ROLE OF PROBIOTIC BACTERIA IN COLORECTAL CANCER METASTASIS

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For Juan, Rosa, and Cookie.

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I. GENERAL INTRODUCTION

COLON CANCER

Cancer currently accounts for one in four deaths in the United States. It was estimated that in the year 2009 colorectal cancer accounted for 10% of new cancer cases (~146,970) and 9% (~49,920) of cancer related deaths among men and women (Centers for Disease Control and Prevention, 2009; Jemal et al,. 2009; National Cancer Institute 2009). Colorectal cancer is both cancer of the colon and rectum combined as one general area of cancer. Cancer of the colon forms in the tissues of the colon, the longest part of the large intestine. Cancer of the rectum forms in the tissues of the rectum, the last inches of the large intestine near the anus (National Cancer Institute, 2009).

Causes of Colon Cancer: Genetic, Dietary, and Gut Flora

The etiology of colorectal cancer is a multistep process that involves specific genetic alterations that can be inherited or caused during the lifetime due to exposure of an individual to exogenous and endogenous chemicals with genotoxic potential (Pool-Zobel and Sauer, 2007). The source for genetic alterations can be within the general environment, the diet, and in various processes of metabolically endogenous conversion (Pool-Zobel and Sauer, 2007).

It is estimated that approximately 15% of all colorectal cancer are due to genetic predisposition, with 60% due to sporadic colorectal cancer that develop from adenomatous polyps (Gill and Rowland, 2002; Ponz de Leon and Percesepe, 2000

Vogelstein et al., 1988). Adenomas and carcinomas develop through inactivation of various tumor-suppressing genes (APC, p53), activation mutation in proto-oncogenes (k-*ras*, *c-myc*), and loss of function in DNA repair genes (hMLH1, hMSH2) (Gill and Rowland, 2002; Lynch et al., 1991; Pool-Zobel and Sauer, 2007; Walther et al., 2009).

Incidence rates globally show a 20-fold variation, with developed nations suffering the highest rates of colorectal cancer (Gill and Rowland, 2002). Fluctuations can be attributed to both genetic and environmental factors (Gill and Rowland, 2002). Preventable factors, such as infection, inflammation, smoking, physical activity, obesity, and diet have been implicated in the etiology of certain cancers (Jong-Eun et al., 2007; Key et al., 2004; Pool-Zobel and Sauer, 2007). Dietary factors alone have been thought to account for approximately 30% of cancers in Western countries, therefore making diet second only to tobacco as a preventable cause of cancer (Key et al., 2004).

Increasing evidence suggests that diet plays a specific role in the etiology of colorectal cancer, which implies that risks are potentially reducible through diet (Commane et al., 2005; Gill and Rowland, 2002; Pool-Zobel, 2005; Pool-Zobel and Sauer, 2007; Rafter et al., 2007). Diets high in fat and red meat have been implicated in the initiation of colorectal carcinogenesis or its enhanced progression (Commane et al., 2005; Gill and Rowland, 2002; Pool-Zobel and Sauer, 2007; WCRF, 1997). In contrast, diets rich in vegetables and fiber have been shown to have protective effects, such as reduction of colorectal cancer risk and toxin dilution (Gill and Rowland, 2002; Harris and Ferguson, 1993; Pool-Zobel, 2005; WCRF, 1997; Wirfalt et al., 2009). In addition, micronutrients (carotenoids, ascorbate, and folate) and phytochemicals (flavonoids, lignans, and anthocyanins) found in vegetables have been examined epidemiologically

for their role in colorectal cancer prevention (Ferguson and Harris, 2003; Slattery et al., 1997).

Diet can also affect the commensal bacteria present in the gut, which in turn can produce diverse, physiologically active metabolites that can influence the normal development and function of the host (Commane et al., 2005). Evidence from numerous studies support the view that endogenous colonic microflora are involved in the etiology of colorectal cancer by producing metabolites from dietary components that have genotoxic, carcinogenic, and tumor-promoting activities (Pool-Zobel and Sauer, 2007; Rafter, 2002; Rafter et al., 2007). Several dietary or biliary compounds can be transformed by anaerobic gut bacteria to gentoxic products (Pool-Zobel and Sauer, 2007). For example, heterocyclic amines are pyrolysis products from fried protein-containing foods that can undergo biotransformation in the liver and can once again be reactivated by eubacteria and clostridia in the colon by the bacterial β-glucuronidase (Knasmuller et al., 2001; Pool-Zobel and Sauer, 2007; Van Tassell et al., 1990). Other bacterial enzymes such as nitroreductase and azoreductase have also been associated with carcinogen production in the gut (Burns and Rowland, 2000; Commane et al., 2005).

HUMAN GUT MICROBIOTA

The microorganims that reside inside and on humans (known as microbiota) are estimated to outnumber human somatic and germ cells by a factor of ten (Kinross et al., 2008; Turnbaugh et al., 2007). Within the human intestine, an "extended genome" of millions of microbial genes (the microbiome) provide a symbiotic landscape within the host (Kinross et al., 2008; Turnbaugh et al., 2007). This complex symbiosis influences

host metabolism, physiology, and gene expression (Kinross et al., 2008; Martin et al., 2008). The composite of human and microbial cells form an aggregate of both human and microbial genes (human genome and microbiome, respectively) that together form a complex biologic "superorganism" (Kinross et al., 2008; Martin et al., 2008; Turnbaugh et al., 2007). Advances in research are now beginning to implicate the gut microbiome in the etiology of diseases due to mammalian-microbial symbiosis, which has a strong role in the metabolism of endogenous and exogenous compounds (Kinross et al., 2008; Martin et al., 2008). The development of diseases such as insulin resistance, Crohn's disease, irritable bowel syndrome, food allergies, gastritis and peptic ulcers, obesity, cardiovascular disease, and gastrointestinal cancers are influenced by the activity of gut microbiota (Kinross et al., 2008; Martin et al., 2008).

