation of colon cancer cells' proliferative activity can be due to an increase in the apoptotic potential of these exposed cells which was attributed to downregulation of apoptosis suppressor protein Bcl-2 and enhancement of apoptotic protein Bax and cytochrome C (Lavi et al., 2012). In addition, confirming our findings, Hu et al., reported that the mushroom *Inonotus obliquus* suppress colon carcinogenesis through apoptosis with different sensitivity in human DLD-1 colon cancer cells which resulted in the decreased expression of the anti-apoptotic protein Bcl-2 and in the elevation of the Bax/Bcl-2 ratio (Hu et al., 2009). Similar to the study which showed that glucan from edible mushroom *Pleurotus pulmonarius,* upregulate cytochrome C and induce apoptosis in mice via intrinsic pathway (Lavi et al., 2012), we observed elevated levels of cytochrome C, an essential component of the mitochondrial respiratory chain in groups treated with milk and/or maitake. The release of cytochrome C, which is usually present in the mitochondrial intermembrane space into the cytosol is a key early step in the apoptotic process. This release into the cytosol was usually associated with an interruption of the normal electron flow at the complex III site of the respiratory chain leading to the generation of superoxide that activates the caspases (Gross et al., 1999, Wei et al., 2001). The caspase cascade is a well-known key pathway in apoptotic signal transduction.

Increased caspase-3 processing has been previously associated with DSS-induced colonic tissue damage and colitis (Joo et al., 2009, Paul et al., 2005). In addition, vitamin D and calcium were found to upregulated caspase 12 to induce apoptosis in colon cancer cell lines (Jeon and Shin, 2018). We found oral feeding of milk and/or maitake can significantly increase expression of cleaved caspase-3 compared to control (**Fig. 3.9**). Further, $1,25D_3$ has strong apoptosis-promoting effects, similar to Ca^{2+}. One study showed that 1 nM of $1,25D_3$ effectively induce Caspase 3/7 dependent apoptosis in colon cancer cells in the presence of 1.8 mM Ca^{2+} and further noticed that the expression is significantly stronger when the cells overexpressed CaSR (Aggarwal et al., 2016). Tumor suppressor, p53 is activated in response to various stresses including DNA damage to promote mutant cell apoptosis by regulating various downstream effectors such as p21 and BAX (O'Brate and Giannakakou, 2003). According to our results, milk and maitake treatments triggered apoptosis, which may be controlled by p53-mediated pro-apoptotic Bad and cytochrome C. This result is consistent with the literature on tamoxifen which describes G1-arrest induced by DNA damage in cancer cells (Ichikawa et al., 2008, Lam et al., 2012). Further, Yoshida et al., in their study concluded that butyric acid can upregulate CITED2, to activate p53 dependent apoptosis in human UCCA cell lines (Yoshida et al., 2011). Even though both maitake and milk treatments showed the apoptotic activity, the effect was apparent in the colon tissues of combination fed animals suggesting that synergistic effects are effective on suppressing colon inflammation and CRC development. Supporting the current results, several other studies also indicated the significance of combination treatments. One study showed a synergistic inhibitory effect against gastric carcinoma cells, when epigallocatechin-3 gallete combined with epicatechin. It was reported that, the synergistic effect was significant than the independent effect (de Kok et al., 2008).

Another study, that was conducted to evaluate the effect of combination of curcumin and catechin on human colon adenocarcinoma HCT 15, HCT 116, and human larynx carcinoma Hep G-2 cell lines, revealed that the synergistic inhibitory effect on the growth of cancerous cell lines was markedly higher than the individual effect (Manikandan et al., 2012). Further, Suganuma et al., revealed that the combination of active catechins with inactive epicatechin is more effective than the independent treatment on induction of apoptosis and inhibition of cell growth of human lung cancer cell line PC-9, and inhibition of TNF-α release from BALB/3T3 cells treated with okadaic acid, a tumor promoter (Suganuma et al., 2009).

3.5 Conclusion

These results suggest that maitake, milk and the combination diets can suppress colon inflammation and upregulated muted cell apoptosis to suppress ACF formation and CRC development. Increased SCFA in colon mucosa, which may have resulted from the stimulated activity of colonic probiotic microorganism, and the presence of bioactive components such as vitamin D, calcium and sphingolipids may be responsible for the bioactivity against colon inflammation. Thus, the synergistic inhibitory effect of maitake in combination with milk may be an effective nutritional chemoprevention agent against colon carcinogenesis.

Table 3.1: Fatty acid composition of the diets

Fatty acids (mol%)	Blank/Control	10MM	10MK	5Mix	10Mix
12:0	0.00 ± 0.0 [a]	0.01 ± 0.0 [a]	0.46 ± 0.0 [c]	0.23 ± 0.0 [b]	0.47 ± 0.0 [c]
14:0	0.00 ± 0.1	0.02 ± 0.0 [a]	1.57 ± 0.1 [c]	0.79 ± 0.0 [b]	1.59 ± 0.1 [c]
15:0	0.00 ± 0.0 [a]	0.04 ± 0.1 [a]	0.10 ± 0.1 [b]	0.07 ± 0.1 [ab]	0.14 ± 0.1 [b]
16:0	0.65 ± 0.0 [a]	2.05 ± 0.0 [b]	4.15 ± 0.0 [c]	3.10 ± 0.0 [b]	5.56 ± 0.0 [c]
16:1	0.01 ± 0.0 [a]	0.06 ± 0.0 [a]	0.17 ± 0.0 [bc]	0.11 ± 0.0 [b]	0.22 ± 0.0 [c]
18:0	0.08 ± 0.2 [a]	0.22 ± 0.2 [b]	1.19 ± 0.1 [c]	0.70 ± 0.2 [b]	1.33 ± 0.2 [c]
18:1 n-9	1.29 ± 0.1 [a]	4.95 ± 0.1 [c]	2.89 ± 0.1 [b]	3.92 ± 0.1 [bc]	6.55 ± 0.4 [d]
18:2 n-6	2.70 ± 0.1 [b]	6.68 ± 0.1 [e]	1.51 ± 0.1 [a]	4.10 ± 0.1 [c]	5.50 ± 0.1 [d]
18:3 n-3	0.04 ± 0.0 [a]	0.05 ± 0.0 [a]	0.02 ± 0.0 [b]	0.04 ± 0.0 [a]	0.04 ± 0.0 [a]
20:4 n-6	0.00 ± 0.0	0.01 ± 0.0	0.01 ± 0.0	0.01 ± 0.0	0.02 ± 0.0
20:5 n-3	0.00 ± 0.0	0.00 ± 0.0	0.01 ± 0.0	0.00 ± 0.0	0.01 ± 0.0

22:6 n-3	0.00 ± 0.0	0.00 ± 0.0	0.01 ± 0.0	0.01 ± 0.0	0.01 ± 0.0
ΣSFA	0.72 ± 0.0 [a]	2.31 ± 0.1 [b]	5.44 ± 0.3 [c]	3.88 ± 0.0 [bc]	7.03 ± 0.0 [d]
ΣMUFA	1.53 ± 0.5 [a]	5.22 ± 0.2 [c]	3.27 ± 2.1 [b]	4.25 ± 1.3 [bc]	6.97 ± 0.1 [d]
ΣPUFA	2.74 ± 1.1 [b]	6.77 ± 0.5 [d]	1.59 ± 1.3 [a]	4.18 ± 0.5 [bc]	5.62 ± 1.1 [c]
Σn-3	0.04 ± 0.01 [a]	0.07 ± 0.0 [b]	0.05 ± 0.0 [a]	0.06 ± 0.0 [ab]	0.08 ± 0.0 [b]
Σn-6	2.70 ± 0.3 [b]	6.70 ± 0.8 [d]	1.54 ± 0.5 [a]	4.12 ± 0.1 [c]	5.54 ± 0.1 [c]
EPA + DHA	0.00 ± 0.0	0.00 ± 0.0	0.01 ± 0.0	0.01 ± 0.0	0.02 ± 0.0
PUFA/SFA	3.79 ± 1.1 [b]	2.93 ± 0.3 [b]	0.29 ± 0.3 [a]	1.08 ± 0.4 [ab]	0.80 ± 0.1 [a]
Σn-3/Σn-6	0.02 ± 0.9 [a]	0.01 ± 0.8 [a]	0.03 ± 0.1 [b]	0.01 ± 0.1 [a]	0.01 ± 0.1 [a]

Blank: DMH negative control with standard AIN-76 diet, Control: DMH positive control with standard AIN-76 diet, 10MM: DMH treated animals fed with 10% maitake diet, 10MK: DMH treated animals fed with 10% milk diet, 5Mix: DMH treated animals fed with combination diet that contains 5% milk and 5% maitake, 10Mix: DMH treated animals fed with combination diet that contains 10% milk and 10% maitake. Values are expressed as mean ± SEM (n=3). Mean values in the same column with different superscripts letter are significantly different ($p < 0.05$) as determined by Tukey-post hoc analysis.

Table 3.2: Sphingolipid composition of maitake and milk

Sphingolipids (nmol/ 100g sample)	**Maitake**	**Milk**
Ceramide	26326	939
GlcCer	489	221
Sphingomyelin	2442	0

Values are expressed as mean ± SEM (n=3). Mean values in the same column with different superscripts letter are significantly different ($p < 0.05$) as determined by Tukey-post hoc analysis.

Table 3.3: Effect of different diets on final body weight, organs weight, cecum pH and colon length 10 weeks after DMH injection

Treatment	body weight (g)	Liver weight (g)	Spleen weight (g)	Cecum weight (g)	Cecum pH	colon length (cm)
Blank	27.8 ± 0.876	1.09 ± 0.030	0.14 ± 0.008 ^{a}	0.18 ± 0.011 ^{a}	8.10 ± 0.060 ^{a}	8.85 ± 0.239 ^{a}
Control	26.1 ± 0.545	1.03 ± 0.015	0.12 ± 0.005 ^{ab}	0.24 ± 0.012 ^{c}	8.61± 0.057 ^{b}	11.52 ± 0.242 ^{b}
10MM	25.9 ± 0.090	1.02 ± 0.030	0.12 ± 0.005 ^{b}	0.26 ± 0.016 ^{bc}	8.20 ± 0.049 ^{a}	10.35 ± 0.125 ^{cd}
10MK	26.1 ± 0.497	1.06 ± 0.041	0.11 ± 0.004 ^{b}	0.23 ± 0.012 ^{c}	8.48 ± 0.073 ^{b}	10.44 ± 0.177 ^{cd}
5Mix	25.8 ± 0.448	0.99 ± 0.015	0.11 ± 0.003 ^{b}	0.24 ± 0.013 ^{c}	8.52 ± 0.042 ^{b}	11.02 ± 0.250 ^{bc}
10Mix	26.2 ± 0.528	0.99 ± 0.029	0.11 ± 0.005 ^{b}	0.30 ± 0.017 ^{b}	8.13 ± 0.056 ^{a}	10.15 ± 0.125 ^{d}

Values are expressed as mean ± SEM (n=10). Mean values in the same column with different superscripts letter are significantly different ($p < 0.05$) as determined by Tukey-post hoc analysis.

Table 3.4: Effect of different diets on short chain fatty acid (SCFA) levels 10 weeks after DMH injection

	Acetate	Propionate	Butyrate	Lactate	Total SCFA
Blank	13.5 ± 1.3 ^{ac}	2.1 ± 0.1 ^{ac}	0.5 ± 0.0 ^{a}	0.6 ± 0.1	16.7 ± 1.4 ^{ac}
Control	8.7 ± 0.8 ^{b}	1.4 ± 0.1 ^{a}	0.1 ± 0.0 ^{b}	0.3 ± 0.1	10.2 ± 0.9 ^{b}
10MM	13.9 ± 0.8 ^{c}	2.8 ± 0.2 ^{b}	0.4 ± 0.0 ^{ad}	0.5 ± 0.1	17.1 ± 1.0 ^{c}
10MK	10.1 ± 0.7 ^{ab}	1.6 ± 0.1 ^{a}	0.2 ± 0.0 ^{bc}	0.2 ± 0.0	11.9 ± 0.8 ^{ab}
5Mix	13.2 ± 0.6 ^{ac}	2.3 ± 0.1 ^{c}	0.2 ± 0.0 ^{cd}	0.5 ± 0.2	15.7 ± 0.8 ^{ac}
10Mix	16.1 ± 0.9 ^{c}	2.6 ± 0.2 ^{bc}	0.5 ± 0.0 ^{a}	0.8 ± 0.3	19.2 ± 1.0 ^{c}

All units are in μmol/g cecum contents. Values are expressed as mean ± SEM (n =10). Mean values in the same column with different superscript letters are significantly different ($p < 0.05$) according to one-way ANOVA with Tukey's post hoc analysis.

Table 3.5: Liver fatty acid profile

Fatty Acid (mol%)	Blank	Control	10MM	10MK	5Mix	10Mix
16:0	25.57 ± 0.5[ab]	20.69 ± 2.5 [ab]	15.46 ± 1.0 [a]	23.17 ± 0.6 [ab]	29.30 ± 4.7 [b]	29.11 ± 4.9 [b]
16:1	3.29 ± 0.3 [a]	4.86 ± 0.8 [b]	7.31 ± 0.4 [c]	8.06 ± 1.3 [c]	7.44 ± 1.2 [c]	10.09 ± 2.2 [d]
18:0	7.19 ± 0.8 [a]	5.06 ± 0.6 [ab]	3.24 ± 0.4 [b]	4.14 ± 1.1 [b]	4.58 ± 0.7 [b]	3.90 ± 0.5 [b]
18:1 n-9	27.75 ± 0.9 [a]	35.46 ± 2.1 [b]	42.80 ± 1.0 [bc]	44.09 ± 1.7 [bc]	45.93 ± 7.7 [bc]	55.02 ± 11.1 [c]
18:1 n-7	2.03 ± 0.2 [a]	1.89 ± 0.1 [a]	2.53 ± 0.1 [b]	2.77 ± 0.1 [b]	2.16 ± 0.3 [a]	4.02 ± 0.7 [c]
18:2 n-6	20.00 ± 0.7 [a]	23.87 ± 0.9 [a]	21.50 ± 0.8 [a]	11.79 ± 0.6 [b]	23.66 ± 4.3 [a]	15.03 ± 4.3 [b]
18:3 n-3	0.60 ± 0.0	0.51 ± 0.0	0.47 ± 0.0	0.67 ± 0.1	0.55 ± 0.1	0.74 ± 0.1
20:3 n-6	0.04 ± 0.0	0.06 ± 0.0	0.05 ± 0.0	0.03 ± 0.0	0.06 ± 0.0	0.06 ± 0.0
20:4 n-6	10.42 ± 0.9 [b]	5.60 ± 0.8 [ab]	5.02 ± 0.4 [ab]	3.52 ± 0.6 [a]	4.65 ± 0.7 [a]	6.27 ± 0.5 [ab]
20:5 n-3	0.02 ± 0.0 [a]	0.01 ± 0.0 [a]	0.02 ± 0.0 [a]	0.03 ± 0.0 [ab]	0.03 ± 0.0 [ab]	0.08 ± 0.0 [b]
22:4 n-6	0.67 ± 0.1[b]	0.49 ± 0.1 [ab]	0.39 ± 0.0 [a]	0.32 ± 0.1 [a]	0.36 ± 0.1 [a]	0.29 ± 0.0 [a]

22:5 n-3	0.03 ± 0.0 [a]	0.02 ± 0.0 [a]	0.04 ± 0.0 [ab]	0.04 ± 0.0 [ab]	0.04 ± 0.0 [ab]	0.08 ± 0.0 [b]
22:6 n-3	1.73 ± 0.2 [b]	1.11 ± 0.2 [a]	0.93 ± 0.1 [a]	1.09 ± 0.3 [a]	1.04 ± 0.2 [a]	1.29 ± 0.2 [a]
ΣSFA	32.77 ± 0.6 [a]	25.75 ± 2.7 [a]	18.70 ± 1.3 [b]	27.32 ± 1.4 [a]	33.88 ± 5.4 [a]	33.00 ± 5.3 [a]
ΣMUFA	33.07 ± 1.2 [a]	42.20 ± 2.9 [a]	52.64 ± 1.2 [ab]	54.92 ± 2.7 [ab]	55.53 ± 9.2 [ab]	69.13 ± 13.9 [b]
ΣPUFA	33.99 ± 0.8 [a]	31.82 ± 1.4 [a]	28.51 ± 0.8 [a]	17.63 ± 1.6 [b]	30.48 ± 5.4 [a]	23.93 ± 4.7 [b]
ω-3	2.85 ± 0.2	1.79 ± 0.2	1.55 ± 0.1	1.96 ± 0.4	1.76 ± 0.3	2.28 ± 0.3
ω-6	31.14 ± 0.6 [a]	30.02 ± 1.2 [a]	26.96 ± 0.8 [a]	15.66 ± 1.2 [b]	28.73 ± 5.1 [a]	21.65 ± 4.4 [b]
EPA + DHA	1.75 ± 0.2	1.12 ± 0.2	0.95 ± 0.1	1.12 ± 0.3	1.07 ± 0.2	1.37 ± 0.2
PUFA/SFA	1.04 ± 0.0 [a]	1.57 ± 0.5 [a]	1.59 ± 0.1 [a]	0.65 ± 0.0 [b]	0.89 ± 0.0 [ab]	0.74 ± 0.1 [b]
ω-3/ω-6	0.09 ± 0.0 [a]	0.06 ± 0.0 [a]	0.06 ± 0.0 [a]	0.12 ± 0.0 [b]	0.06 ± 0.0 [a]	0.11 ± 0.0 [b]
(EPA+DHA)/AA	0.17 ± 0.0 [a]	0.20 ± 0.0 [a]	0.19 ± 0.0 [a]	0.30 ± 0.0 [b]	0.23 ± 0.0 [a]	0.22 ± 0.0 [a]

Values are expressed as mean ± SEM (n=10). Mean values in the same column with different superscripts letter are significantly different ($p < 0.05$) as determined by Tukey-post hoc analysis.