The human intestine contains approximately 100 trillion microorganisms that represent about 1000 separate bacteria, yeasts, and parasites (Kinross et al., 2008). These microbiota vary along the digestive tract with density increasing from the mouth to the anus. The human gut can be classified into three different regions: the stomach, small intestine, and colon (large intestine) (Rastall, 2004). The stomach contains very low facultative anaerobe (*Lactobacillus* sp., *Streptococci*, and yeast) bacterial numbers, ~100 colony forming units (CFU) per milliliter (ml) due to the low pH present in the stomach (Rastall, 2004). The small intestine increases in bacterial load containing facultative anaerobes and aerobes (*Lactobacillus* sp., *Streptococci, Enterobacteria*, *Bifidobacterium* sp., *Bacteroides* sp., and *Clostridia*) at ~10^4-10^8 CFU/ml (Rastall, 2004). The large intestine is the most heavily colonized region of the gut and harbors the most complex and dense array of microorganisms than any other part of the human body (Rastall, 2004;

Rambaud et al., 2007). Bacterial densities are greater in the colon with populations of 10^9-10^{12} colony forming units (CFU) per gram of contents (Rastall, 2004; Rambaud et al., 2007). The microflora residing within the colon is dominated by strict anaerobes that include *Bacteroides* sp., *Clostridia* and other families within the *Clostridium* mega-genus (*Ruminococcus* sp., *Butyrovibrio* sp., *Fusobacterium* sp., *Eubacterium* sp., *and Peptostreptococcus* sp.), *Bifidobacterium* sp., *Atopobium* sp., and peptococci (Rastall, 2004). Facultative anaerobes that include *Lactobacillus* sp., *Enterococci, Streptococci,* and *Enterobacteriaceae,* occur in numbers of ~1000-fold lower (Rastall, 2004) (Fig. 1.1.).

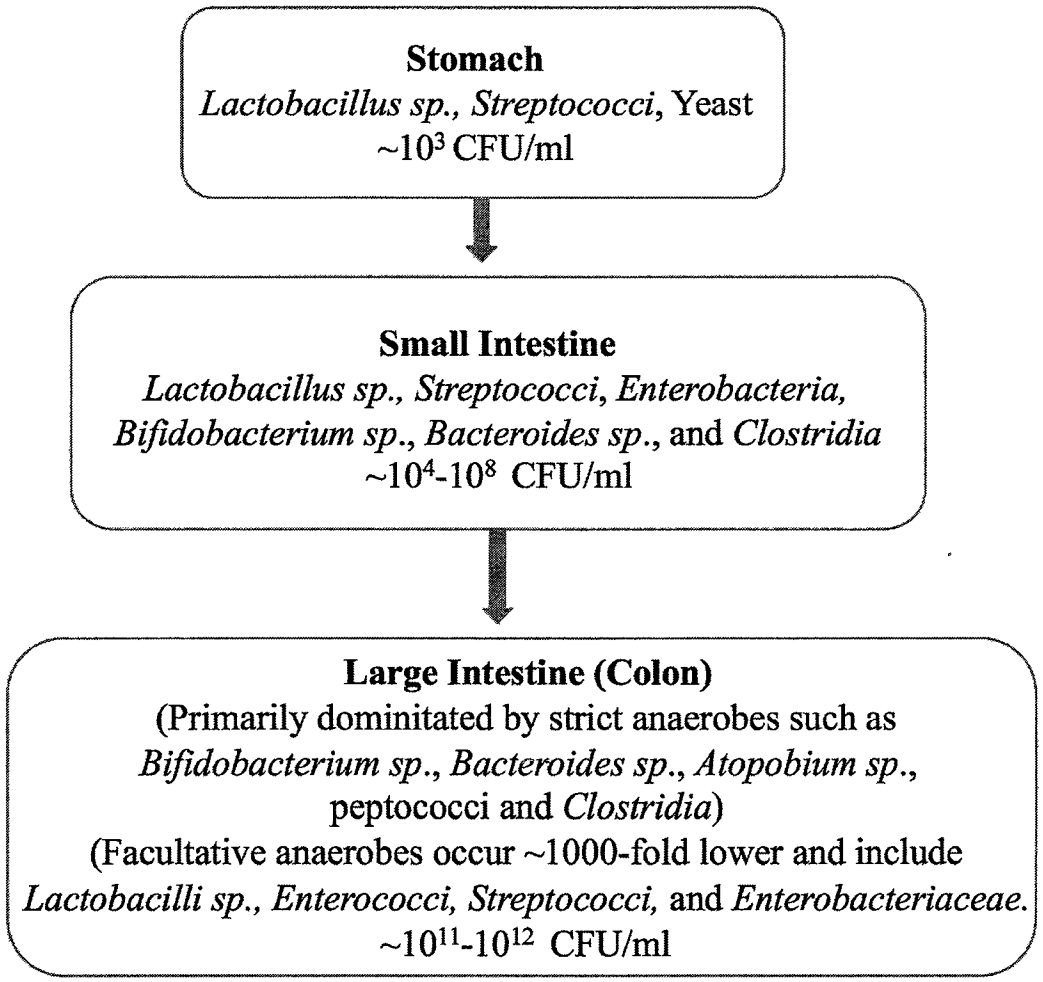

Figure 1. 1. Overview of microflora within the human gut.