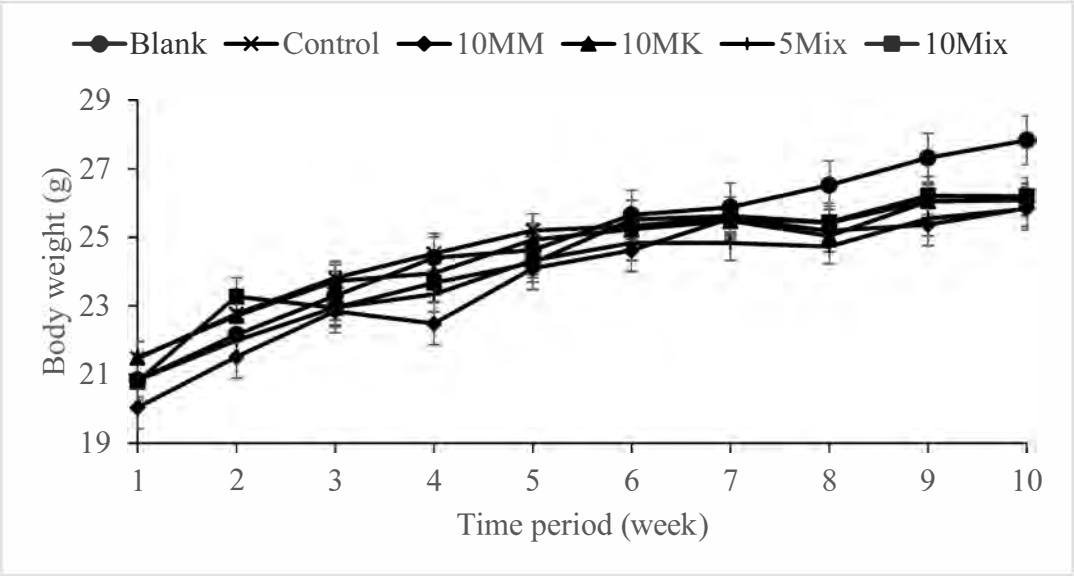

Figure 3.1: Effect of different diets and DMH treatments on body weights. Values are expressed as mean ± SEM (n = 10). Time points with different superscript letters are significantly different ($p < 0.05$) according to one-way ANOVA with Tukey's post hoc analysis.

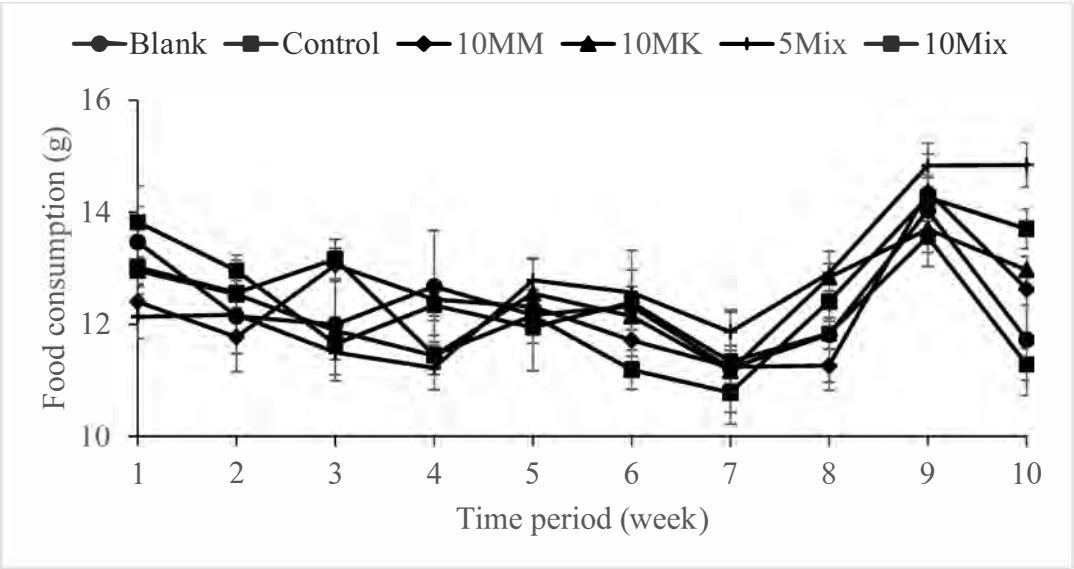

Figure 3.2: Effect of different diets and DMH treatments on weekly food consumption. Values are expressed as mean ± SEM (n = 10). Time points with different superscript letters are significantly different ($p < 0.05$) according to one-way ANOVA with Tukey's post hoc analysis.

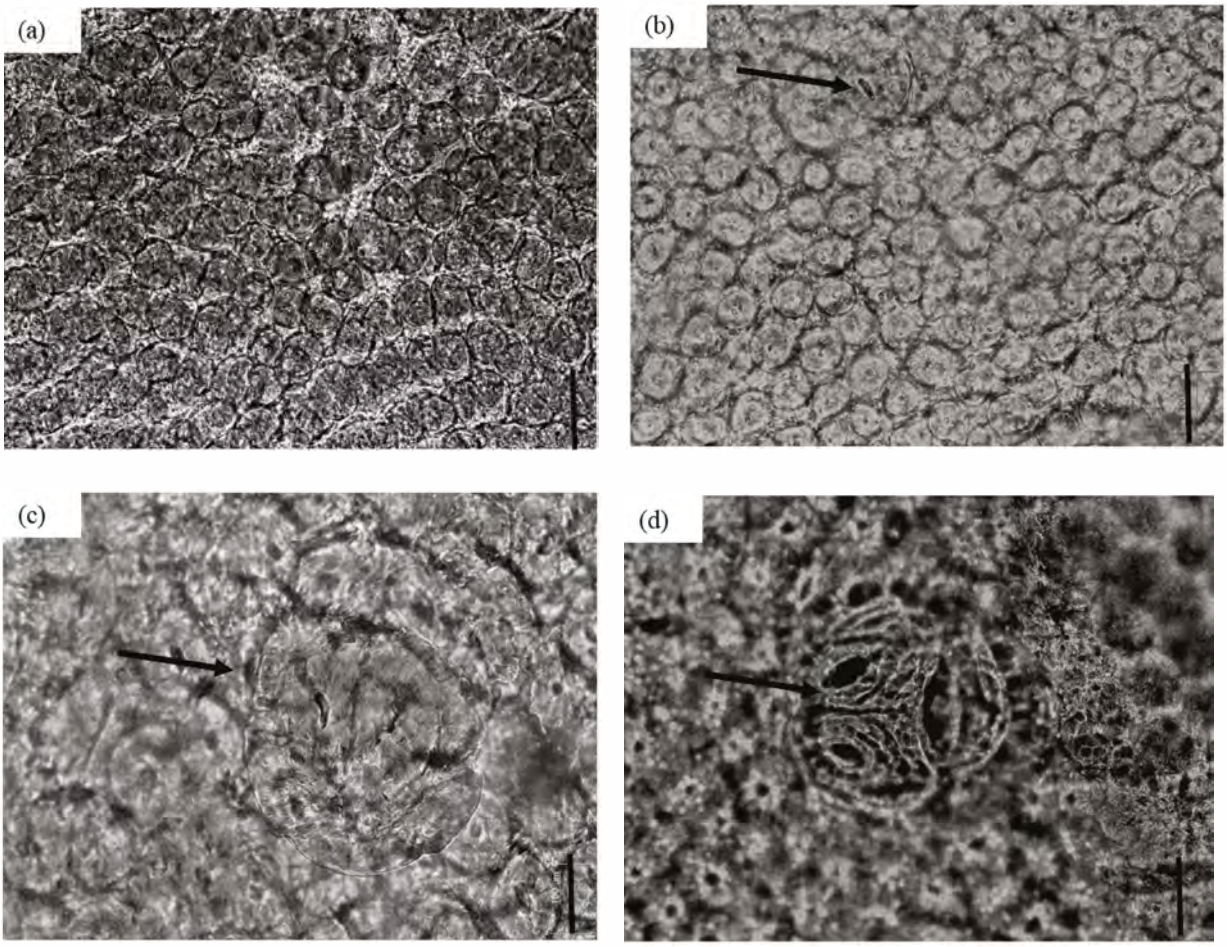

Figure 3.3: Aberrant crypt foci (ACF) with different number of crypts. (a): normal, (b): 1 crypt (AC1), (c): 2 crypts (AC2), (d): 3 crypts (AC3). (methylene blue staining, ×100). Scale bars indicate 100 μm.

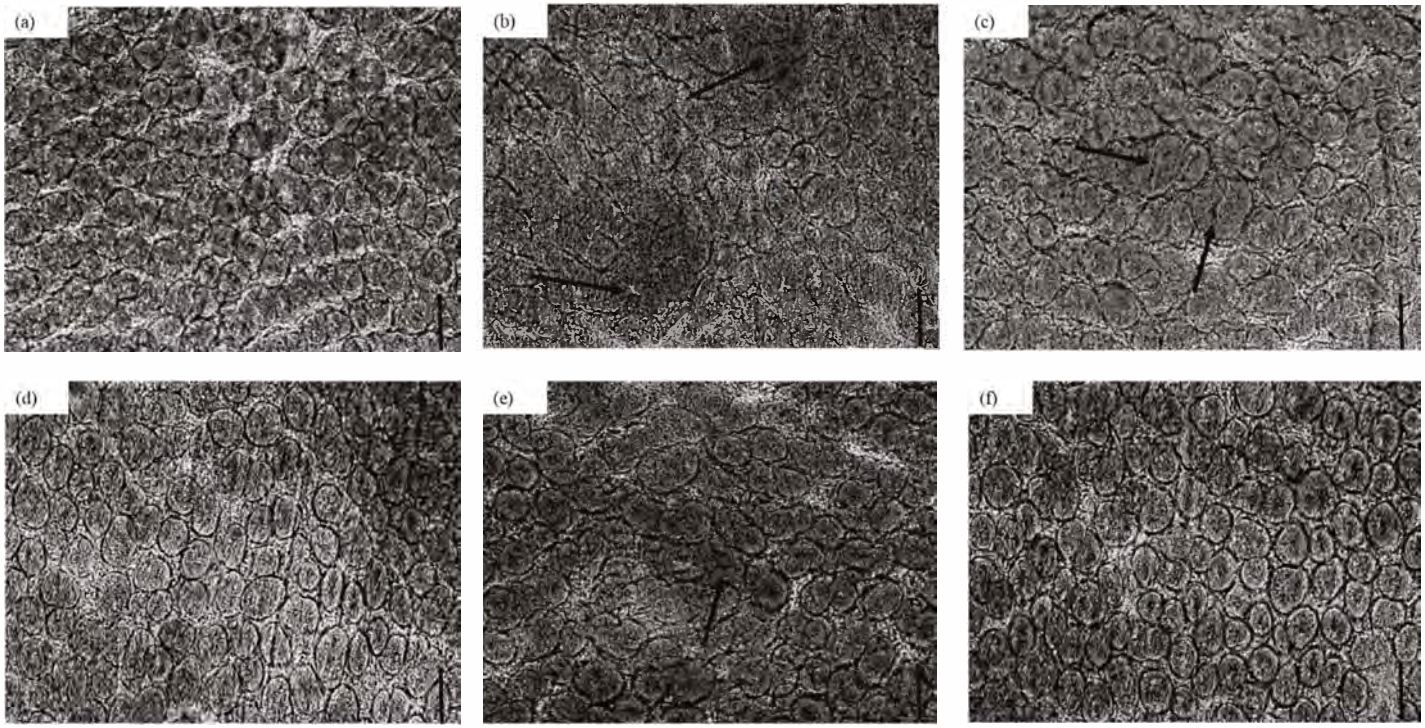

Figure 3.4: B Effects of oral feeding of maitake, milk and mixture of maitake and milk on DMH-induced ACF in mice colon. (a): blank group, (b): control group, (c): 10% milk group (10MK), (d): 10% maitake group (10MM), (e): 5% milk and 5% maitake group (5Mix), (f): 10% milk and 10% maitake group (10Mix). (methylene blue staining, ×100). The arrows indicate ACF with single or multiple crypts. Scale bars indicate 100 μm.

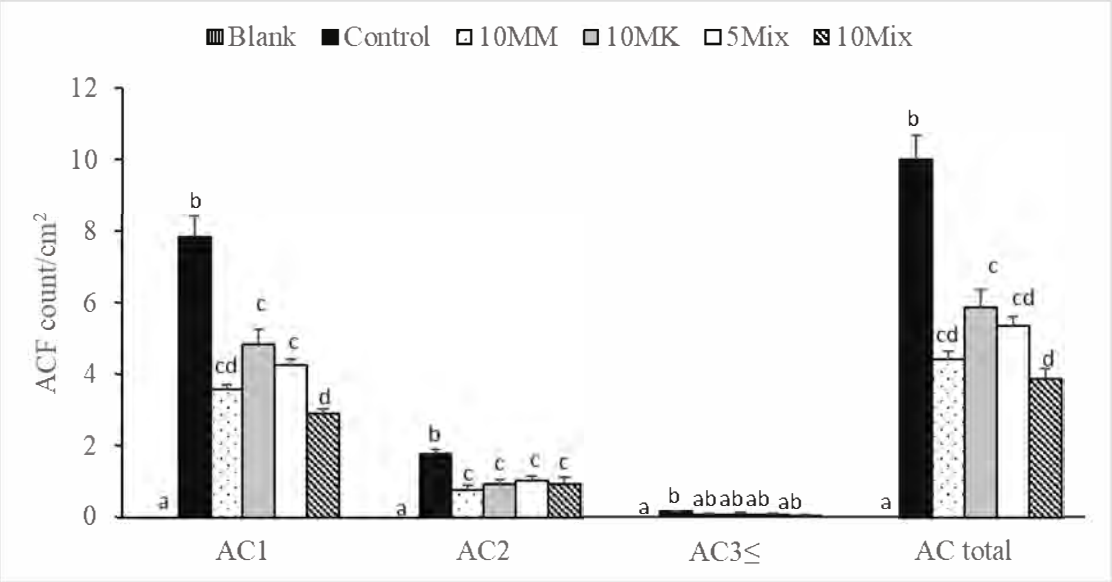

Figure 3.5: Effect of different dietary treatments on formation of aberrant crypt foci (ACF) in mice after 10 weeks of DMH treatments. Values are expressed as mean ± SEM (n=5). ACF and AC in colons were divided by degree of AC and counted as AC1, AC2, and AC3 which indicate ACF with 1, 2, and 3 or more crypts, respectively. Bars with different superscripts letter are significantly different ($p < 0.05$) as determined by Tukey-post hoc analysis.

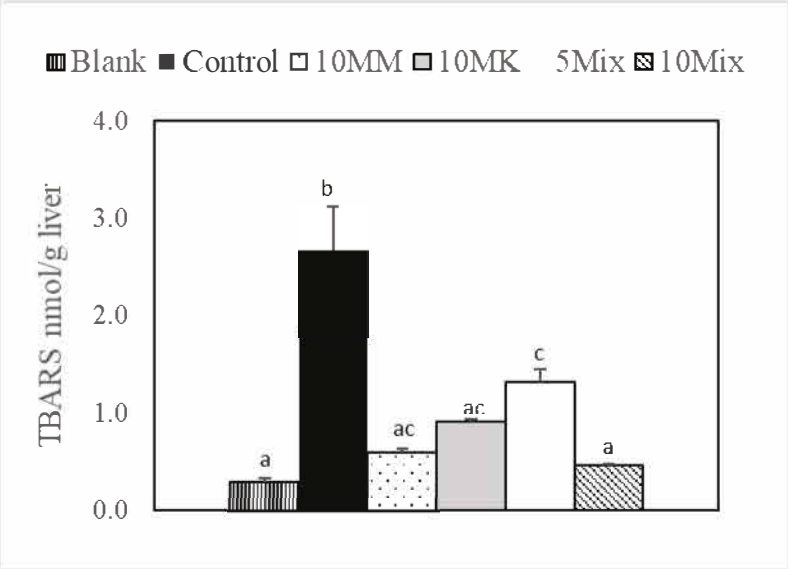

Figure 3.6: Effect of different dietary treatments on liver TBARS levels of mice after 10 weeks of DMH treatments. Values are expressed as mean ± SEM (n = 10). Bars with different superscript letters are significantly different ($p < 0.05$) according to one-way ANOVA with Tukey's post hoc analysis.

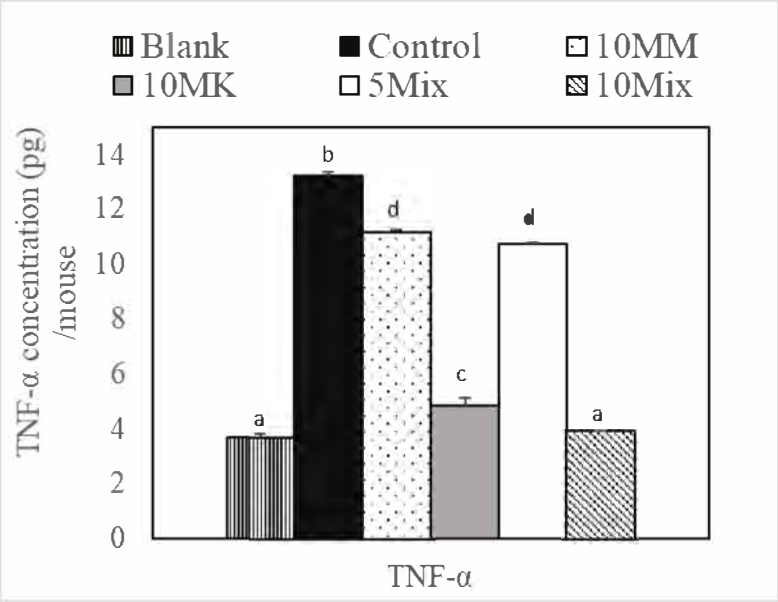

Figure 3.7: Effect of different dietary treatments on colon TNF-α levels of mice after 10 weeks of DMH treatments. Values are expressed as mean ± SEM (n = 4). Bars with different superscript letters are significantly different ($p < 0.05$) according to one-way ANOVA with Tukey's post hoc analysis.