The ecological environment of the large intestine plays an important role in the maintenance and presence of these microorganisms. The intestinal microbiota can change in response to diet, pharmaceutical input, age, disease, and medical or surgical intervention (Kinross et al., 2008). Therefore the role of dietary intervention in gut ecology has been implicated in having a positive effect when modifying the intestinal microbiota to that of a more beneficial environment (Davis et al., 2009; Ng et al., 2009; Rastall, 2004). As transient bacteria, probiotics have demonstrated tremendous potential in treating gastrointestinal diseases (Davis et al., 2009; Rabot et al., 2010). Probiotics provide additional enzymatic activities as well as interact with the resident microbial community, thus changing the ecosystem and metabolic characteristics of the gut (Rabot et al., 2010). It is becoming more evident that some bacterial groups (*Lactobacillus* sp. and *Bifidobacterium* sp.) have much lower activities of enzymes that can generate carcingogens than do other GI microflora (*Clostridia* and *Bacteroides*) (Rafter et al., 2007). Therefore it may be theorized that factors that influence the modulation of the intestinal microflora composition and activity may inhibit tumor development and colorectal cancer progression (Commane et al., 2005). Thus considerable attention has been focused on dietary supplements that can influence gut microflora (Commane et al., 2005; Rafter, 2002). In particular, numerous studies have demonstrated the protective role of probiotics in colon cancer prevention (Commane et al., 2005; Fooks and Gibson, 2002; Gill and Rowland, 2002; Rafter, 2002; Rafter et al., 2007).

Probiotics

It has been demonstrated that incorporation of probiotics as part of a balanced diet may serve as a promising strategy for reducing the risk of colon cancer (Commane et al.,

2005; Jong-Eun et al., 2007; Rafter el al., 2007; Teitelbaum and Walker, 2002). The beneficial effects of probiotic species within the GI tract include pathogen resistance, suppression of allergies, control of blood cholesterol, cancer chemoprevention, and modulation of immune function (Commane et al., 2005; Teitelbaum and Walker, 2002). Probiotics are defined as: "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (Joint Food and Agriculture Organization (FAO)/World Health Organization 2002).

Precise bacterial types associated with colorectal cancer risk have not been elucidated, however several studies support reduction in risk with an increase in beneficial bacterial groups such as *Lactobacillus* sp. and *Bifidobacterium* sp. (Cole et al., 1985; Rafter et al., 2007; Saito et al., 1992). Overall, the GI tract may be divided into species that exert either harmful or beneficial effects on the host (Gibson and Roberfroid, 1994). Pathogenic effects include diarrhea, infections, liver damage, carcinogenesis, and intestinal putrefaction; GI health promoting effects include inhibition of growth of harmful bacteria, stimulation of immune functions, improved digestion and absorption of nutrients, and synthesis of vitamins (Gibson and Roberfroid, 1994). Therefore the balance of bacterial types within the gut is imperative in terms of overall GI health and colorectal cancer risk (Rafter et al., 2007). Lactic acid bacteria (LAB) have been shown to be an effective chemopreventative food ingredient against many cancer types (Jong-Eun et al., 2007). Probiotics, specifically LAB, e.g. *Lactobacillus* sp. and *Bifidobacterium* sp. have well defined gut survival properties and associated biological activities, and can be ingested in fermented milk products or supplements (Rafter, 2002). Epidemiological studies show that consumption of large quantities of dairy products

(yoghurt, and fermented milk products) containing *Lactobacillus* sp. or *Bifidobacterium* sp., may be related to a lower incidence of colon cancer (Rafter, 2002).

LAB are characterized as Gram-positive, nonsporing, nonrespiring, catalase negative cocci or rods that produce lactic acid during fermentation of carbohydrates within the large intestine (Fooks and Gibson, 2002; Jong-Eun et al., 2007). The LAB species *Lactobacillus* and *Bifidobacterium* are commonly used probiotic bacteria chosen specifically for their ability to withstand gastric and bile digestion (Teitelbaum and Walker, 2002). The survival of LAB through the gastrointestinal tract and colonization of the colon is due to decreased sensitivity to low stomach pH and production of bile salt hydrolase (BSH) (Lambert et al., 2008; Moser and Savage, 2001; Teitelbaum and Walker, 2002). The *Lactobacillus* species are divided into three groups that are based on fermenting abilites, these include: obligately homofermentative species, facultatively heterofermentative species, and obligately heterofermentative species (Dicks et al., 2000). Some *Lactobacillus* strains have been shown *in vitro* to exhibit adhesive factors that aid in their ability to interact with human enterocytes and inhibit potential pathogenic bacteria (Rambaud et al., 2007). *Bifidobacterium* is not related to LAB phylogenetically and is known as a saccharolytic bacterium that uses a unique pathway for sugar metabolism (Jong-Eun et al., 2007; Teitelbaum and Walker, 2002). *Bifidobacterium* accounts for approximately 25% of total bacteria present in the adult colon, it also accounts for 95% of total bacteria in breastfed newborns.