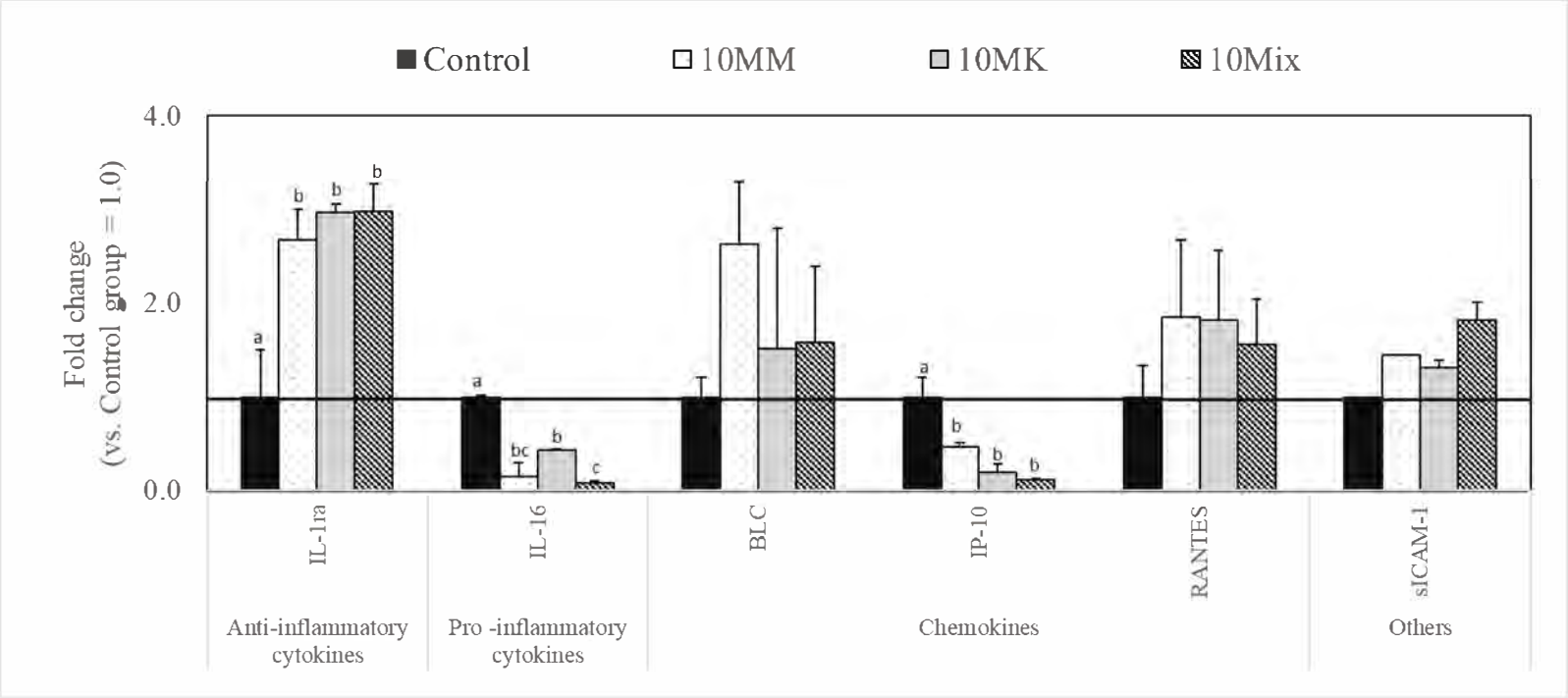

Figure 3.8: Effect of dietary treatments on inflammation-related cytokines levels in mice colon after 10 weeks of DMH treatments. Values are expressed as mean ± SEM (n = 4). Blank = 1.0. Bars with different superscript letters are significantly different ($p < 0.05$) according to one-way ANOVA with Tukey's post hoc analysis

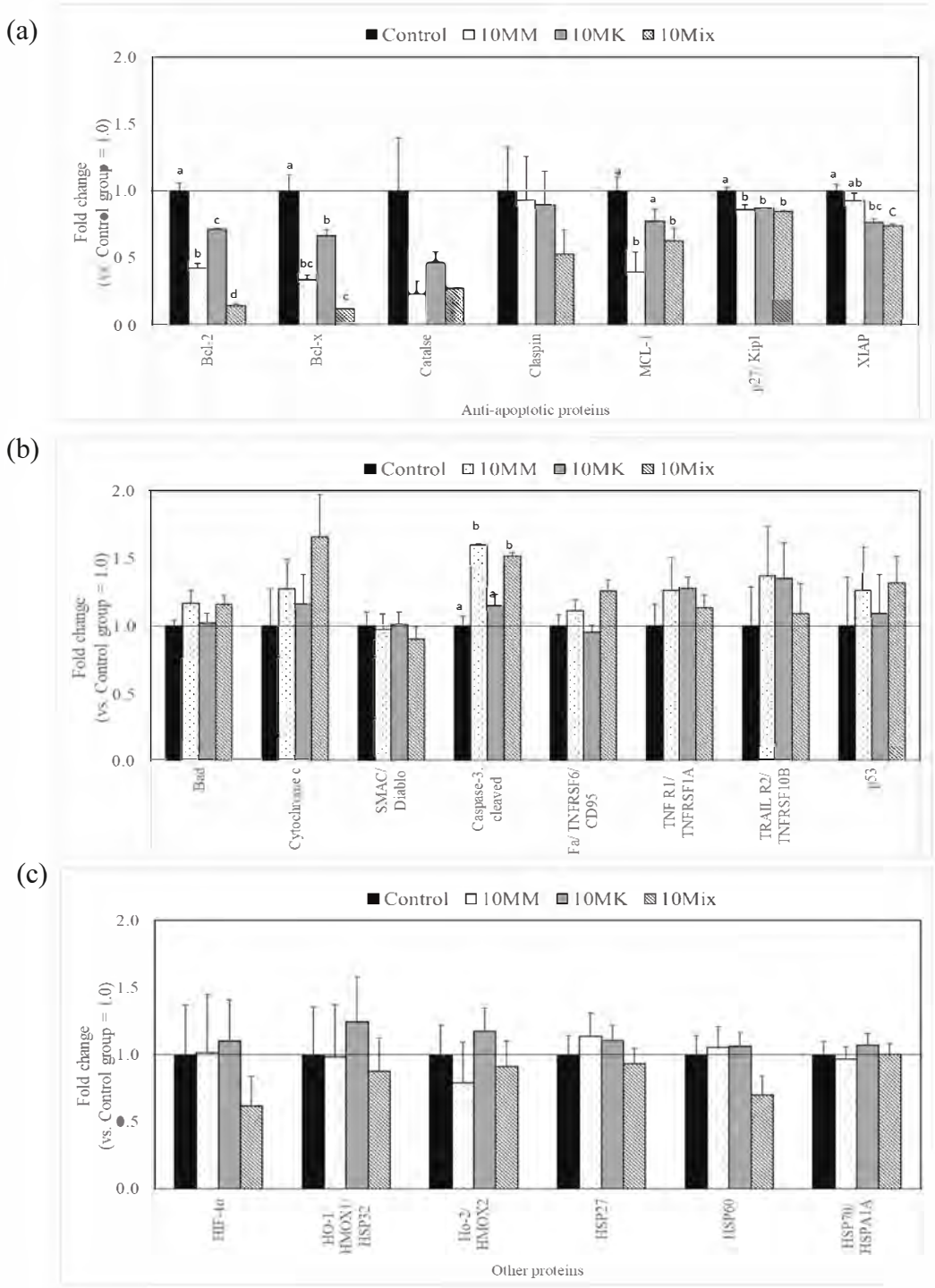

Figure 3.9: Effect of different dietary treatments on apoptosis related protein levels (a) anti apoptotic proteins, (b) pro-apoptotic proteins (c) other proteins in mice colon after 10 weeks of DMH treatments. Values are expressed as mean ± SEM (n = 4). Control = 1.0. Bars in the same protein with different superscript letters are significantly different ($p < 0.05$) according to one-way ANOVA with Tukey's post hoc analysis

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4. Extraction, structural elucidation and evaluation of potential prebiotic activity of major polysaccharides from maitake

4.1 Introduction

Mushrooms have been used for many decades not only in gourmet cuisines but also in traditional medicine (Lindequist et al., 2005, Wasser et al., 2011). There is a vast body of evidence indicating that mushrooms and derived substances demonstrate immunomodulation, anti-viral, anti-diabetic, anti-tumor, antioxidant, anti-bacterial and hypocholesterolemic effects and are used as dietary supplements or drugs (Kalač et al., 2013, Nowacka et al., 2014, Roupas et al., 2012). These activities result from the presence of biologically active compounds, including polysaccharides, which have recently generated growing interest. The dry matter content of the mushroom fruit bodies varies from 5-15% while 19-35% are protein and the fat content in most of the edible mushrooms is negligible (Manzi et al., 1999, Mattila et al., 2000). The content of carbohydrates ranges from 50-90% and the most abundant polysaccharides are chitin, hemicellulose, xylan, mannan, galactan, and α, β-glucan. These polysaccharides mostly present as linear and branched glucans with different glycosidic linkages but some are as heteroglycans and depending on the source and the structure, molecular mass (Mw) of theses polysaccharides varies from 5-2000 kDa (Bohn & BeMiller, 1995, and Wasser,2002).

Mushroom polysaccharides extending from homopolymers to high complex heteropolymers (Ooi & Lui, 1999) are known to have many biological activities including anti-tumor, anti-inflammatory, hyperglycemic, and hypocholesterolemic (Bobek and Galbavy, 2001, Smiderle et al., 2008, Yuan et al., 2015). However, theses biological actives are correlated with water solubility, molecular size, branching rate and structure (Wasser, 2002). Several studies have found that β-1,3-glucan is more effective in immunomodulation and suppression of tumors than chitin-glucan complexes, the main source of biologically active polysaccharides (Mizuno et al., 1995, Maeda et al., 1988).

Grifola frondosa (maitake) has been subjected to a vast range of pharmacological studies and, most of the studies have focused on bioactive polysaccharides (He et al., 2017). The average carbohydrate content in maitake fruiting bodies and mycelia are around 33.53% and 47.84% respectively (Huang et al., 2011). Maitake contains a markedly high level of water soluble polysaccharides (3.8%) on a dry weight basis, and among different water soluble polysaccharides, β-1,3-1-6 D-glucans account for around 13.2% (Su et al., 2016). In addition, maitake is a rich source of water soluble α-glucan and highly available in fruiting body (Ohno et al., 1985). The molecular mass of water soluble polysaccharides shows diverse distribution and varied from 722.7 kDa to19.6 kDa (Su et al., 2016). Bioactive fractions obtained from maitake with different polysaccharides structures and molecular mass such as MD-fraction (Kodama et al., 2002), MZ-fraction (Masuda et al., 2006), SX fraction (Konno et al.,2013), and Grifolans (Suzuki et al., 1987) have been identified to possess therapeutic abilities against diseases and metabolic disorders (He et al., 2017). Particularly, MD-fraction (a glyco-protein) has glucan/ protein ratio ranging from 80:20 to 99:1 (Nanba and Kubo,

1987) and the D-fraction has been developed by further purification of MD fraction. The D fraction, which was approved by the U.S Food and Drug Administration for phase II studies on patients with advanced breast and prostate cancers, is currently using as complementary and alternative medicines against various tumors and cancer (Glauco et al., 2004). The Sx-fraction is also a glycoprotein with protein: saccharide ratio ranging from 75:25 to 90:10 and known to have biological activities. Various purification methods such as precipitation, ion-exchange chromatography, gel filtration, and affinity chromatography are used in purification of maitake polysaccharides (Zhuang et al., 1994). Pharmacological results have revealed that some crude maitake polysaccharides and fractions possess various promising bioactivities including anti-tumor, immunomodulation, anti-oxidation, hepatoprotection, and anti-hyperglycemia. Polysaccharide fraction of maitake, named GF-1 showed anti-tumor effect via host-mediated mechanism in particular, modulating the antibody response and carbon clearance activity (Suzui et al., 1985). Grifolan NMF-5N, another β-1,3 glucan from maitake showed anti-tumor effect via activation of macrophages and T-cells (Suzuki et al., 1987, Takeyama et al., 1987). In addition, maitake Z fraction was also shown to promote the infiltration of CD4+ and CD8+T cells in tumor sites (Masuda et al., 2009). A study performed with SGC-7901 cells have suggested that a polysaccharide-peptide and a glucan sulfate from maitake can significantly induce cell apoptosis and the apoptotic mechanism was shown to be associated with a drop in mitochondrial transmembrane potential, Bax upregulation, Bcl-2 downregulation, and caspase-3 activation (Cui et al., 2007). Similarly, another experimental finding showed cancer metastasis can be prevented by 91.3% by the oral administration of maitake D-fraction (Nanba, 1995). D-Fraction was shown to exert pro-apoptotic effects and reduce breast cancer cell viability. Further in depth study showed that certain genes, such as IGFBP-

7, ITGA2, ICAM3, SOD2, CAV-1, Cul-3, NRF2, Cycline E, ST7, and SPARC, are responsible for the suppression of the tumoral phenotype mechanism induced by D-Fraction in breast cancer cells (Alonso et al., 2013). In addition, maitake is known to suppress colon inflammation, via modulating pro, anti-inflammatory cytokines particularly TNF-α (He et al., 2017).

Fungal polysaccharides as well as the partially hydrolyzed products have potential prebiotic functions. The major components rendering prebiotic action in mushroom are non-digestible polysaccharides such as glucan, chitin, and heteropolysaccharides. Similar to other prebiotics, mushroom also exert several beneficial effects for the host through enhancing the growth and activity of probiotics. Particularly, several mushroom polysaccharides such as pleuran, lentinan, schizophyllan, α and β-glucans, mannans, xylans, galactans, chitin, inulin and hemi celluloses can be considered as well studied promising prebiotics agents (Singdevsachan et al., 2016). Synytsya et al., showed that water extract mushroom glucans from *Pleurotus ostreatus* and *Pleurotus eryngii* were able to stimulate the growth of probiotics: 3 strains of *Bifidobacterium* spp, 4 strains of *Lactobacillus* spp and *Enterococcus faecium* (Synytsya et al., 2009). In another study the crude polysaccharide extract from *Ganoderma lucidum* fermented in batch culture fermentation of human feces culture unit showed a growth promotion of *Bifidobacterium* spp, *Lactobacillus* spp and growth inhibition of *Salmonella* spp (Yamin et al., 2012). An experimental findings revealed that β-1,3 glucan from bamboo shoot extract stimulate the growth of selected *Bifidobacterium* and *Lactobacillus* spp (Azmi et al., 2012).

Grifola frondosa, contains natural oligofructoses, fructo-oligosacharides (FOS), lactulose, galactomannan, and indigestible polydextrose, mannan, xylose, indigestible dextrin and β-glucan as polysaccharides (Jayachandran et al., 2017). The β-glucan fraction of maitake, known as grifolan has triple helix structure and has the ability to stimulate the growth of cultured *Bifidobacterium* and *Lactobacillus* while suppressing the growth of *Salmonella*. This suggest that grifolan may have the prebiotic effect on intestinal microbiota.

Therefore, the present study was conducted to evaluate the effect of maitake fruiting bodies, maitake extracts, and purified fractions on the growth of colon probiotic microorganisms using *in vitro* bacterial culture models and to isolate and elucidate the responsible maitake polysaccharides.

4.2 Experimental section

4.2.1 Extraction and Isolation of polysaccharides

Maitake fruiting body was extracted as described by Synytsya et al., 2009 with some modifications (**Fig. 4.1**). After the successive extraction with hot water, 99.5 % ethanol, 1 M NaOH at RT, 1 M NaOH with 20 mM $NaBH_4$ at 70 °C, four soluble crude extracts namely: hot water soluble extract (ER1), ethanol soluble extract (ER2), cold alkali soluble extract (ER3), and hot alkali soluble extract (ER4) were obtained after each centrifugation at 7500 rpm for 10 min at 4 °C or RT, leaving behind an alkali insoluble (ER5) residue. Alkali soluble extracts and the insoluble residue were neutralized with 4 M HCl solution. Extracts at pH 7, were incubated with α-amylase (Sigma) in a boiling water bath with continuous agitation for 30 min to remove α- glucan. The crude extracts

(sample/Sevag 5:1) were then treated with Sevag reagent (chloroform: *n*-butanol 4:1, v/v) for three times and centrifuged at 7500 rpm for 15 min to remove all free proteins. Deprotenized supernatants were dialyzed against deionized water (cut off 3500 Da) overnight at 4 °C and lyophilized as described previously (Wang et al., 2014) to obtained each extracts of ER1~ER5.

4.2.2 Evaluation of Chemical composition

Total carbohydrate content in the extracts was determined using phenol-sulfuric acid method (DuBois et al., 1956). Briefly, 250 µL of samples were mixed with phenol sulfuric solution (5% v/v phenol and 98% v/v sulfuric acid) and incubated at 30 °C for 30 min to complete acid degradation. The absorbance was measured at 490 nm and carbohydrate content was determined using D-glucose (0-100 ppm) standard curve.

Protein content of the extracts was evaluated using Bio-Rad protein assay in accordance with manufacturer's instructions using bovine serum albumin (0-100 ppm) as the standard.

Uronic acid content was estimated colorimetrically with *m*-hydroxybiphenyl-sulfuric acid method at 540 nm as described by van den Hoogen et al., (1998) with some modifications. 40 µL of sample was mixed with 200 µL of concentrated sulfuric acid (98% v/v) containing 120 mM sodium tetraborate and incubated at 80 °C for 1 h. After cooling to the ambient temperature background absorbance of the samples was measured. Then 40 µL of *m*-hydroxybiphenyl-sulfuric acid was added, mixed and incubated at room temperature for 15 min prior to the measurement of absorbance. The

background absorbance was subtracted and uronic acid content was calculated using D-galactouronic (0-100 ppm) as the standard.

4.2.3 Isolation and Purification of polysaccharide fractions

The crude polysaccharide extracts (ER1~ER5) were dissolved in 20 mM phosphate (pH 7.2) buffer to a concentration of 10 mg/mL and centrifuged at 3000 rpm for 30 min. The supernatant was filtered through 0.22 μm syringe filter and injected into a Toyopearl DEAE-650M anion exchange column (5.3 cm × 37 cm) and eluted with two column volumes of 20 mM phosphate buffer followed by two column volumes of gradient NaCl aqueous solution (0- 2 M) at a flow rate of 0.4 mL/min. Collected fractions were dialyzed against distilled water (cut-off 3500 Da) for 24 h and lyophilized to obtain purified fractions of ER1, ER2, ER3, ER4, and ER5. As the next step, hot water soluble crude polysaccharide extract was purified and 5 mL of each fractions were collected with a fraction collector and combined based on the total carbohydrate contents calculated by using phenol-sulfuric acid method to obtain three sub-fractions of ER1-F1, ER1-F2, ER1-F3 (**Fig. 4.2a**). Combined fractions were dialyzed and lyophilized.

4.2.4 Determination of molecular mass

Molecular weight (Mw) of the maitake polysaccharide in purified fractions (ER1, ER2, ER3, ER4, and ER5) were determined by high performance liquid chromatography (HPLC) equipped with TSKG5000PW$_{XL}$ (7.8 × 300 mm) gel column (Tosoh Corporation, Japan) at 30 °C. The purified polysaccharide fractions were dissolved in 0.1 M NaCl to a concentration of 10 mg/mL and passed through a 0.22 μm syringe filter prior to analysis. The pre-degassed 0.1 M NaCl aqueous solution was used as mobile

phase at a flow rate of 1 mL/min and detected by refractive index detector RI-8020 (Tosoh Corporation, Japan). The molecular weights were calculated by a calibration curve developed with a series of standard pullan of known molecular mass (Fukuda et al., 2010).

4.2.5 Analysis of monosaccharide composition

Monosaccharide composition of purified fractions was analyzed by thin layer chromatography (TLC) and HPLC after acid hydrolysis (Fukuda et al., 2010). Briefly, 5 mg of samples (ER1 and ER5) was hydrolyzed with formic acid (HCOOH) followed by trifluoroscetic acid (TFA) at 100 °C for 5 h. Excess TFA was removed by rotary evaporation and the hydrolysate was then washed thoroughly with distilled water, lyophilized and re-dissolved in 100 µL deionized water and 5 µL of aliquots were used for TLC. Samples were developed twice in silica gel TLC plates (20 × 20 cm) using developing solvent of *n*-butanol: ethanol: water (2:1:1 v/v). After overnight drying, 5% (v/v) sulfuric acid in ethanol was sprayed over the TLC plate and heated to visualize the carbohydrates. D-glucose, D-galactose, D-lactose and corn were used as standards.

4.2.6 NMR spectroscopy

Five milligrams of purified fractions were exchanged by deuterium in 99.99% D_2O (Merck Corporation, USA), lyophilized and re dissolved in 0.6 mL of 100.00% D_2O (Sigma, Japan). 1D ^1H NMR spectra were recorded in D_2O at 500 or 600 MHz with a JEOL ECP-500 Fourier transform-NMR spectrometer (Jeol, Japan) or a Varian INOVA 600 spectrometer (Varian Inc, USA) with the probe temperature at 70 °C. Suppression of the HOD signal was achieved by applying a WEFT pulse sequence for 1D experiments and by a pre-saturation of 1 s during the relaxation delay for 2D

experiments. The carrier frequency was set at the downfield edge of the spectrum in order to minimize TOCSY transfer during spin-locking. Natural abundance 2D ^{13}C–^{1}H HSQC experiments (^{1}H frequency 500.0821 MHz, ^{13}C frequency 125.7552 MHz) were recorded without decoupling during acquisition of the ^{1}H FID. Chemical shifts were expressed in ppm by reference to internal acetone (δ 2.225 for ^{1}H and δ 31.08 for ^{13}C).

4.2.7 Methylation analysis

The purified polysaccharide ER1 and ER5 fractions were methylated as described by Hakomori (Hakomori et al., 1964). Methylated samples were further purified using Wakogel S-1 silica gel chromatography and the alditol acetate derivative of partially methylated sugar was prepared from the pre-methylated polysaccharide using the method described by Hakomori. The obtained partially methylated alditol acetates were analyzed by gas chromatography-mass spectrometer equipped with ULBON-HR1 (non-polar), 0.25mm× 50m column and the temperature program of 330 °C Partially methylated alditol acetates in the GC-MS chromatogram were identified by their retention time in the total ion chromatogram and by comparison with the mass spectrum patterns from the literature database (Chen et al., 2014) and with the β-1-3-glucan standard (Sigma, Japan)

4.2.8 Evaluation of prebiotic activity

The extracts and the purified fractions were dialyzed overnight against distilled water at 4 °C to remove excess salt and lyophilized prior to use as substrates. Potential prebiotic activity was analyzed along with glucose and FOS (Wako, Japan) as described previously (Azmi et al., 2012). The MRS medium supplied with 0.05% L-cysteine was prepared without glucose and mixed with either 2% of purified fractions (ER1, ER2,

ER3, ER4, and ER5), sub-fractions (ER1-F1, ER1-F2, and ER1-F3) and FOS prior to sterilization. Modified MRS broth supplemented with or without glucose were used as positive and negative controls, respectively. Prepared mediums were then inoculated with selected colonic probiotics (**Table 2 Appendix**) at the initial biomass of 0.3 (OD_{600} of 0.3) under aseptic condition and incubated anaerobically using Aneropack Kenki system (Mitsubishi Gas Chemical Co. Inc., Japan) at 37 °C. Sampling was done at 0, 6, 12, 24, 36, 48, 60 and 72 h to evaluate pH, optical density ($OD_{600\ nm}$) and viable cell count. The pH of fermented broth was determined using a pH meter (AS ONE, Japan). The biomass was evaluated as optical density using UV-Visible spectrophotometer at 600 nm and results were expressed as unit of cell densities. Growth of microorganism (viable cell counts) were calculated using number of visible colonies within the range of 2-200. All the experiments were done in triplicate.

4.2.9 Statistical analysis

Experiments were performed in triplicate from three independent cultures and expressed as the mean ± standard deviation. Cell viability was analyzed by Student's t-test using the general linear model (GLM) procedure of the SAS System (SAS, 2001). P values of <0.05 were considered to be statistically significant.

4.3 Results

4.3.1 Isolation and purification of polysaccharides

Drying and milling 800 g of fresh maitake fruiting bodies resulted in 100 g (12.5 % w/w dry weight) of dried maitake powder (DMP). Five crude fractions (ER1, ER2, ER3, ER4, and ER5) were obtained from the maitake dried powder (MDP) of fresh fruiting bodies. Hot water soluble fraction (ER1) accounted for 15.5% dry weight (DW), and

ethanol soluble fraction (ER2) was negligible accounting only to 0.22%, whereas the two alkali soluble fractions (ER3 and ER4) constituted 41.9% of the DMP (14.7% and 27.2% for ER3 and ER4, respectively). The insoluble fraction: ER5 constituted 38.7% of DMP. All the extracts were predominantly composed of carbohydrates (**Table 4.1**) and the ethanol fraction contained around 98% of carbohydrates. Low levels of proteins were determined in all the fractions and the highest level of protein was observed in hot alkali soluble fraction accounting for around 1% of dry weight while relatively lower levels of proteins (0.32%) were found in ER1. The levels of uronic acids vary among the MDP fractions (**Table 4.1).** However, after purification, no uronic acids were detected in the purified fractions (ER1, ER2, ER3, ER4 and ER5) (**Fig. 4.2**).

4.3.2 NMR analysis

The ^{1}H NMR spectra of the purified fractions (ER1, ER2 ER3, ER4 and ER5) were quite similar (**Fig. 4.3**). In the present study, none of the purified fractions showed signals at $\delta = 5.233$ confirming the absence of α-configurations of the glucan residue. However, the presence of signals at $\delta = 4.733$, $\delta = 4.716$, and at $\delta = 4.671$ confirmed the β-anomeric configuration of the D-glucose residue. In addition, all the purified fractions showed signals at $\delta = 4.53$ confirming the presence of 1,3-β-linked back bone chain. Further, the signals at $\delta = 4.22$ and at $\delta = 3.331$ indicated the presence of 1,3-β-linked glucose polymer with 1,6- β linked side chains in ER1 fraction.

4.3.3 Molecular mass analysis of maitake polysaccharide

High performance liquid chromatogram was employed to determine the molecular mass of crude fractions and the purified fractions. Water soluble purified fraction has the

molecular weight of approximately 2.1×10^4 Da while all the other fractions (ER2, ER3, ER4 and ER5) showed molecular weight of approximately 1.3×10^4 Da (**Table 4.2**).

4.3.4 Monosaccharide composition of maitake polysaccharide

Based on the preliminary studies, only ER1F and ER5F were used to evaluate the monosaccharide composition. The main polysaccharide of the fractions was glucose. No other tested monosaccharide was detected on TLC (**Fig. 4.4**)

4.3.5 Linkage analysis of maitake polysaccharide

Only ER1 and ER5 were used for linkage analysis. The partially methylated alditol acetates in the GC-MS chromatogram of ER1 and ER5 were identified by their retention time in the total ion chromatogram and by comparison with the mass spectrum patterns from the literature database and β-1-3-glucan standard. Methylation analysis of ER1 revealed the presence of non-reducing terminal glucose, 1,3-D-Glcp and 1,6-D-Glcp while ER5 consisted only of 1,3-D-Glcp (**Table 4.3**).

4.3.6 Effect of maitake and maitake polysaccharide on the growth of probiotic bacteria

All the strain tested which include *Lactobacillus. rhamnosus* (ATCC 53103), *Lactobacillus acidophilus* (JCM 1132), *Lactobacillus delbrueckii* subsp bulgaricus (ATCC 11842), *Lactobacillus casei* (ATCC 393), *Lactobacillus fermentum* (ATCC 14931), *Bifidobacterium longum* (BB 536), *Bifidobacterium adolescentis* (ATCC 1275), and *Bifidobacterium animalis* subsp *animalis* (ATCC 1253) showed a positive growth with the supplementation of crude polysaccharides extracts from maitake (**Fig. 4.5**).

Among the maitake crude extracts, ER1 showed remarkably different growth characteristics, which were similar to that of the positive control (glucose). The color of fermented broth was noticed to change from colorless to cloudy and deep yellow in all tubes, indicating polysaccharide fermentation by the bacterium. All the purified crude extracts were found to have significant effect on the growth of probiotics, comparable to FOS. Among the further purified ER1 extracts (ER1-F1, ER1-F2, and ER1-F3), ER1-F2 and ER1-F3 showed remarkably different effects on the growth of probiotics compared to ER1-F1 (**Fig. 4.6**). The ER1-F2 and ER1-F3 fractions with molecular weight >10 kDa showed significant effects on the growth of *L. rhamnosus* (ATCC 53103), *B. longum* (BB 536) and *B. adolescentis* (ATCC 1275) (**Fig. 4.7**).

4.4 Discussion

Mushroom has an emerging identity as a potential prebiotic. The major components rendering prebiotic action in mushroom are non-digestible polysaccharides such as glucan, chitin, and heteropolysacchrides. Particularly, several mushroom polysaccharides such as pleuran, lentinan, schizophyllan, α and β-glucans, mannans, xylans, galactans, chitin, inulin and hemi celluloses are considered as promising prebiotics agents (Singdevsachan et al., 2016). However, purified polysaccharide with promising prebiotic activities are in high demand as an alternative to probiotics or to use with probiotics to overcome the possible survival difficulties of probiotics. In addition, purified polysaccharides have recently gained a higher demand in pharmacological sector since prebiotics-based treatments can be one of the major therapeutic approaches in the near future, particularly for chronic gastrointestinal disorders (Davis and Milner, 2009, DeFilippo et al., 2010, Derikx et al., 2016). In addition, isolation and purification of polysaccharides from its original source helps to

identify the responsible compound and its characteristic. The suitable procedures for isolation of polysaccharides from the maitake fruiting bodies were summarized in **Fig. 4.1**. The homogenized samples were washed with 90% ethanol to remove small molecules including mono and oligo saccharides and fat (Synytsya et al., 2009, Azmi et al., 2012). The presence of fat in the raw material can limit water penetration and interfere with the extraction process, thus prior fat removal is necessary to increase the extraction efficiency (Azmi et al., 2012). Lipid substances can be defatted using polar solvents such as choloform-methanol (95:5 v/v), ethanol (80-90% v/v) or dioxane and hexane (Izydorczyk, 2005). However, mushroom contains a very low quantity of fat and the fat content in maitake is around 0.3-0.5 mg/ 100g (Mori et al., 2008). Polysaccharide extraction from plant materials are usually carried out using hot water. However, the yield depends on extraction temperature, extraction time, water to raw material ratio and number of extraction (Cai et al., 2008). According to several studies, extraction of polysaccharide at high temperatures (over 80 °C) may lead to hydrolysis of polysaccharides, thus reduce the yield and the optimum temperature for polysaccharide extraction ranged from 70-80 °C (Azmi et al., 2012). Lengthening the time of extraction showed increased polysaccharide yield, however, excessive extraction time (more than 4 h) was known to cause structural changes in polysaccharides, thus affecting the yield. In addition, water to raw material ratio also influences the extraction yield. In particular, water to raw materials within the 3-4 range was shown to have higher extraction yield and studies showed that usage of excessive water did not increase the yield. The number of extraction that have optimum yield is two to three and the increased number of extraction did not increase the yield significantly (Azmi et al., 2012). Therefore, in our study a maximum of 80 °C, for 4 h was used for the subsequent extraction. In addition, the number of extraction time was

limited to three based on previous studies (Chen et al., 2014, Synytsya et al., 2009). The supernatants after extraction with hot water were isolated by centrifugation. In the study conducted by Synytsya et al., it was stated that water insoluble extract contains a high amount of glucan even after extraction at 100 °C, for 6 h (Synytsya et al., 2009). Thus, they included alkali extraction step to extract further polysaccharides from water insoluble fraction (Synytsya et al., 2009). In another study two alkali extraction steps: cold alkali extraction at RT and hot alkali extraction at 80 °C, were included to extract mushroom polysaccharides from water insoluble extract (Chen et al., 2014). Therefore, the present study employed an ethanol extraction step for further removal of fat and free protein followed by two alkali extraction steps to extract polysaccharide from water insoluble crude extract. All the extracts were subjected to purification process in order to remove free protein and ballast polysaccharides (starch). Starch was fragmentized by α-amylase to release maltose and the monosaccharides were removed by dialysis step. Prior to incubation with α-amylase alkali extracts were neutralized with 4 M HCl solution. The proteins are easily extracted with polysaccharides during water extraction and can be free or combined with polysaccharides. Multiple application of Sevag reagent removed free protein and based on the Braford test conducted, all the crude extracts contained less than 1% protein (**Table 4.1**). This was used for deproteinization of lentinan (β-1,3-d-glucan) (Zhang et al., 2011) and water-soluble polysaccharides (Yu et al., 2010) isolated from *Lentinus edodes* mushrooms. Maitake fruiting body conteained around 87.5% of water and the isolated crude extracts contained around 96% dry matters. Hot water soluble crude extract (ER1) contained around 16% of dry matter of the maitake dried powder (MDP), whereas two alkali soluble crude extracts (ER3 and ER4) constituted 42% of the MDP (14.7% and 27.2% for ER3 and ER4, respectively). The dry matter distribution among extracts of *Pleurotus* tuber-regim

however was different from the present study (Chen et al., 2014). In that study the cell wall content of *Pleurotus* tuber-regim fruiting body was used for the subsequent extraction while in the present study fruiting body was used. The free protein content in all the crude extracts were below 1% with the highest amount in ER3 (0.99%) and lowest in ER1 (0.32%). One study conducted using fruiting bodies of *Pleurotus ostreatus* and *Pleurotus eryngii* stated that the free protein from the extracts were totally removed after multiple application of Sevag reagent (chloroform: butanol 4:1 v/v) (Synytsya et al., 2009), however, in our study we could not completely remove the free protein even after several applications of Sevag reagent. Another study conducted using *Gigantochloa levis* shoots also confirmed the presence of protein (<1%) in the extracts (Azmi et al., 2012). The levels of uronic acids were vary among the MDP crude extracts. The ER1 crude extracts showed the highest amount of uronic acid (4.85%) which was similar to the study conducted by Lee et al., and in that study they found around 4% of uronic acid in the hot water extracts of *Hericium erinaceus* (Lee et al., 2009). In another experimental research conducted using *Pleurotus eryngii* the presence of 1.25% of uronic acid in hot alkali extract which was comparable with the current results was found (He et al., 2016). Crude extracts were purified using anion exchange chromatography on a DEAE-cellulose column and eluted with 20 mM phosphate buffer followed by two column volumes of gradient NaCl aqueous solution following a previously described protocol (Azmi et al., 2012). Each purified fractions were checked for uronic acids and no absorption was detected at 540 nm confirming that the purified fractions were free from uronic acids. Similarly, in a previous study *Grifola frondosa* water extracts showed absence of uronic acid after purification with DEAE cellulose column using water as elution medium (Wang et al., 2014). Only ER1 crude extracts were purified using anion exchange chromatography on a DEAE-cellulose column and

collected fractions were divided into three sub-fractions namely: ER1-F1, ER1-F2, and ER1-F3 (**Fig. 4.2a**) based on the carbohydrate contents calculated by using phenol-sulfuric acid method.

The ^{1}H NMR spectra of the purified fractions (ER1, ER2, ER3, ER4 and ER5) were quite similar (**Fig. 4.3**). Since the carbohydrate signal appeared at δ 4.73, which is in the close neighborhood of the HOD signal at 300 K, all NMR spectra were recorded at 334 K (HOD signal at 4.40 ppm). The anomeric regions ^{1}H signals for standard Laminablose denoted at δ = 5.233, δ = 4.733, δ = 4.716, and at δ = 4.671, respectively, according to decreasing chemical shift values. The δ values suggest the presence of α-configurations at δ = 5.233 and β configuration at δ = 4.733, δ = 4.716, and at δ = 4.671. Previous studies also confirmed the presence of α-configuration of the glycan residues at δ = 5.22 (Wang et al., 2014, Molinaro et al., 2002, Perepelov et al., 2007, Ye et al., 2008). However, in the present study none of the purified fractions showed signals at δ = 5.233 confirming the absence of α-configurations of the glucan residue. The β configuration of the D-Glc residues was indicated by ^{1}H resonance at 4.57- 4.26: the reducing terminal ^{1}H at δ = 4.42, non-reducing terminal ^{1}H at δ = 4.46, ^{1}H of the second 1,3-β-linked anhydro **d**-glucose units (AGUs) next to reducing terminus at δ = 4.53, and ^{1}H of the branched single or terminal AGU in the side chain at δ = 4.26 (Kim et al., 2000, Tada et al., 2007). In the present study all the purified fractions showed signals at δ = 4.53 confirming the presence of 1,3-β-linked back bone chain which was confirmed by previous studies (Lowman et al., 2011). In addition, the signals at δ = 4.22 and at δ = 3.331 indicated the presence of 1,3-β-linked glucose polymer with 1,6-β linked side chains in maitake fractions. The experimental research designed to evaluate the glucan structure of *Candida glabrata also showed the presence of* 1,3-β-

linked glucose polymer with 1,6-β linked side chains confirms our results (Lowman et al., 2011). However, the present findings also confirmed that even after successive extraction with hot water, 99.5 % ethanol, 1 M NaOH at RT, 1 M NaOH with 20 mM $NaBH_4$ at 70 °C, β-1,3 D glucose still remained in the insoluble fraction.