Foods known as prebiotics contain protective factors such as inulin-type fructans and other non-digestible food ingredients that enhance the production of fecal compounds such as short chain fatty acids (SCFA) that have anti-cancer properties (Kindler et al.,

2004; Pool-Zobel and Sauer, 2007). Gibson and Roberfroid defined a prebiotic in 1995 as a "non-digestible food ingredient which beneficially affects the host by selectively stimulating the growth of and/or activating the metabolism of one or a limited number of health promoting bacteria in the intestinal tract (Gibson and Roberfroid, 1995; Teitelbaum and Walker, 2002). Prebiotics benefit the host by increasing the growth and activity of *Bifidobacteria* and *Lactobacilli*, and decreasing the numbers of bacteroides, fusobacteria and clostridia, this effect is known as the 'bifidogenic' nature or prebiotic effect (Burns and Rowland, 2000; Fooks and Gibson; 2002; Gibson and Roberfroid, 1995; Pool-Zobel, 2005; Rafter et al., 2007; Rastall, 2004). Foods such as garlic, onion, artichoke, chicory root, and asparagus have high levels inulin-type fructans (Pool-Zobel, 2005). When fermented in the gut by commensal bacteria or probiotics to SCFA such as lactate, acetate, propionate, and butyrate, these combined can counteract the effects of carcinogens and tumorgenic cell growth (Fooks and Gibson, 2002; Gibson and Roberfroid, 1995; Pool-Zobel, 2005; Pool-Zobel and Sauer, 2007; Rafter et al., 2007). The use of synbiotics (probiotics and prebiotics combined) can result in a synergistic effect on overall gastrointestinal function (Burns and Rowland, 2000; Fooks and Gibson, 2002). Live microbial additions combined with a specific substrate for growth such as fructooligosaccharide and *Bifidobacterium* can result in improved survival of the probiotic as well as individual advantages of each strain (Burns and Rowland, 2000; Fooks and Gibson, 2002).

METASTASIS

Despite advances in surgical technique, patient care, prevention, and local and systemic adjuvant therapies, death by colorectal cancer is generally a result of metastasis of the primary tumor to other tissues of the body, primarily the liver and lungs (Langley and Fidler, 2007; Poste and Fidler, 1980; Zucker and Vacirca, 2004). With one of the worst prognoses of distant cancer metastasis, colorectal cancer patients diagnosed with metastatic disease have a median survival rate of 1 year with only a 4% survival rate of 5 years (Centers for Disease and Prevention, 2009; National Cancer Institute, 2009).

Metastasis is a process in which malignant cells are released from the primary tumor and invade through the extracellular matrix and migrate to distant areas of the body forming new colonies. The formation of these new tumor populations in other tissues of the body (metastasis) is the cause for 90% of human cancer deaths (Hanahan and Weinberg, 2007; Poste and Fidler, 1980).

Metastasis involves a series of sequential steps in which the initial first step in the development of metastasis is the seperation of single tumor cells from the primary tumor (Bohle and Kalthoff, 1999) (Fig 1.2.). The separation involves the disruption of intercellular/extracellular adhesions (E-cadherin/catenin complex) and tight junctions (occludin, ZO-1, ZO-2, ZO-3, claudin), followed by escape from anoikis (Bohle and Kalthoff, 1999). Once detached the metastatic cell can then invade the extracellular matrix (ECM), a dense latticework of collagen and elastin, embedded in a ground viscoelastic substance (Bohle and Kalthoff, 1999). The ECM must first be degraded in order to allow migration and invasion of the metastatic cell (Bohle and Kalthoff, 1999; Poste and Fidler, 1980; Yoon et al., 2003; Zucker and Vacirca, 2004). In the context of

cancer, the degradation of the extracellular matrix is a key event in tissue invasion and metastasis (Waas et al., 2002). Therefore understanding its role in tumor cell invasion is imperative.

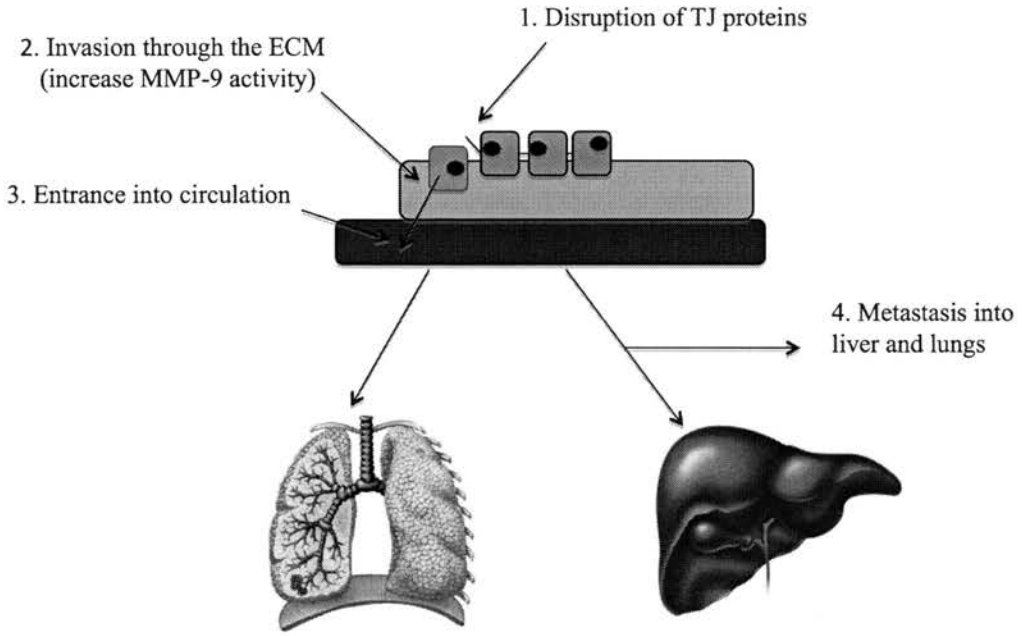

Figure 1.2. Schematic illustration of colon cancer metastasis (Bohle and Kalthoff, 1999; Hanahan and Weinberg, 2007).