In order to determine the molecular mass of each purified fraction, calibration curve developed with a series of standard pullan of known molecular mass was employed as described previously (Azmi et al., 2012, Fukuda et al., 2010). The polysaccharides showed only one single and symmetrical narrow peak with elution time in the HPLC, indicating the presence of homogeneous polysaccharides. Meng et al., also found the presence of single and symmetrical peak after analyzing the purified polysaccharide from *Grifola frondosa* water extract (Meng et al., 2017). In that study, dextrans were used as calibration standard and the average molecular weight of purified polysaccharide from *Grifola frondosa* was estimated as 155 kDa (Meng et al., 2017). In the present study estimated molecular weight of ER1 was around 22 kDa while all the other extracts showed molecular weight of approximately 13.5 kDa (**Table 4.2**). The molecular weight of polysaccharide usually related to certain biological activities. Particularly, higher activity in biological complement system of polysaccharides was noted with the increase molecular mass (Camel et al., 2001). Studies of the biological activity of 1,3-β-D-glucans become more complicated due to the influence of both size and the shape of the molecules on the activity. Several studies have been conducted to compare the activity of β-D-glucans with same molecular mass but different structure (Sletmoen and Stokke, 2008). In one study, high molecular weight oat β-glucan was shown to increase the total antioxidant status effectively than low molecular weight β-glucan (Suchecka et al., 2016). Similarly, a study conducted to evaluate the impact of

low and high molecular weight of oat β-glucan on oxidative stress in mice injected with *Escherichia coli* lipopolysaccharides found out high molecular weight β-glucan is effective on suppressing oxidative stress than low molecular weight counterpart (Błaszczyk et al., 2015). Supporting the above findings several other studies found that prebiotic activity, SCFA production, and cholesterol lowering effect of high molecular weight β-glucans are moreeffective than low molecular weight counterpart (Kalra and Jood, 2000, Theuwissen and Mensink, 2008a, Tong et al., 2015, Wood et al., 1994, Zheng et al., 2011).

After complete hydrolysis with formic acid (HCOOH) and trifluoroscetic acid (TFA), monosacchride composition of ER1 and ER5 was evaluated using thin layer chromatography technique and D-glucose, D-galactose, D-lactose and corn were used as standards. Both ER1 and ER5 shared the same spots as glucose (**Fig. 4.4**) but not the others, confirming the results observed in NMR chromatogram. Even though Wang et al., indicated the presence of galactans in water extract of *Grifola frondosa* (Wang et al., 2014), our NMR data did not show any peak relevant to galactan and this was confirmed by the TLC (**Fig. 4.4**). Then, to determine the detailed structured features, ER1 and ER5 were subjected to methylation analysis and the results were compared to the standard figure and literature as described previously (Miao et al., 2011, Qiu et al., 2010, Xu et al., 2010). Seven colonic probiotic microorganisms; *Lactobacillus. rhamnosus* (ATCC 53103), *Lactobacillus acidophilus* (JCM 1132), *Lactobacillus delbrueckii* subsp bulgaricus (ATCC 11842), *Lactobacillus casei* (ATCC 393), *Lactobacillus fermentum* (ATCC 14931), *Bifidobacterium longum* (BB 536), *Bifidobacterium adolescentis* (ATCC 1275), and *Bifidobacterium animalis* subsp animalis (ATCC 1253) were used in this study. All tested purified maitake

polysaccharides fractions facilitate the growth of tested probiotics (**Fig. 4.5**) indicating the polysaccharide fermentation by colonic probiotics. In particular, ER1 purified fraction showed a remarkable effect on growth of colonic probiotic bacteria compared to other purified maitake fractions. The growth rate of both *Lactobacillus* and *Bifidobacterium* species in ER1 fraction were comparable to the bacteria grow in positive control. The complexity of ER1 fraction might be responsible for the growth effect. Synytsya et al, found out that water extraction of *Pleurotus eryngii* support the probiotic growth rate, biomass and SCFA production of colonic *Lactobacillus* species (Synytsya et al., 2009). Further, the study confirmed that, the water extraction of *Pleurotus eryngii* consist of highly branched 1,3-1,6 β-glucan, which might be responsible for prebiotic activity. The NMR and linkage analysis results of the present study confirmed that ER1 fraction consist of branched 1,3-1,6 β-glucan while the other fractions consist of unbranched 1,3 β-glucan. Thus, the branched 1,3-1,6 β-glucan in ER1 fraction might be the responsible compound of higher prebiotic activity. In addition, several other studies confirmed that higher molecular weight β-glucan is effective in SCFA production and colonic fermentation than low molecular weight β-glucan (Kalra and Jood, 2000, Theuwissen and Mensink, 2008a, Tong et al., 2014, Wood et al., 1994, Zheng et al.,2011). The purified ER1 fraction had the highest molecular weight (approximately 22.2 kDa) among the other extracts (molecular weight of other extracts were approximately 13.5 kDa). This may explain the reason for higher prebiotic activity of ER1 fraction. The ER1 fraction was then divided into three sub-fractions (ER1-F1, ER1-F2, and ER1-F3) based on the carbohydrate contents and tested for prebiotic activity. All three sub-fractions stimulated the growth of tested probiotics (7 species). However, ER1-F2 and ER1-F3 showed a remarkable effect on the growth, biomass and cell viability of probiotics compared to ER1-F1 (**Fig 4.5**).

Gullon et al., indicated that the purity of fraction does not play any significant role in fermentation (Gullón et al., 2011). Contradictory to the above findings, a study conducted to evaluate the cytokine stimulating activity of 1,3 β-D-glucans of same extract with different fractions which have different molecular weights showed thatthe bioactivity differ with the molecular weight though they share the similar liner ripple helical structure (Falch et al., 2000). In addition, Sanz et al., also indicated that glycosidic linkages and molecular weight of the maltose-based oligosaccharides can be affected the fermentation of human gut microbiota (Sanz et al., 2006). In the present study each purified fraction showed only one single and symmetrical narrow peak in the HPLC chromatogram, indicating the presence of homogeneous polysaccharides. Thus, this results suggest that different fractions of ER1 may consist of the same structured β-glucan with different molecular weights. To confirm the above findings, the ER1-F2 and ER1-F3 sub-fractions were further divided using a molecular weight cutoff value of 10 kDa. The sub-fractions with molecular weight >10 kDa showed significant effects on the growth of *Lactobacillu. rhamnosus* (ATCC 53103), *Bifidobacterium longum* (BB 536) and *Bifidobacterium adolescentis* (ATCC 1275) confirming that molecular weight influence the bioactivity (**Fig. 4.7**). Since the quantity obtained was very low, and three replicate were carried out per each experiment, only three bacteria were selected for this experiment by considering the viable cell count and biomass concentration.

4.5 Conclusion

These findings indicate that maitake fruiting bodies contain neutral polysaccharides with β 1,3-glucan as the backbone which leads to its higher bioactivity. The hot water extracted polysaccharides consist of branched β-1,3-1,6-glucan with an average

molecular weight of 22 kDa. Synbiotic test showed that ER1 fraction, and its sub fractions with over 10 kDa molecular weight facilitated the growth of colonic probiotic microorganism similar to that of commercially available prebiotics (FOS). Thus, ER1 and its sub fractions may be a very promising candidate for synbiotic construction with selected colonic probiotic strains to improve the intestinal environment.

Table 4.1: Composition of the polysaccharide extracts of maitake fruiting bodies

Sample	% DW	Total carbohydrate (wt %)	Protein (wt %)	Uronic acid (wt %)
ER1	15.51 ± 0.12	91.11 ± 0.72	0.32 ± 0.22	4.25 ± 0.03
ER2	0.22 ±0.16	98.00 ± 0.25	0.75 ± 0.07	1.78 ± 0.12
ER3	14.7 ± 0.52	93.30 ± 0.08	0.66 ± 0.71	2.87 ± 0.38
ER4	27.19 ± 0.74	89.6 ± 0.72	0.99 ± 0.63	0.97 ± 0.05
ER5	38.72 ± 0.08	72.73 ± 0.12	0.74 ± 0.20	2.21 ± 0.43

Values are the mean ± SD (n=3). DW: dried weight. Total carbohydrate, protein and uronic acids are based on the total dry weight. ER1: hot water soluble fraction, ER2: ethanol soluble fraction, ER3: cold alkali soluble fraction, ER4: hot alkali soluble fraction, ER5: insoluble fraction

Table 4.2: Molecular weight of the purified maitake extracts

Sample	Retention time (min)	Molecular weight (kDa)
ER1	9.3677	21.7 ± 0.2
ER2	11.17	13.4 ± 0.1
ER3	11.1233	13.6 ± 0.1
ER4	11.1833	13.4 ± 0.1
ER5	11.1433	13.5 ±0.1

Values are the mean ± SD (n=3).

Table 4.3: Retention times and mass spectra primary fragments of methl alditol acetates

Sample	Position of methylation	Retention time (min)	Primary fragments in the mass spectra
ER1	1,3-Glc	5.67	43, 57, 73, 87, 103, 116, 187
	1,3,6-Glc	6.97	43, 73, 103, 116, 189,234
ER5	1,3-Glc	5.67	43, 57, 73, 87, 103, 116, 187

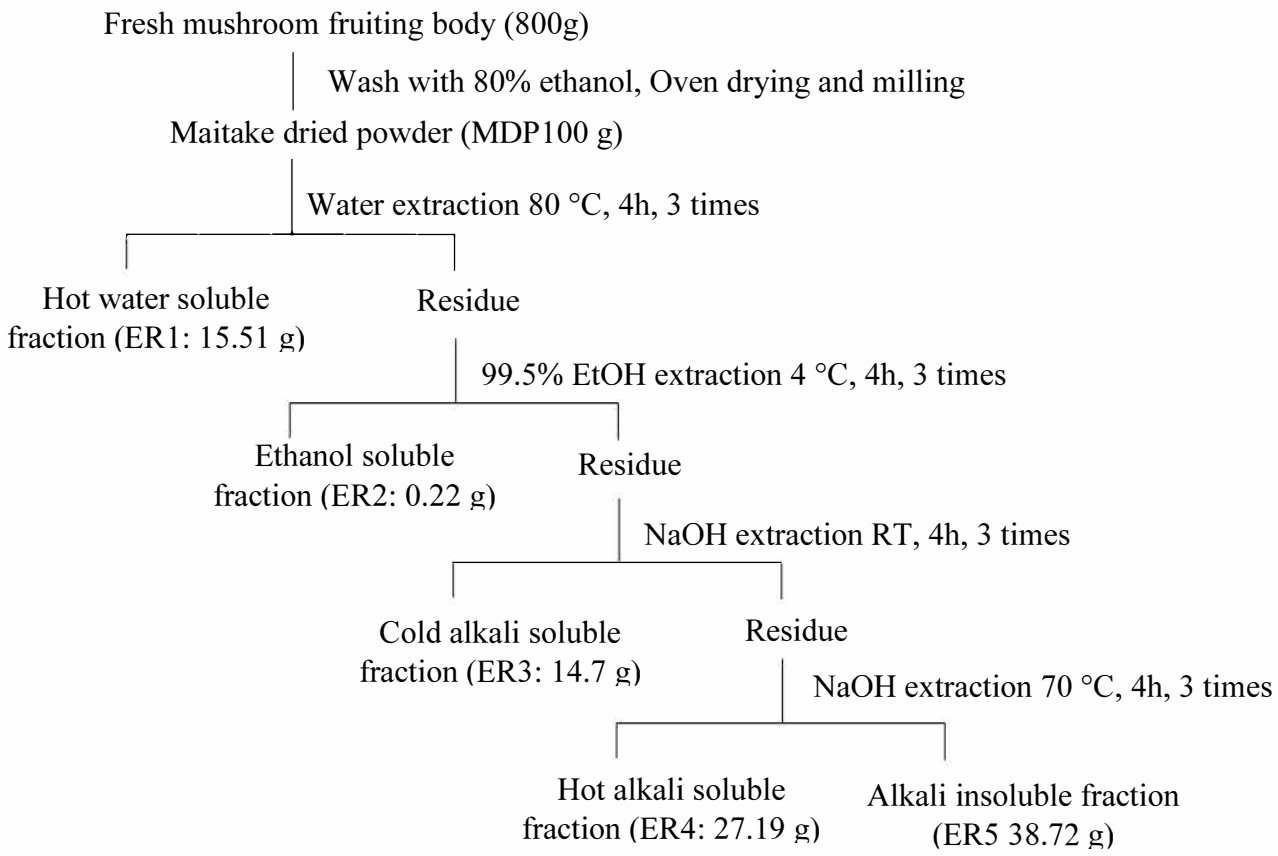
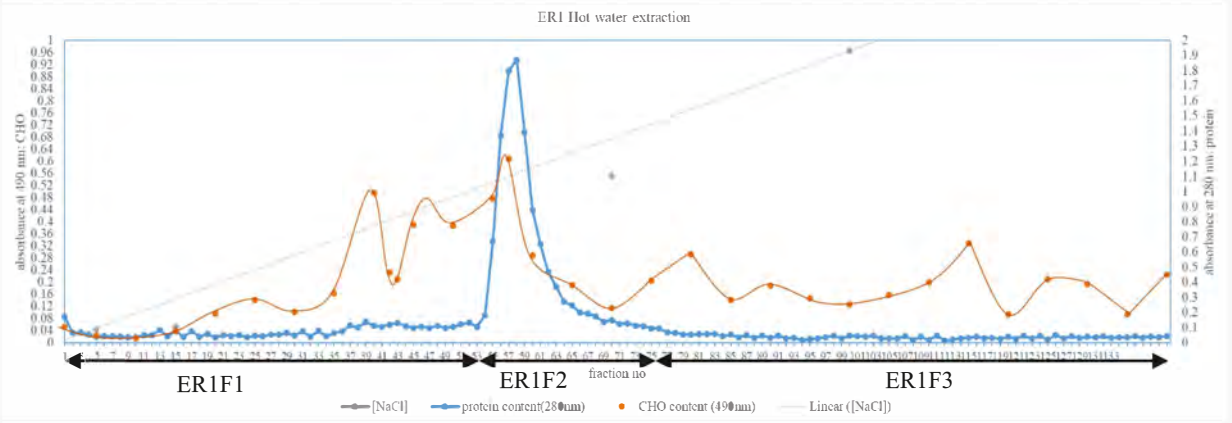
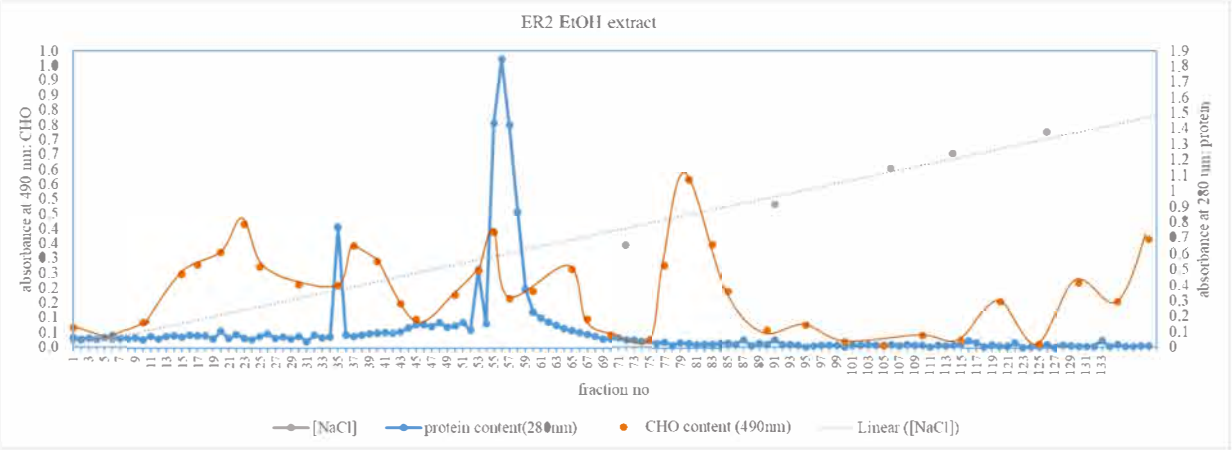


Figure 4.1: Schematic diagram of extraction and isolation of polysaccharides from fresh fruiting bodies of *Grifola frondosa*

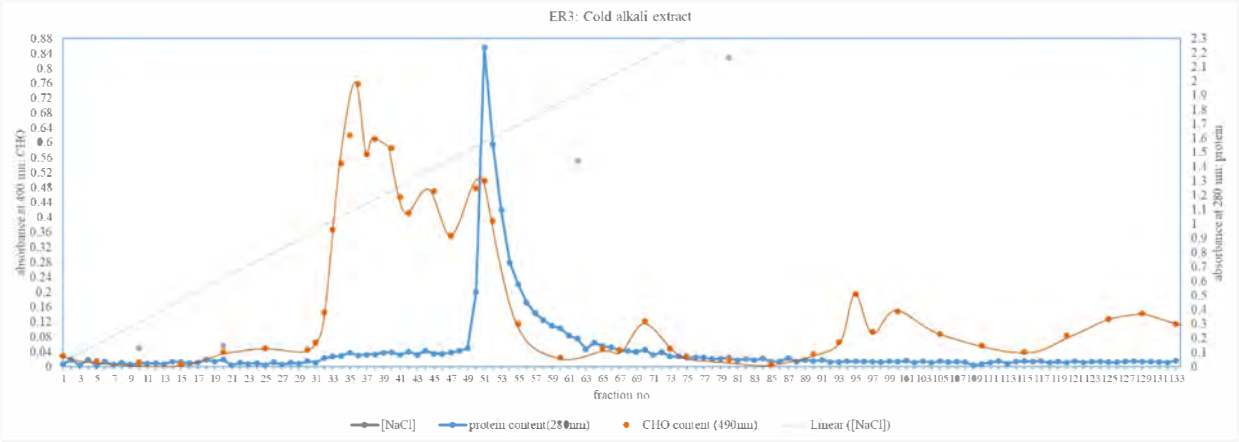
(a)


ER1 Hot water extraction
absorbance at 490 nm: CHO
absorbance at 280 nm: protein
fraction no
ER1F1
ER1F2
ER1F3
[NaCl]
protein content(280nm)
CHO content (490nm)
Linear ([NaCl])

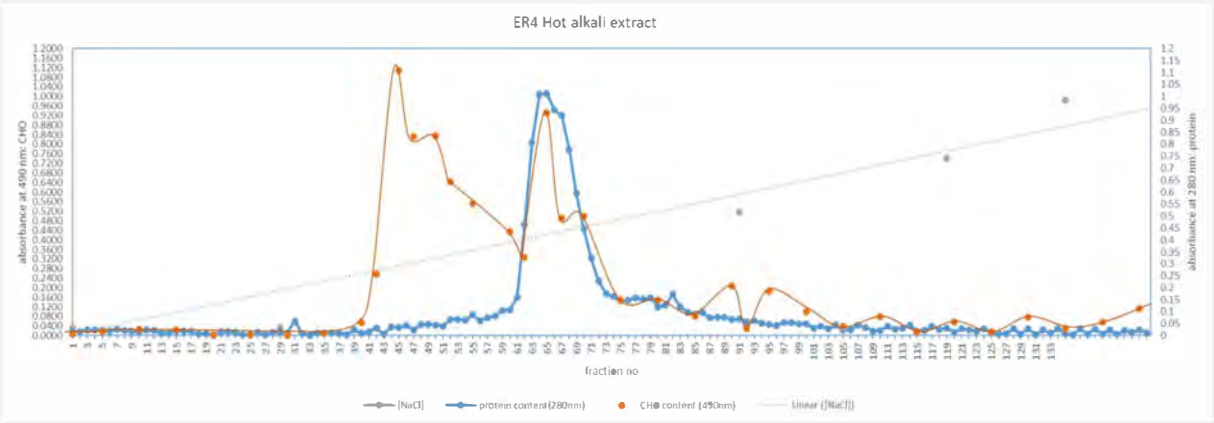
(b)


ER2 EtOH extract
absorbance at 490 nm: CHO
absorbance at 280 nm: protein
fraction no
[NaCl]
protein content(280nm)
CHO content (490nm)
Linear ([NaCl])

(c)


ER3: Cold alkali extract
absorbance at 490 nm: CHO
absorbance at 280 nm: protein
fraction no
[NaCl]
protein content(280nm)
CHO content (490nm)
Linear ([NaCl])

(d)



(e)

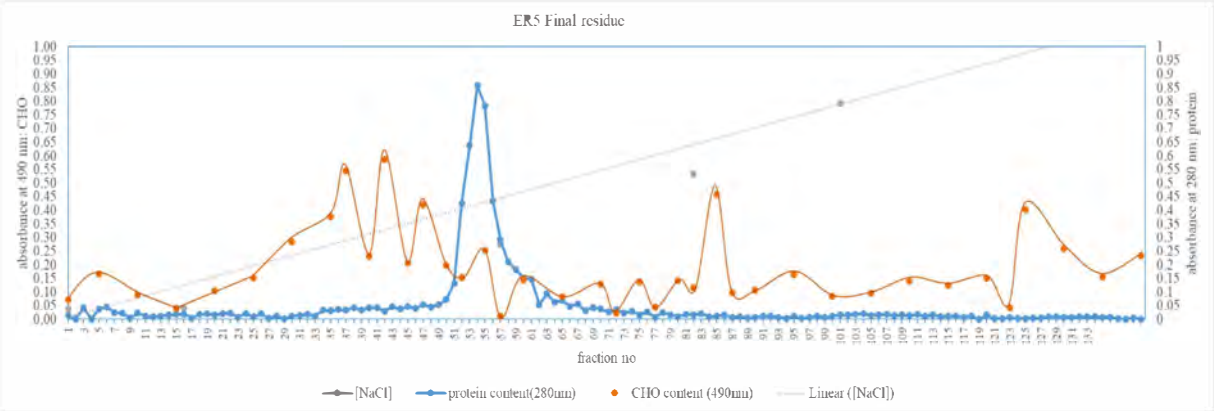


Figure 4.2: Anion exchange chromatograms of (a) ER1: hot water soluble, (b) ER2: ethanol soluble, (c) ER3: cold alkali soluble (d) ER4: hot alkali soluble (e) ER5: insoluble fractions

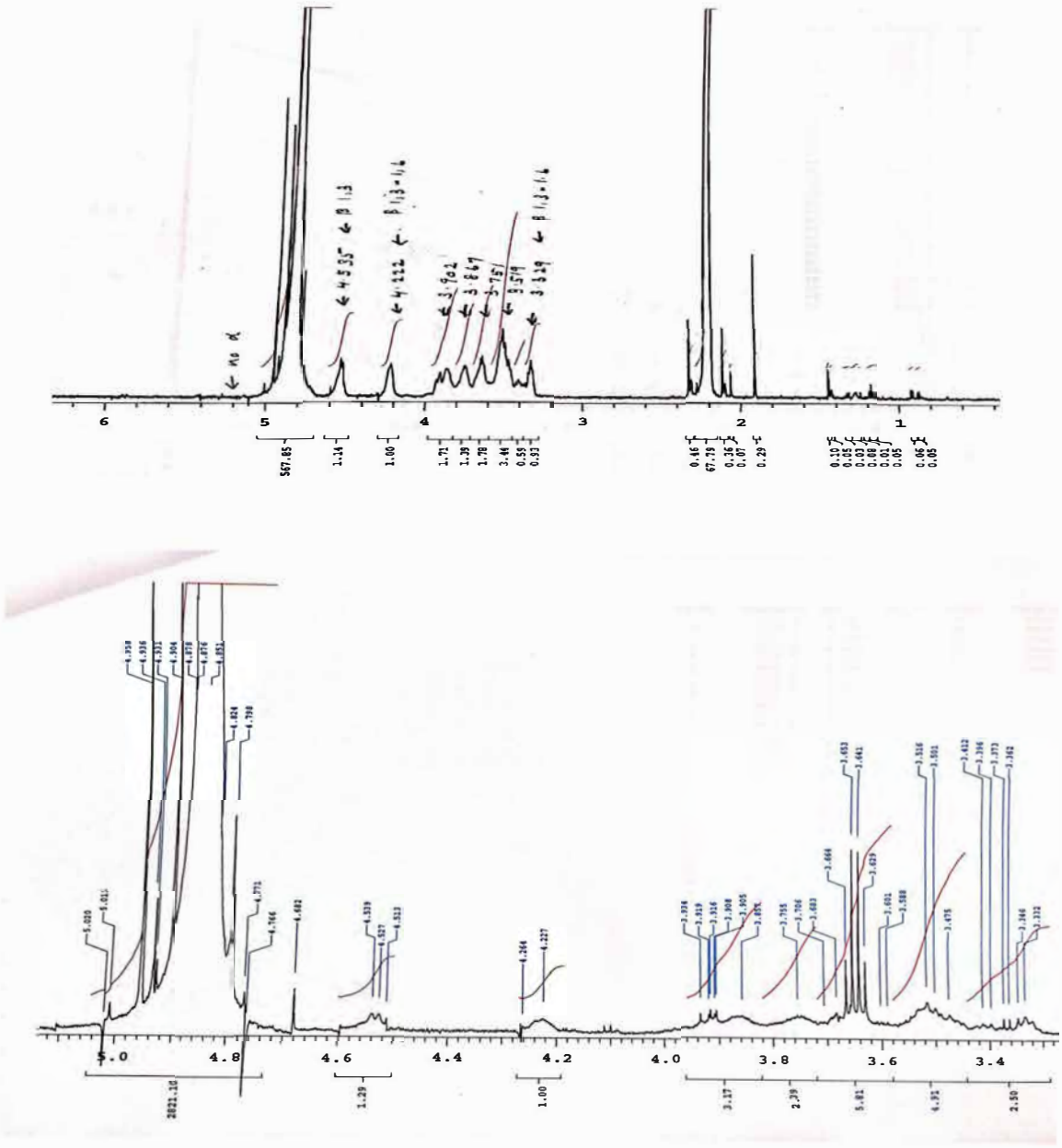


Figure 4.3: 1H NMR Spectra of the ER1 fraction

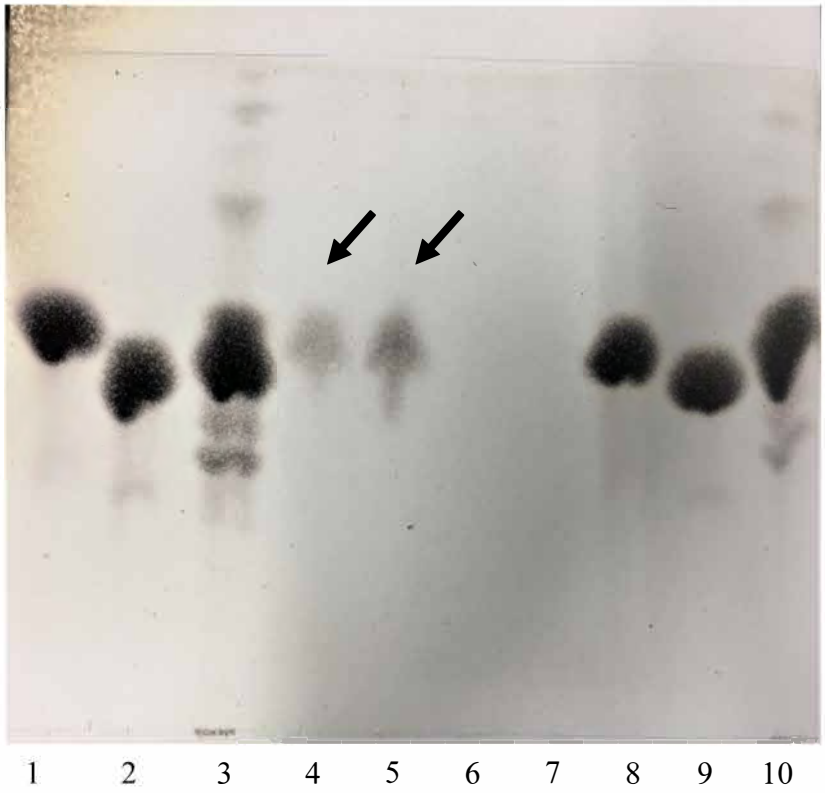
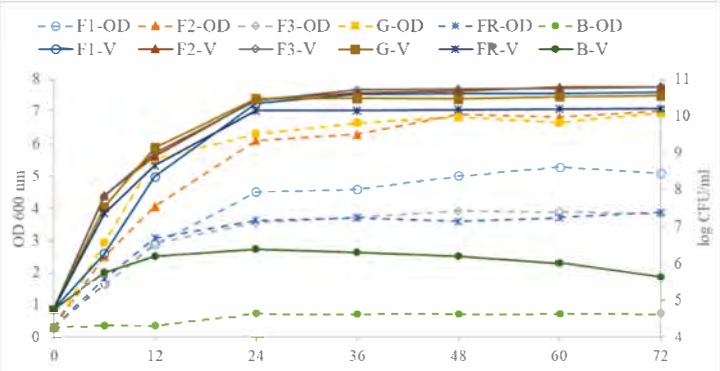


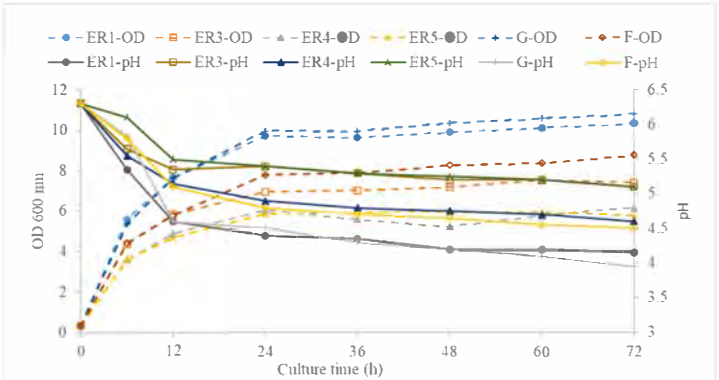
Figure 4.4: Monosaccharide composition of ER1 and ER5 by TLC (lane 1: D-glucose, lane 2: D-galactose, lane 3: corn, lane 4: ER1 with formic acid treatment lane 5: ER5 with formic acid treatment, lane 6: ER1 without formic acid treatment, lane7: ER5 without formic acid treatment, lane 8: D-glucose, lane 9: D-galactose, lane 10: corn). Presence of glucose in ER1 and ER5 samples treated with formic acids are shown by the arrows.

(a)



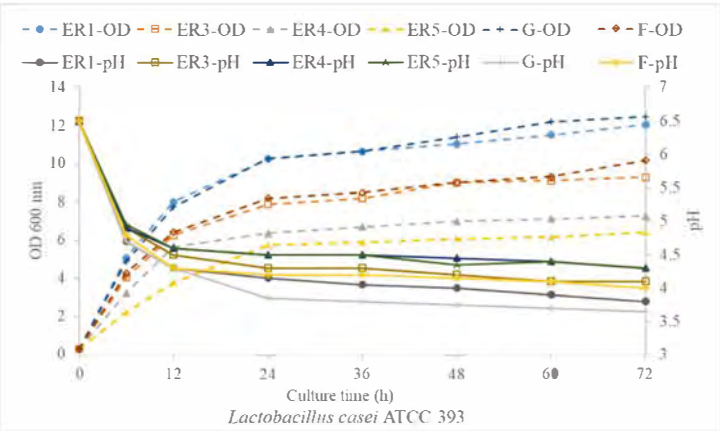
Lactobacillus rhamnosus ATCC 53103

(b)



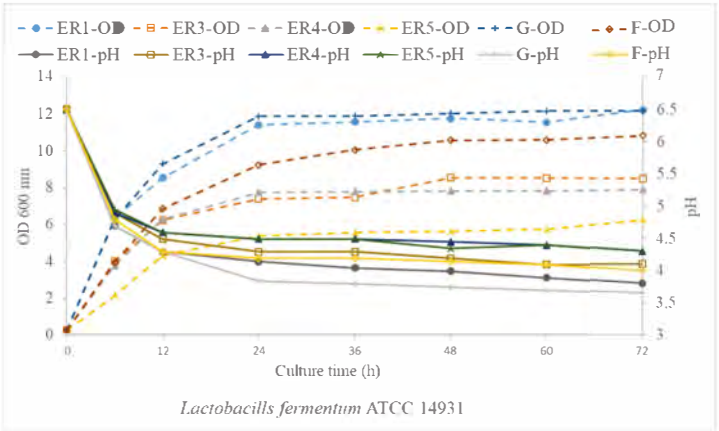
Lactobacillus acidophilus JCM 1132

(c)



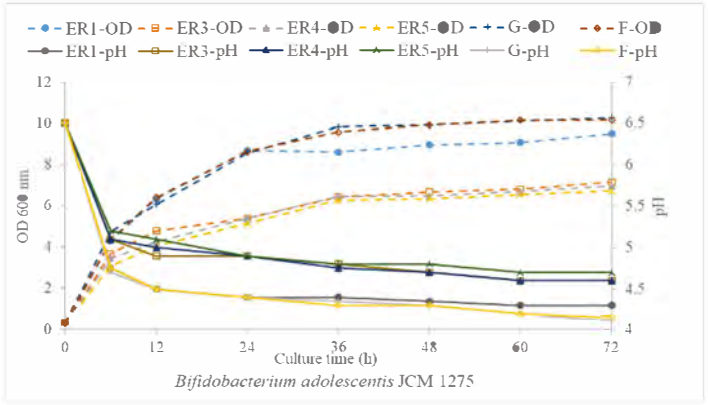
Lactobacillus casei ATCC 393

(d)

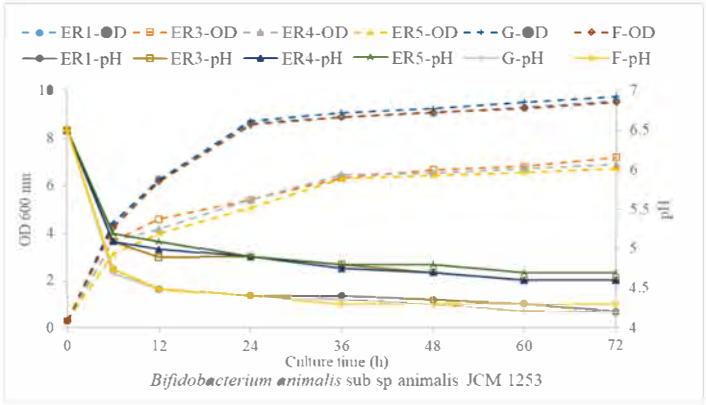


Lactobacills fermentum ATCC 14931

(e)



(f)



(g)

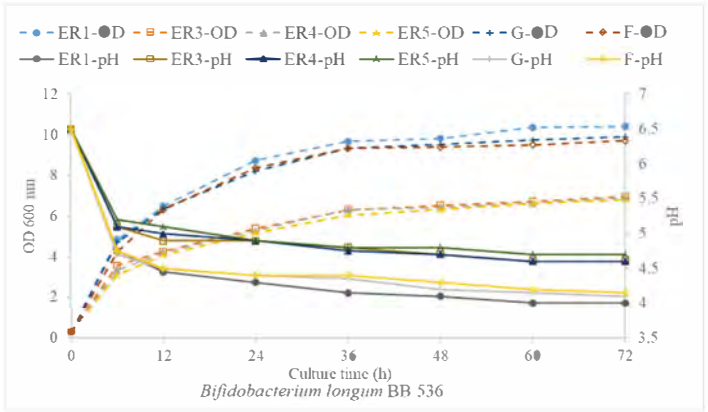
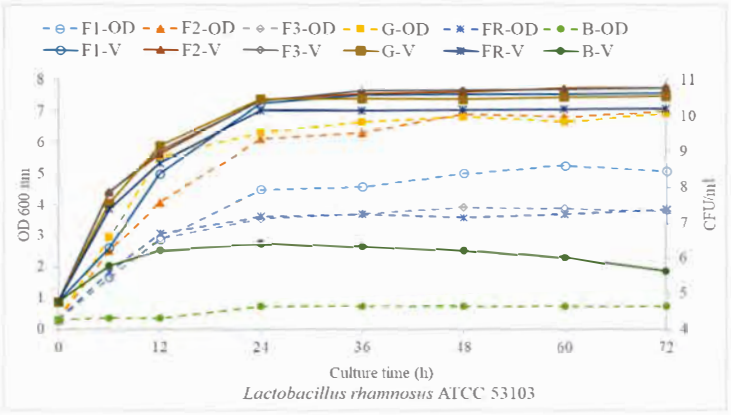


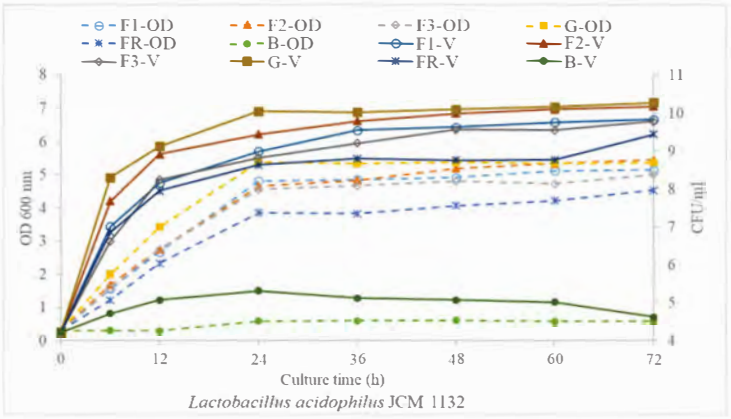
Figure 4.5: Changes in cell density ($OD_{600\ nm}$) and pH of selected probiotics over fermentation time with purified maitake extracts, G: glucose, and F: frutooligosacchride. (a) *Lactobacillus rhamnosus* (b) *Lactobacillus acidhophilus* (c) *Lactobacillus casei* (d) *Lactobacillus fermentum* (e) *Bifidobacterium adolescentis* (f) *Bifidobacterium animalis sp animalis* (g) *Bifidobacterium longum.* All the strains were grown under anaerobic condition at 37 °C for 72 h in triplicates.