The Extracellular Matrix

Tissues within the human body are comprised mainly of extracellular space and cells. Cells residing within these tissues secrete molecules that interact and form a complex network known as the extracellular matrix (ECM) (Rhodes and Simons, 2007). As cells vary within tissues, so does the ECM (Rhodes and Simons, 2007). Connective tissue for example harbors very few cells and mostly ECM, whereas the epithelium

contains cells tightly packed together resting on a thin sheet of ECM (Rhodes and Simons, 2007).

The ECM plays several important physiological roles in the human body such as providing structural support, a guide for the production and assembly of specific tissues, and direct signaling to specific cells, allowing for adaptive responses from environmental stimuli (Rhodes and Simons, 2007).

The ECM is formed primarily of two classes of molecules, the fibrous proteins and glycoaminoglycans (GAGs). The fibrous proteins consist of collagen, laminin, and elastin, and make up the scaffold that is embedded in GAGs (Rhodes and Simons, 2007). The chemical structure of GAGs allow them to be hydrated, while giving them gelatinous properties that provide a 'ground substance' allowing for the diffusion of vital nutrients between the blood and tissue (Rhodes and Simon, 2007). Assembling of the ECM is only one aspect of ECM formation, cells must also attach to the matricellular environment (Rhodes and Simon, 2007). A majority of animal cells bind to the ECM by utilizing anchoring proteins known as integrins (Rhodes and Simon, 2007).

Connective and epithelia tissue integrity depends on the balance between degradation and repair of the ECM, this is also important in its role in colon cancer metastasis (Loftus et al., 2002; Waas et al., 2002). The activity of matrix metalloproteinases; proteolytic enzymes, is the rate-limiting step in ECM degradation and an important step in tissue invasion and metastasis (Loftus et al., 2002; Waas et al., 2002).

Matrix Metalloproteinases

Tissue invasion and metastasis is dependent on malignant cell invasion through the colonic epithelial basement membrane, a process mediated by the degradation of the ECM. The degradation of the ECM involves a group of secreted matrix-degrading proteases known as matrix metalloproteinases (MMPs) that degrade the ECM during the metastatic process (Bohle and Kalthoff, 1999; Hanahan and Weinberg, 2007; Yoon et al., 2003; Zucker and Vacirca, 2004).

These zinc-dependent proteinases are classified by their substrate activity, amino acid sequence, and protein structure (Leeman et al., 2003; Yoon et al., 2003). MMPs are synthesized as pre-proenzymes (mostly secreted from the cell as proenzymes) and belong to a large group of proteins capable of degrading a majority, if not all the components within the extracellular matrix structure (Bohle and Kalthoff, 1999; Leeman et al., 2003). Under normal physiological conditions MMPs are involved in a variety of functions such as embryonic development, wound repair, ovulation, bone remodeling, macrophage function, and neutrophil function (Yoon et al., 2003). Their activity is controlled by specific inhibitors, such as tissue inhibitors of metalloproteinases (TIMP), however, in malignant diseases, MMPs can be overexpressed and TIMPs underexpressed, leading to increased proteolytic activity (Bohle and Kalthoff, 1999).

MMP-9, a gelatinase has been implicated in playing a significant role in colorectal cancer invasion and metastasis (Kumar et al., 2000; Zucker and Vacirca, 2004). Over expression of MMP-9 has been correlated with increased activity and synchronous metastasis in colorectal cancer, furthermore inhibition of MMP-9 has been shown to decrease proliferation of tumor cells in a three-dimensional collagen matrix

(Kumar et al., 2000; Leeman et al., 2003; Waas et al., 2002; Yu and Stamenkovic, 1999; Zucker and Vacirca, 2004).

Tight Junction: Zonula Occluden 1

Tumor formation and metastasis has been associated with loss of epithelial barrier function, specifically tight junction (TJ) structures in epithelial cancers (Martin et al., 2007; Soler et al., 1999). Increased permeability across the epithelial mucosa, arises from a weakening of TJs (Commane et al., 2005). Epithelial cells lining the lumen of the digestive tracts form cell-cell interactions that are mediated by junctional complexes comprised of tight junctions, adheren junctions, desmosomes and gap junctions (Martin el al., 2007; Yu and Yang, 2009). TJs contain both transmembrane proteins (occludin, claudin, and junctional adhesion molecule) and cytoplasmic plaque proteins (zona occludens: ZO-1, ZO-2 and ZO-3, cingulin, and 7H6) that are directly involved in the formation of paracellular occlusions on the apical side of intestinal epithelial cells (Martin et al., 2007; Yu and Yang, 2009) (Fig. 1.3.). TJs regulate in large part epithelial barrier function by the apical intercellular junctions formed, occluding the extracellular space between cells (Yu and Yang, 2009). Apart from maintaining epithelial cell-cell adhesion, TJs also maintain both internal and external electrical gradients preventing permeation by ions and larger solutes, play a role in cell differentiation and maturation, as well as regulate paracellular permeability and invasion by pathogenic microorganisms (Madara 1998; Martin et al., 2007, Yu and Yang, 2009). Studies have indicated an association between increased TJ permeability of the colon epithelium and tumor progression. The loss of TJ integrity has also been implicated as one of the factors in

breast cancer progression, specifically loss of ZO-1 and ZO-2 (Martin et al., 2007; Soler et al., 1999).

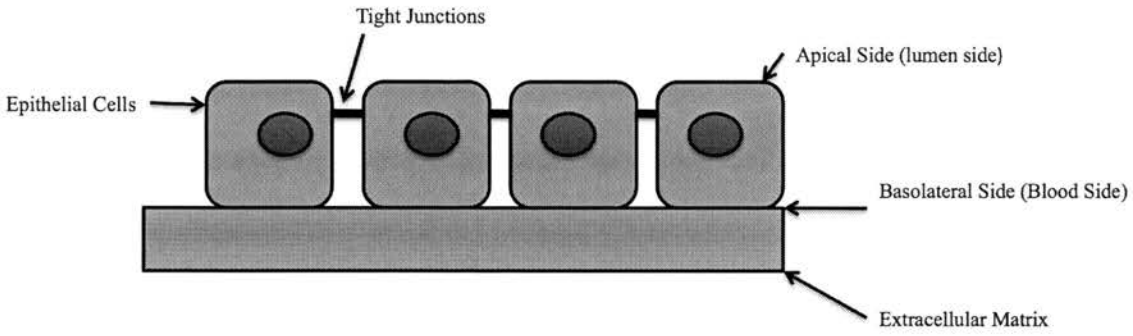

Figure 1.3. Epithelial cell structure within the intestinal lumen (colon).

ALTERNATIVE THERAPIES FOR METASTASIS PREVENTION AND CONTROL

The use of alternative therapies other than conventional medicine for the treatment of cancer has been gaining momentum and popularity internationally (Astin, 1998; Paltiel et al., 2001; Richardson et al., 2000). This phenomenon can be attributed to the dissatisfaction of cancer patients with conventional treatment due to their intolerance of more aggressive chemotherapies, adverse effects, high costs, and ineffectiveness (Astin, 1998; Hurwitz et al., 2005). Both *Lactobacillus* sp. and *Bifidobacterium* sp. have shown tremendous potential as chemopreventive agents against colorectal cancer (Burns and Rowland, 2000; Commane et al., 2005; Fooks and Gibson, 2002; Rafter et al., 2007; Rastall, 2004; Wollowski, et al., 2001). For example these LAB have been shown to prevent carcinogen-induced preneoplastic lesions and tumors, decrease pro-carcinogenic enzymes, modulate intestinal microflora and metabolism, and provide anti-genotoxic activity (Burns and Rowland, 2000; Commane et al., 2005; Rafter et al., 2007; Wollowski

et al., 2001). To date several studies examining *Lactobacillus* sp. and *Bifidobacterium* sp. *in vivo* and *in vitro* have shown positive chemopreventive effects at initiation and early stages of colorectal cancer. However, currently no data exists on the potential effects of *Lactobacillus* and *Bifidobacterium* as inhibitors of the metastatic process in later stages of colorectal cancer.

LITERATURE RELEVANT TO THE APPLICATION OF PROBIOTICS IN COLON CANCER METASTASIS

Previous *in vitro* and *in vivo* studies have examined the effects of *Lactobacillus* sp. and *Bifidobacterium* sp. on various human carcinoma cell and animal models. A study done by Moorthy et al. demonstrated the protective role of *Lactobacillus rhamnosus* and *Lactobacillus acidophilus* in *Shigella dysenteria* 1-induced diarrhea in rats. *Shigella dysenteria* 1 alone increased levels and activity of MMP-9, however when combined with both *Lactobacillus* sp., MMP-9 levels and activity were attenuated. Furthermore, in a study done by Ulisse et al., patients were given a probiotic mixture VSL#3 containing a mixture of both *Lactobacillus* sp. and *Bifidobacterium* sp. prior to collecting pouch biopsy samples. Patients given the VSL# 3 regimen had less tissue levels of inflammatory cytokines and increased levels of IL-10 (anti-inflammatory cytockine), as well as decreased activity of MMP-9.

A study done by Miyauchi et al. examined the effects of *L. rhamnosus* on impaired intestinal barrier function and paracellular permeability in Caco-2 cells and BALB/c mice. *In vitro* results showed a significant increase in transepithelial resistance (TER) (an indicator of tight junction permeability), an increase in ZO-1 expression, and a decrease in IL-8 (chemotactic cytokine) in cells treated with *L. rhamnosus* in the presence

of TNF-α (inflammatory cytokine) (Baggiolini et al., 1994; Miyauchi et al., 2009). *In vivo* results showed similar effects. Mice treated with both live and heat killed bacteria showed a decrease in myeloperoxidase activity (indicator of inflammation) in the distal colon, and an increase in ZO-1 expression (Miyauchi et al., 2009).

Commane et al. 2005 further examined the method by which probiotic bacteria aid in epithelial integrity and increased TER by examining the various metabolites formed during bacterial fermentation. *L. rhamnosus GG, L. plantarum, L. casei Shirota, B.Bb 12, Enterococcus faecium,* and *B. lactis* sp. *420* were grown in select carbohydrates. Metabolites produced during bacterial fermentation were identified through gas chromatography; short chain fatty acids (SCFA) lactate and acetate were observed in high concentrations in all bacterial fermentations (Commane et al., 2005). Both lactate and acetate treatments showed a significant increase in TER in treated Caco-2 cells. Lactate resulted in a dose-dependent increase in TER with values significantly above those found in untreated cells (Commane et al., 2005). Similar dose-dependent significant increases in TER were also observed with acetate treatments. Interestingly, there was a poor correlation between optical density of final bacterial cell counts and their effects on TER, demonstrating that bacterial metabolites and not bacterial numbers affect TER (Commane et al., 2005).