(a)



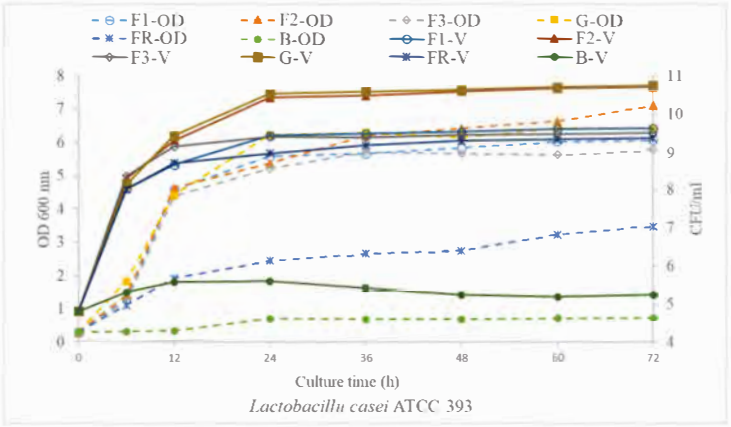
Lactobacillus rhamnosus ATCC 53103

(b)



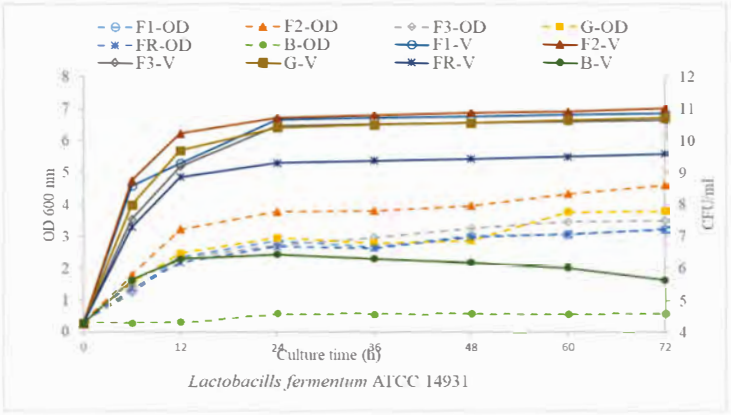
Lactobacillus acidophilus JCM 1132

(c)



Lactobacillu casei ATCC 393

(d)



Lactobacills fermentum ATCC 14931

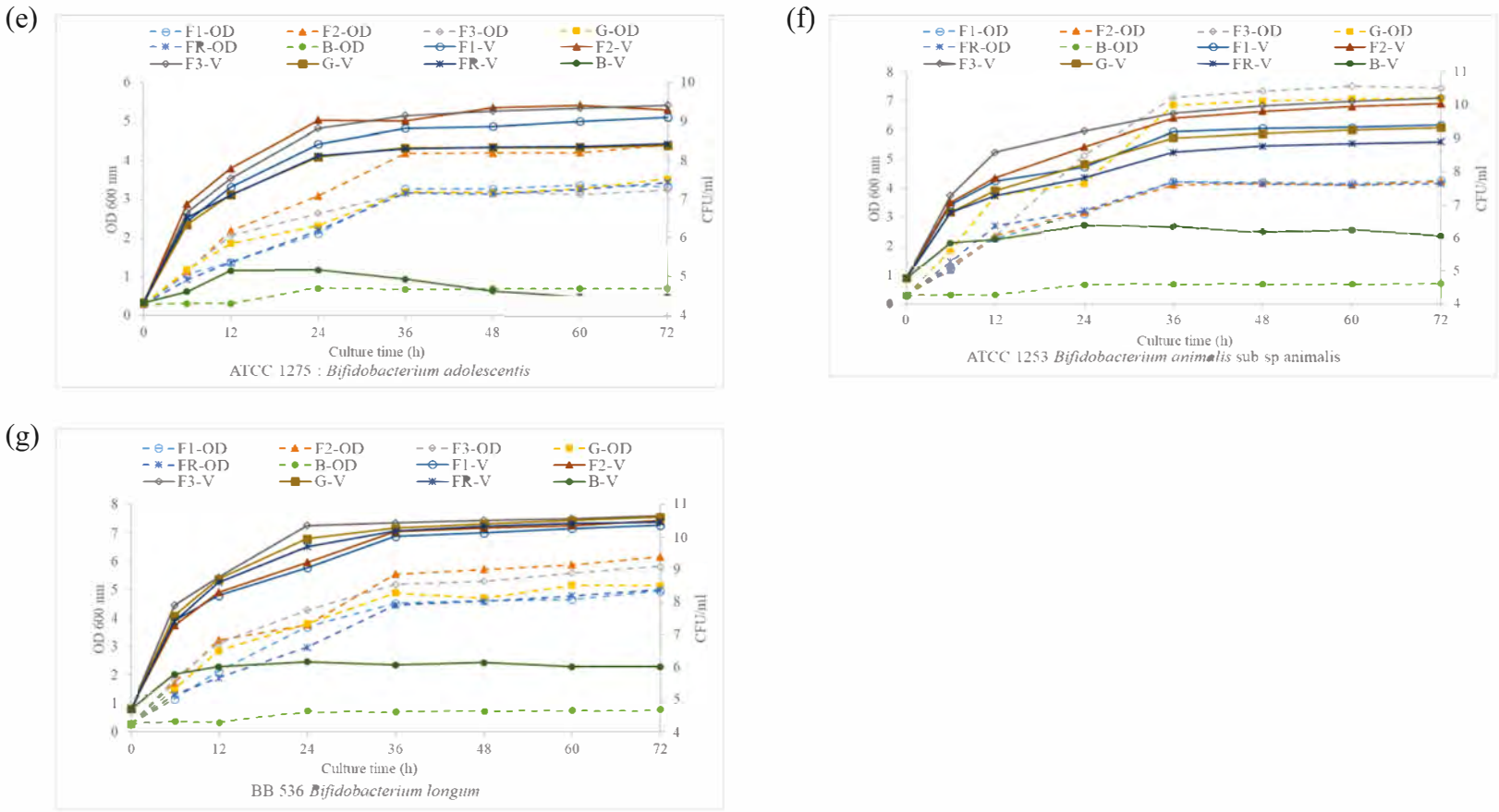
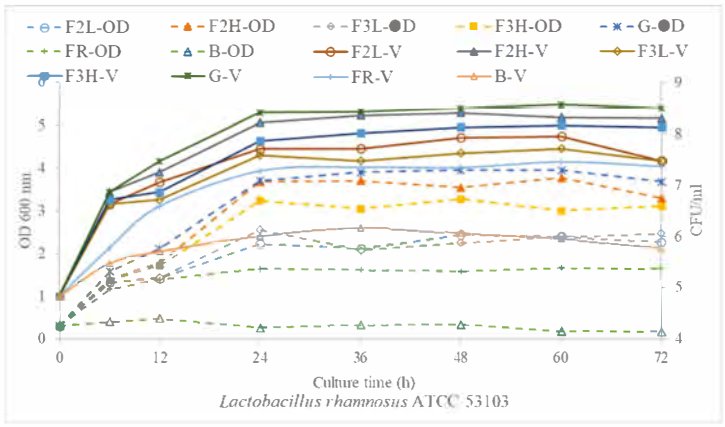
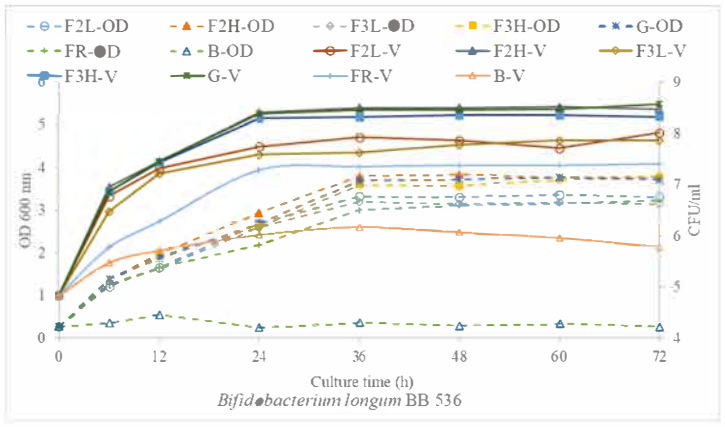


Figure 4.6: Changes in cell density and viable call count of selected probiotics over fermentation time with fractionated ER1 (ER1-F1, ER1-F2, ER1-F3), G: glucose, and FR: frutooligosacchride. (a) *Lactobacillus rhamnosus* (b) *Lactobacillus acidhophilus* (c) *Lactobacillus casei* (d) *Lactobacillus fermentum* (e) *Bifidobacterium adolescentis* (f) *Bifidobacterium animalis sp animalis* (g) *Bifidobacterium longum.* All the strains were grown under anaerobic condition at 37 °C for 72 h in triplicates.

(e)



(f)



(g)

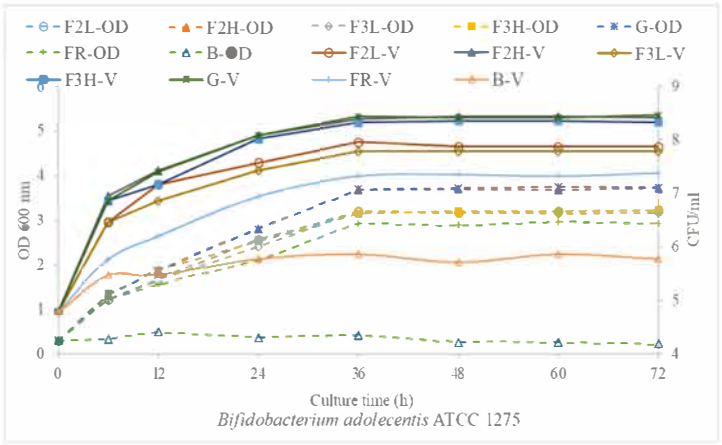


Figure 4.7: Changes in cell density and viable call count of selected probiotics over fermentation time with fractionated ER1F2: F2L: molecular weight <10 kDa, F2H: molecular weight >10 kDa and ER1F3: F3L: molecular weight <10 kDa, F3H molecular weight >10 kDa, G: glucose, and FR: frutooligosacchride. (a) *Lactobacillus rhamnosus* (b) *Bifidobacterium longum* (c) *Bifidobacterium adolescentis*

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5. General Discussion

Intestinal homeostasis which depends on complex interactions between gut microbiota, intestinal epithelium, and host immune system is important not only in the maintenance of intestinal health but also on the systemic health. The dysregulation of commensal microbial population or intestinal immune responses can cause elevated intestinal inflammation, altered intestinal environment and development of colorectal cancer (Arshad and Visweswariah, 2012). CRC which is a malignant tumor with high morbidity and mortality rate (Center et al., 2009). However, limited resources, side effects of currently available drugs and chemotherapy make CRC as one of the most problematic non communicable disease around the world (Half and Arber, 2009). Furthermore, currently available drugs are monotargeted which make them less efficient on suppressing CRC since, growth and progression of cancer is a multiple-step process which include dysregulation of multiple cell signaling pathways. Recent researches have revealed that most naturally occurring compounds in fruits and vegetables including phytochemicals and secondary metabolites have pleiotropic anticancer effect and could modulate multiple cell signaling pathways and thus are effective on preventing and suppressing CRC (Xu et al., 2010). Diets play a vital role in maintenance of gut homeostasis through modulating gut microbiota composition, specificity and diversity. Specially, diets rich in non-digestible carbohydrates, which commonly known as prebiotics can increase the growth and activity of beneficial microbiota such as those who belong to probiotic group. Probiotic bacteria involved in maintenances of gut homeostasis through the production of secondary metabolites such as SCFA and BCFA, inhibition of the activity of pathogenic bacteria and immunomodulation. Most

recent studies were therefore, focused on how to facilitate the growth and activity of probiotics through supplementation of prebiotics. In this study we investigated the effect of maitake on maintaining gut homeostasis, colorectal cancer development, and growth of probiotics.

Mice treated with DMH-induced colon carcinogenesis were employed to evaluate the effect of maitake and synergistic effect of maitake when combined with milk on ACF development, colonic inflammation and suppression of CRC. ACF formation was observed in animals treated with DMH which is considered as an ideal biological indicator on evaluating development of CRC (Furukawa et al., 2002, Katyama et al., 2002, Luebeck and Moolgavkar, 2002, Tanaka et al., 2000). Pathological analysis of CRC showed infiltration in the cells such as neutrophils, mast cells, natural killer (NK) cells, dendritic cells (DC), and tumor-associated macrophages that are involved in innate immunity, associate with CRC development (Atreya and Neurath 2008). These cells not only suppress tumor growth and angiogenesis but involve in adaptive immune responses which leads to balance immune surveillance with tumor-promoting inflammatory functions (Janakiram and Rao 2014). Furthermore, immune surveillance helps in early detection of ACF and elimination of aberrant cells, especially, before they develop into higher multiplicity (dysplastic) ACF which may progress into adenomas and adenocarcinomas in CRC. However, persistence of chronic inflammation creates microenvironment that favors inhibition of anti-tumor immune responses and leads to the formation of tolerogenic DCs, infiltration of T regulatory (T reg) cells and conversion of primary/single ACF into multiplicity which help in the progression of tumor cells (Janakiram and Rao 2014). In the present study, development of ACF multiplicity were noticed in all the DMH treated groups, indicating the alleviated

inflammation in the colon epithelia (**Fig. 3.5**). Macrophages which play a critical role in the initiation, maintenance and resolution of inflammation, accumulate at the site of inflammation and activate production of pro-inflammatory cytokines including IL-10, TNF-α and MCP-1 during early stages of CRC development or by T cells during late-stage of tumor progression (Becker et al., 2004, Majumdar and Aggarwal, 2001, Terzic et al., 2010). Confirming the above findings, in parallel to the ACF development our results shows release of pro-inflammatory cytokines TNF-α and IL-16 from colonic tissue samples of the mice in the DMH positive control group which significantly elevated as compared with DMH negative control (**Fig. 3.7**). Increased levels of TNF-α, IL-1β, IL-6 and IL-8 have been reported in CRC patients at different tumor developmental stages (Aggarwal, 2003, O'Shea et al., 2002, Pullman et al., 1992, Raab et al., 1993) and among them, TNF-α is known as a critical component in cell death and inflammation (Sedger and McDermott, 2014). The tissue levels of TNF-α protein were correlated with the degree of inflammation and macroscopic score, supporting previous as well as more recent reports (Akazawa et al., 2002, Olsen et al., 2016). In addition, in line with the present results, recently published clinical reports show that treatment with anti-TNF-α, a specific antibody against TNF-α, induces remission in 30–40 % of ulcerative colitis patients (Lavi et al., 2012, Rutgeerts et al., 2005). On the other hand, studies have showed that the fibroblasts play a role in tissue repair by secreting pro-inflammatory cytokines IL-6, IL-8, and prostaglandin E2 (PGE2), which help in the neutrophil response. In addition, epithelial cells and stroma cells help in repairing the injury. Resolution of inflammation can lead to complete remission of inflammation and stop the aberrant proliferation, which can extend into tumor growth. However, if the process of resolution of inflammation is impaired, it can lead to chronic inflammation, creating a favorable microenvironment that eventually can lead to tumor cell growth,

proliferation, and metastases (Janakiram and Rao, 2014). Suppression of colonic inflammation at the early phase of cancer development, is one of the promising way to prevent tumor progression. Theses natural food ingredients include non-starch polysaccharides, particularly dietary fiber, unsaturated n-3 fatty acids, vitamins (vitamin D), minerals (calcium and selenium) and phytochemicals (resveratrol, curcumin) (Kim and Milner, 2007). Although the molecular mechanisms of these compounds are not well known and predicted to be numerous, the recent evidences suggest that they reduce inflammation and suppress the activity of oncogenic signaling pathways (Terzic et al., 2010). In a previous study the dietary complex sphingolipids were able to suppress the upregulated TNF-α and induced inflammation in colon of DMH administrated mice (Yamashita et al., 2017). Confirming above findings, both milk and maitake showed anti-inflammatory effect by suppression of increased levels of TNF-α (**Fig. 3.7**). Furthermore, the lowest levels of TNF-α were observed in mice treated with 10Mix combination diet. In addition, total and dysplastic ACF count were significantly lower in the dietary treatments group compared to DMH positive control (**Fig. 3.5**) suggesting that anti-inflammatory activity of maitake and milk may have the ability to suppress the tumor progression. Interestingly, TNF-α levels in milk alone treated group was significantly lower than that of maitake alone treated group. This may be due to higher bioavailability of anti-inflammatory compounds such as calcium, vitamin D, and sphingomyelin in milk. Sphingolipids which are available in both maitake and milk, particularly sphingomyelin and GlcCer are known to suppress inflammatory cytokines including TNF-α. The sphingolipids available in both maitake and milk (GlcCer and sphingomyelin respectively) also known to have suppressive effect not only against ACF development but also colonic inflammation (Yamashita et al., 2019). Our results suggest the presence of sphingomyelin in milk is higher than