Ewaschuck et al. (2008) further elucidated the effects of bioactive metabolites produced by bacteria by examining *Bifidobacteria infantis in vitro* and *in vivo. B. infantis* was studied to determine how metabolites released from *B. infantis* influence epithelial permeability, tight junction proteins, and whether these secreted metabolites retain their bioactivity when administered to IL-10 deficient mice (Ewaschuck et al.,

2008). *In vitro* studies using *B. infantis* conditioned media (BiCM) showed an increase in TER and ZO-1 protein levels. BiCM also prevented a decrease in TER and ZO-1 protein levels in the presence of inflammatory cytokines TNF-α and IFNγ. Oral administration of BiCM in IL-10 deficient mice decreased inflammation and normalized colonic permeability (Ewaschuck et al., 2008).

OBJECTIVES

The long-term goal of our research is to investigate the ability of probiotic bacteria to prevent colon cancer metastasis and investigate the mechanisms behind it. Previous studies done *in vitro* and *in vivo* using probiotic bacteria have demonstrated a decrease in MMP-9 protein levels and activity as well as an increase in barrier function by improving tight junction integrity and increasing expression of the protein zona occludens-1 (ZO-1) (Commane et al., 2005; Ewaschuk et al., 2008; Miyauchi et al., 2009; Moorthy et al., 2007; Ulisse et al., 2001). We explored if these observations could translate into an anti-metastatic role for probiotic bacteria in the context of cancer.

The objectives of the proposed study were to investigate if probiotic cell-free supernatants (CFS) from selected probiotic bacteria in this study: (1) decrease cell invasion, (2) decrease MMP-9 protein levels and activity, and (3) promote colonic epithelial barrier function. Our central hypothesis is that probiotic bacteria will decrease colon cancer cell invasion by promoting colonic epithelial barrier function and decreasing MMP activity. This hypothesis is based on previous studies investigating the overall effects of probiotic bacteria on gastrointestinal health in relation to colorectal cancer.

The proposed research will examine the ability of probiotic CFS to decrease metastasis resulting in a novel approach in the non-invasive chemotherapeutic treatment of colorectal cancer. The results of the proposed research may lead to the development of new preventive and treatment approaches for colorectal cancer that incorporate probiotic metabolites and help understand the targets of probiotic action relevant to metastasis prevention.

II. CELL-FREE SUPERNATANTS FROM PROBIOTIC LACTOBACILLUS SP. AND BIFIDOBACTERIUM SP. DECREASE COLON CANCER CELL INVASION

ABSTRACT

Probiotics have been shown to have chemopreventative effects in early stages of colorectal cancer. Research however is lacking on how probiotics affect later stages of colorectal cancer, specifically metastasis. The objectives of the current study were to determine if probiotic CFS collected from *Lactobacillus* sp. and *Bifidobacterium* sp. inhibited colon cancer cell invasion and matrix metalloproteinase [(MMP), MMP-9] activity in the human colorectal carcinoma HCT-116 cell line *in vitro*. *Lactobacillus* sp. CFS and *Bifidobacterium* sp. CFS decreased cell invasion. *Lactobacillus* sp. CFS decreased MMP-9 protein levels and activity, whereas *Bifidobacterium* sp. had no effect on MMP-9 activity or protein levels. For preliminary investigation of the secreted bacterial factor responsible for a decrease in colon cancer cell invasion, *Lactobacillus* sp. CFS were fractionated into different defined molecular weight (MW) ranges and tested for cell invasion. Cell invasion was inhibited by fractions of MW > 3 kDa but not by small MW fractions of < 3 kDa. Further investigation of the > 3 kDa fraction revealed that the inhibitory activity was contained in the > 100 kDa and 50-100 kDa fractions, suggesting that the potential nature of the inhibitory compound was a protein, nucleic acid or polypeptide, rather than a small metabolite.

INTRODUCTION

Colorectal cancer accounts for approximately 10% of cancer related deaths in the United States among men and women (Jemal et al., 2009). At diagnosis, cancers are localized to the colon and in more complex cases metastasis to either the lymph nodes or distant organs, such as the liver and lungs occurs (Zucker and Vacirca, 2004). Metastasis is a process found among malignant cancer cells and involves a series of steps in which primary tumor cells are released, invading and migrating to distant areas forming new tumor populations (Hanahan and Weinberg, 2000; Poste and Fidler, 1980). Tissue invasion and metastasis is dependent on cell invasion through the extracellular matrix (ECM) and involves matrix metalloproteinases (MMPs) that degrade the ECM during the metastatic process (Hanahan and Weinberg, 2007; Zucker and Vacirca, 2004). Excess ECM degradation is one of the hallmarks of cancer and an important step in tumor progression (MCCawley and Matrisian, 2000). MMPs play a key role in promoting tumor invasion by inducing proteolysis of many of the components of the ECM (McCawley and Matrisian, 2000; Egeblad and Werb, 2002; Yu and Stamenkovic, 1999). MMPs are zinc-dependent proteinases that currently consist of over24 members that are classified according to their modular domain structure and ECM substrate specificity (Egeblad and Werb, 2002; McCawley and Matrisian, 2000; Yoon et al., 2003).