GlcCer in maitake (**Table 3.2**) and also the bioavailability of milk sphingomyelin is higher than plant or fungi GlcCer (Yamashita et al., 2019). In addition, calcium also known to play a significant role on regulating colonic inflammation, inflammation related diseases and intestinal homeostasis. Calcium content in milk used in the present study was 890 mg per 100 g dry weight while maitake has only trace amount. This suggest the contribution of calcium and sphingomyelin on suppression of pro-inflammatory cytokines and colonic inflammation in milk treated mice. Further, low calcium intake is a recognized risk factor for total cancer incidence (Park et al., 2009, Peterlik et al., 2013). The availability of extracellular calcium determines the activity of CaSR which is associated with suppressed colonic inflammation and cancer development (Tennakoon et al., 2016). On the other hand, Mine and Zhang, suggested that CaSR suppress TNF-α-induced inflammation in intestinal epithelial cell to suppress CRC development (Mine and Zhang, 2015). However, the CaSR expression not only depends on the bioavailability of extracellular Ca^{2+} but also on vitamin D (Aggarwal et al., 2016, Bennette et al., 2000, Canaff and Hendy, 2002, Chakrabarty et al., 2005). Confirming the above findings, Canaff and Hendy showed that the CaSR expression was significantly reduced in the vitamin D-depleted rats (Canaff and Hendy, 2002). The involvement of vitamin D in colorectal tumor development was revealed by Horvath et al., showing significantly higher expression of CYP24A1, vitamin D degrading enzyme in tumor cells compared with the adjacent normal tissue (Horváth et al., 2010). Besides, several other studies showed that vitamin D reduce the risk of CRC by maintaining normal calcium gradient in the colon epithelial crypts, decreasing proliferation of noncancerous but high risk epithelial cells, and stimulating mutual adherence of epithelial cells (Bobek and Galbavy, 2001, Lavi et al., 2012). Maitake fruiting bodies contains around 28.1 µg/100 g of vitamin D and while milk contain around 1 µg/100 g

of vitamin D (Japanese Standard Tables of Food Composition) which possibly explain the reason behind higher bioactivity of 10 Mix diet. In addition, SCFA is known to involved in regulation of TNF-α induced inflammation. A SCFA mixture with different proportions of butyrate (5%, 20% and 50%) was used to examine the effect of treatment on barrier function with concomitant addition of pro-inflammatory lipopolysaccharide or TNF-α and authors found out that when the proportion of butyrate was higher in the SCFA mixture (20% or 50%), TER increased significantly despite TNF-α and lipopolysaccharide treatment, suggesting that butyrate incubation can exert protective effect against inflammation (Chen et al., 2017). In addition, Zhao et al., showed increased levels of SCFA in AOM treated rats fed high amylose corn starch suggesting the involvement of SCFA on suppressing ACF occurrence (Zhao et al., 2011). In human colonic mucosa, butyrate was found to regulate several pathways such as fatty acid metabolism, electron transport, TNF-α signaling and oxidative stress pathways to suppress colon carcinogenesis (Vanhoutvin et al., 2009). In our study, we observed decreased SCFA level in the DMH-induced groups, however, it was reversed by maitake and milk treatments (**Table 3.4**) suggesting the involvement of milk and maitake on SCFA production. Bovine milk contains oligosaccharides that are analogue to human milk oligosaccharides and bioactive peptides (Zivkovic and Barile, 2017). Fermentation of milk oligosaccharides and bioactive peptides by probiotic microorganism, particularly *Bifidobacterium* and *Lactobacillus* decrease colon pH while increasing SCFA and lactate (Yu et al., 2012). In addition, oligosaccharides, FOS, lactulose, galactomannan and indigestible polysacrides such as mannan, xylose and β-glucan in maitake are considered as promising prebiotics agents (Singdevsachan et al., 2016). The stimulation of growth and activity of probiotics might be the reason behind increased SCFAs levels and decreased cecal pH in dietary treatments group. However,

total SCFA concentration including butyrate was comparatively lower in milk only treated group (**Table 3.4**) than in maitake or combination treated groups. Morita et al., reported that when a highly purified and digestible protein such as casein is the sole protein source or available in excess in the diet with the presence of large amount of fermentable carbohydrates, nitrogen supply may become insufficient to sustain a rapid bacterial proliferation, thus resulting in a decline fermentation capacity (Xu et al., 2010). This may explain the decline in SCFA production in milk, as well as the influence of SCFA on suppression of ACF. High concentrations of sphingolipids, milk calcium and vitamin D and increased production of SCFA in 10Mix group might be the possible reason for higher bioactivity on suppressing ACF development and colon inflammation. Liver fatty acid profiles were different among groups. The levels of liver PUFA which depends on the permeability and fluency of cell membranes (Gutierrez et al., 2004). were higher in standard diet fed animals despite of the dietary PUFA content. In addition, liver n-3 and n-6 PUFAs which are important in colon cancer development, progression and inhibition (Agnihotri et al., 2016) were different among groups (**Table 3.5**). The liver n-3/n-6 ratio which is crucial in cancer prevention was highest in milk only fed animals following the dietary patterns. However, this pattern was not observed with other dietary treatment groups suggesting that the bioavailability of PUFA depend on body tissue and lipid peroxidation.

Lipid peroxidation is an indicator of oxidative stress which increased with malignant alterations (Das, 2002). DMH, which ultimately metabolize into dizonium ion in the liver is not only colon carcinogen but also a potent necrogenic hepatic tumorigenesis which could elicit an oxidative stress (Devesena and Menon, 2007). Lipid peroxidation (LPO) can be measured by evaluating the levels of liver and colon TBARS and

according to the Devesena and Menon, enhanced liver TBARS in DMH treated animals could be attributed to oxidative stress and production of reactive oxygen metabolites (ROMs) (Devesena and Menon, 2007). In the current study, elevated lipid peroxidation was observed in DMH treated animals compared to DMH negative group (**Fig. 3.6**). However, oral ingestion of milk or maitake were able to suppress increased oxidative stress which is indicated with lower TBARS levels in dietary treatment groups. Previous studies suggest, phytochemicals in natural food sources can suppress oxidative stress related colon carcinogenesis through increasing the activities of detoxification enzymes (Sreedharan et al., 2009). Following the same, our study also showed near to normal TBARS levels in 10Mix group which suggest the activity of phytochemicals, particularly vitamin E and D in milk and maitake.

Colonic inflammation eventually regulates apoptosis and anti-apoptosis by multiple pathways through binding to TNF R1 which is considered as an effective method for a wide variety of cancer treatments (Fesik, 2005, Sedger and McDermott, 2014). Previous several studies have shown significant apoptosis in colonic epithelial cells suffering mild acute inflammation induced by DSS (Elmore 2007). In another study, sphingoid which was digested from complex sphingolipids markedly induced apoptosis in colorectal cancer cells but with no changes in normal cells (Yamashita et al., 2019). Pro-apoptotic mechanisms are activated in different ways such as direct or indirect activation of caspases, the executor of apoptosis, stress activated protein kinases (SAPK/JNK) pathway, and through upregulation of TNF receptor 1 and Fas receptors (Selzner et al., 2001).

It is well known that the ratio of pro-apoptotic (Bad) and anti-apoptotic mediators (Bcl-2, Bcl-x) determine the relative permeability of the mitochondrial membrane to cytochrome c. Increased levels of free cytosolic cytochrome c interacts with apoptosis-activating factor-1 (Apaf-1), adenosine triphosphate, and procaspase 9 to form the apoptosome. The apoptosome cleaves and activates caspase 9, which leads to caspases 3, 6, and 7 activities, thus stimulating apoptosis (Huerta et al., 2006). In our present study, significantly decreased expression levels of the anti-apoptotic proteins Bcl-2 and Bcl-x were evident by milk and maitake, in contrast to the increased expression level of the pro-apoptotic protein Bad (**Fig. 3.9**). other than that, increased levels of cytochrome c in the groups treated with milk or maitake were also observed suggesting the involvement of maitake and milk in apoptosis. High fiber diets which favors production of SCFA known to suppress ACF formation and CRC incidences through activation of caspases cleavage of the cyclin-dependent kinase inhibitor p21, Bcl-2 protection and DNA fragmentation (Avivi Green et al., 2001, Bingham, 1990, Jan et al., 2002, Perrin et al., 2001). Further, a study conducted using fruiting body and mycelia extracts of the edible mushroom *Pleurotus pulmonarius showed decrease proliferative potential in colon cancer cell lines suggesting the upregulated apoptotic potential of exposed cells* (Lavi et al., 2012). Several other studies conducted to evaluate the CRC suppressive effects of plant materials particularly mushroom, are also showed the results which were similar to present study (Hu et al., 2009, Lavi et al., 2012). The caspase cascade is a well-known key pathway in apoptotic signal transduction. Increased caspase-3 processing has been previously associated with DSS-induced colonic tissue damage and colitis (Joo et al., 2009, Paul et al., 2005). Vitamin D and calcium known to upregulated caspase 12 to induce apoptosis in colon cancer cell lines (Jeon and Shin, 2018). Another study showed the treatment of 1 nM of $1,25D_3$

effectively induce Caspase 3/7 dependent apoptosis in colon cancer cells in the presence of 1.8 mM Ca^{2+} (Aggrwal et al., 2016). Confirming these findings our study showed the oral feeding of milk and maitake can significantly increase expression of cleaved caspase-3 compared to control (**Fig. 3.9**) which might be due to the presence of vitamin D and calcium in maitake and milk respectively. In addition, tumor suppressor, p53 is activated in response to various stresses including DNA damage to promote mutant cell apoptosis by regulating various downstream effectors such as p21 and Bax (O'Brate and Giannakakou, 2003). According to our results, milk and maitake treatments triggered apoptosis, which may be controlled by p53-mediated pro-apoptotic Bad and cytochrome c. Even though both maitake and milk treatments showed the apoptotic activity, the effect was apparent in the colon tissues of combination fed animals suggesting that synergistic effects are effective on suppressing colon inflammation and CRC development. Supporting the current results, several other studies also indicated the significance of combination treatments. One study showed a synergistic inhibitory effect against gastric carcinoma cells, when epigallocatechin-3 gallete combined with epicatechin. There, the synergistic effect was more significant than the independent effect (de Kok et al., 2008). Another study, that was conducted to evaluate the effect of combination of curcumin and catechin on human colon adenocarcinoma HCT 15, HCT 116, and human larynx carcinoma Hep G-2 cell lines, revealed the synergistic inhibitory effect on growth of cancerous cell lines was markedly higher than the individual effect (Manikandan et al., 2011).

The findings in chapter 3 revealed that, maitake plays a vital role in the maintenance of intestinal homeostasis, suppression of colonic inflammation and CRC which may possibly be through stimulating the activity of colonic probiotic microorganism.

Therefore, to identify the effect of maitake fruiting bodies on the growth of colon probiotic microorganisms *in vitro* bacterial culture were used and the responsible maitake polysaccharides were identified. Purified maitake polysaccharide fractions (fractionation was done as in the **Fig. 4.1**) were tested using 7 probiotic species and found out purified maitake polysaccharides fractions stimulate the growth of tested probiotics (**Fig. 4.4**) indicating the polysaccharide fermentation by colonic probiotics. In particular, ER1 purified fraction showed a remarkable effect on growth of colonic probiotic bacteria compared to other purified maitake fractions. Confirming present results, Synytsya et al, found out that water extraction of *Pleurotus eryngii* facilitate the probiotic growth rate, biomass and SCFA production of selected colonic *Lactobacillus* species (Synytsya et al., 2009). Further, the study confirmed that, the water extraction of *Pleurotus eryngii* consist with highly branched 1,3-1,6 β-glucan, which might be the responsible for prebiotic activity. The NMR and linkage analysis results of the present study confirmed that ER1 fraction consist with branched 1,3-1,6 β-glucan while the other fractions consist with unbranched 1,3 β-glucan (**Fig. 4.2**), thus, the branched 1,3-1,6 β-glucan in ER1 fraction might be the responsible compound of higher prebiotic activity. In the present study, purified ER1 fraction had the highest molecular weight (approximately 22 kDa) than the other extracts (molecular weight of other extracts were approximately 13.5 kDa) (**Table 4.2**). Previous studies confirmed that, higher molecular weight β-glucan is higher in bioactivity (Kalra and Jood, 2000, Theuwissen and Mensink, 2008a, Tong et al., 2014, Wood et al., 1994, Zheng et al.,2011). In particular, Tong et al., showed higher molecular β-glucan is effective in SCFA production and colonic fermentation than low molecular weight β-glucan (Tong et al., 2014). The high molecular mass of ER1 fraction may be a reason for the higher prebiotic activity. The ER1 fraction was then further divided into three sub-fractions

(ER1-F1, ER1-F2, and ER1-F3) based on the carbohydrate contents to test for prebiotic activities (**Fig. 4.1a**). Sub fractions also stimulated the growth of probiotics (7 species), however ER1-F2, and ER1-F3 showed a remarkable effect on the growth, biomass and cell viability of probiotics compared to ER1F1(**Fig. 4.5**). Even though, one previous study reported that the purity of fraction does not play any significant role in fermentation (Gullaon et al., 2011), another study conducted to evaluate the cytokine stimulating activity of 1,3 β-D-glucans of same extract with different fractions which have different molecular weights showed the different bioactivity though they share the similar structure (Falch et al., 2000). Further, Sanz et al., also indicated that glycosidic linkages and molecular weight of the maltose-based oligosaccharides can be affected the fermentation of human gut microbiota (Sanz et al., 2006). In the present study each purified fraction showed only one single and symmetrical narrow peak in the HPLC chromatogram, indicating the presence of homogeneous polysaccharides. Thus, this results suggest that different fractions of ER1 may consist same structured β-glucan with different molecular weights. To confirm above findings, the ER1-F2 and ER1-F3 sub-fractions were further divided using a molecular weight cutoff value of 10 kDa. The sub-fractions with molecular weight >10 kDa showed significant effects on the growth of *Lactobacillu. rhamnosus* (ATCC 53103), *Bifidobacterium longum* (BB 536) and *Bifidobacterium adolescentis* (ATCC 1275) confirming the influence of molecular weight on prebiotic activity (**Fig 4.6**). This would further strengthen the hypothesis that high molecular weight 1,3 β-D-glucans has higher effect on stimulating the growth of probiotics thus the production of SCFA. Further research is under way to isolate the highly pure maitake 1,3 β-D-glucan, and evaluate the molecular weight of the responsible pure 1,3 β-D-glucan. In addition, the synbiotic activity of purified 1,3 β-D-

glucan compared to commercially available 1,3 β-D-glucan may provide an insight for future directions.

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6. Conclusion and future aspects

These research findings have revealed the protective effect of *Grifola frondosa* on the maintenance of gut homeostasis, suppression of colon inflammation and CRC development. DMH colon carcinogenesis induced mouse models showed that maitake has the ability to conquer formation of pre-cancerous lesions, through suppression of colon inflammation and upregulation of cancer cell apoptosis. Particularly, our study showed maitake directly or indirectly involved in the maintenance of colonic inflammation through upregulation of SCFA levels and downregulation of pro-inflammatory cytokines production. Further, simultaneous intake of maitake with milk synergistically increased the inhibitory effect against ACF development and colon inflammation. In addition, *in vitro* bacterial culture studies also confirmed that maitake involved in the maintenance of gut homeostasis through stimulation of the growth of colonic probiotics. Further, the β-1,3-1,6-glucan in maitake with an average molecular weight of 22 kDa facilitated the growth of tested probiotics suggesting the possibility of maitake hot water extract to be used as a synbiotic agent with selected colonic probiotic strains to improve the host intestinal environment.

The next step of this study would be to test the synergistic effects of maitake and combination of maitake and milk with colonic probiotics on suppression of colon inflammation and CRC development using *in vivo* colon carcinogenesis models and *in vitro* cell culture models. Based on the present studies we speculate that the inhibitory effect of maitake against ACF formation is mainly due to the increased levels of SCFA in which colonic probiotics play a vital role. Thus, the evaluation of synergistic inhibitory effect of simultaneous intake of maitake, combination of milk and maitake

and probiotics against ACF development may provide a new insight on CRC researches. In particular, isolated maitake β-1,3-1,6-glucan with colonic probiotics may be an effective source of therapeutics in the treatment of early development of CRC. In addition, synergistic effect of maitake and milk on suppression of ACF formation and colon inflammation suggested the involvement of different cell signaling pathways thus, the evaluation of efficacy and safety of combination diet with commercially available chemopreventive agents may open a new direction in the treatments of CRC with minimum side effects.

Appendix

Table 1: Composition of feed used in *in vivo* experiment

g/1000 g in Diet)	Control diet (Blank & Control)	10% Maitake diet (10MM)	10% Milk diet (10MK)	5% milk + 5% maitake diet (5Mix)	10% milk + 10% maitake diet (10Mix)
Casein	200	178	174	176	152
Corn Starch	150	126	111	115	87
Cellulose	50	9	50	30	9
Corn oil	50	46	24	35	20
Sucrose	500	500	500	500	500
dl-Methionine	3	3	3	3	3
Choline	2	2	2	2	2
Mineral Mix *	35	35	35	35	35
Vitamin Mix *	10	10	10	10	10
milk	-	-	100	50	100
maitake	-	100	-	50	100

All units are g/kg diet.

*AIN-76 vitamin and mineral mixtures contains 500 g of calcium hydrogen phosphate dehydrate /kg mix.

Table 2: Probiotic bacteria used in the *in vitro* bacterial culture experiment

Species/strain	Species/strain number
Lactobacillus rhamnosus	ATCC 53103
Lactobacillus acidophilus	ATCC 4356
Lactobacillus caseis	ATCC 393
Lactobacillus fermentum	ATCC 14931
Bifidobacterium adolescentis	JCM1275
Bifidobacterium animalis sub sp animalis	JCM1253
Bifidobacterium longum	BB536