It has been demonstrated that poor prognosis in many human cancers are correlated with increased activity of MMP-9 (Kumar et al., 2000; Zeng et al., 2006). MMP-9, a gelatinase has also been associated with significant shorter disease-free and overall survival in patients with colorectal carcinoma (Kumar et al., 2000, Zeng et al.,

1996). Increased MMP-9 mRNA expression has also been demonstrated from early colorectal carcinoma through invasive and metastatic stages (Park et al., 2007; Yoon et al., 2003; Zeng et al., 1996).

Epidemiological studies have shown that diet plays a large role in the etiology of most large bowel cancers, therefore implying that it is a potentially preventable disease (Rafter, 2002). The colonic microflora has been suggested to have a key role in maintaining a healthy bowel, including decreasing the risk for developing colorectal cancer (Davis and Milner, 2009). Bacterial populations residing in the large intestine contribute many factors to the development of colonic tumors, this has led to the theory that endogenous bacterial populations are involved in the formation of carcinogens, tumor promotors and anti-carcinogens in the gut (Gill and Rowland, 2002). Thus, considerable attention has focused on dietary supplements that can modulate the large intestine microlfora and its activities as a preventative approach to colorectal cancer (Burns and Rowland, 2000; Commane et al., 2005; Fooks and Gibson, 2002; Gibson and Roberfroid et al., 1994; Gill and Rowland, 2002; Rafter, 2002; Rowland et al., 1998; Wollowski et al., 2001).

Previous studies have provided evidence that manipulating the large intestine microbiota with probiotics can influence host health (Ng et al., 2009). In addition, evidence supporting the protective role of probiotics, specifically lactic acid bacteria (LAB) against the development of colon tumors has been demonstrated (Davis and Milner, 2009; Gill and Rowland, 2002; Rafter, 2002; Wollowski et al., 2001). Specific strains of bacteria have been implicated in the pathogenesis of large bowel cancers, these include: *Steptococcus bovis, Bacteriodes, Clostridia* and *Helicobacter pylori* (Davis and

Milner, 2009; Moore and Moore, 1995; Rastall, 2004). Conversely, LAB strains, including *Lactobacillus acidophilus* and *Bifidobacterium longum* have been shown to inhibit carcinogen-induced colon tumor development (Davis and Milner, 2009; Moore and Moore, 1995; Rafter, 2002; Rastall, 2004; Rowland et al., 1998). Therefore, a balance between "detrimental" and "beneficial" bacteria may play a role in the development of colorectal cancer (Davis and Milner, 2009; Rastall, 2004).

The protective role of probiotics has primarily been implicated in preventing colorectal carcinogenesis; however no data have been reported regarding the effects of LAB on later stages of colorectal cancer, specifically metastasis. Previous studies, outside the context of cancer, have shown that probiotic LAB *Lactobacillus* sp. can influence MMP-9 by attenuating protein levels and activity (Moorthy et al., 2007; Stamatova et al., 2007; Ulisse et al., 2001). Therefore the objective of our study is to determine the effects of probiotic cell free supernatants (CFS) on colon cancer cell invasion and MMP-9 activity *in vitro* using the HCT-116 cell line as a model.

MATERIALS

Cell lines and bacteria

The human colon carcinoma cell line HCT-116 and bacterial strains *Lactobacillus casei* (ATCC 334), *Lactobacillus rhamnosus* (ATCC 53103), *Bifidobacterium longum* (ATCC 15707), and *Bacteroides thetaiotaomicron* (ATCC 29148) were obtained from American Type Culture Collection (ATCC) (Manassas, VA). The bacterial strain *Bifidobacterium lactis Bb12* (2816638) was obtained from CHR Hansen (Denmark).

Size-Fractionation of bacterial cell-free supernatants (CFS)

Vivaspin Columns (Sartorius Stedim Biotech, France) were used for the fractionation of bacterial CFS.

Cell Invasion

For the analysis of *in vitro* cell invasion, Matrigel-coated Boyden chambers (Becton Dickinson, Franklin Lakes, NJ) were used.

MMP-9 Zymography

Centricon 30 kDa MWCO columns (Millipore, Temcula, CA) were used for the concentration and collection of extracellular MMP-9 protein. For the purpose of protein quantitation Bradford Reagent (Bio-Rad Laboratories, Hercules, CA) was used. For analysis of MMP-9 enzyme activity, Zymogram Developing Buffer (Invitrogen, Carlsbad, CA) was used to develop zymogels.

MMP-9 Western Blot

Blotto (Santa Cruz Biotechnology, Santa Cruz, CA) dissolved in tris buffered saline (TBS) (Boston BioProducts, Ashland, MA) was used for blocking non-specific antibody-binding. Primary anti-MMP-9 (#AB19016, Millipore, Temcula, CA) antibody and its corresponding secondary antibody, IgG-HRP goat-anti-rabbit (Millipore, Temcula, CA) were used for the detection of extracellular MMP-9 protein levels. Pierce Horseradish Peroxidase Super Signal West Pico Chemiluminescent Substrate Kit (Rockford, IL) was used as a chemiluminescence for the analysis of extracellular MMP-9.

METHODS

Tissue Culture

The HCT-116 human colon carcinoma cell line was grown in Dulbecco's modified Eagle's medium (DMEM) in a humidified atmosphere at 37°C with 5% CO_2. Media was supplemented with 10% fetal bovine serum (FBS) and antibiotics (1,000 U/ml penicillin and 1,000 g/ml streptomycin).

Bacterial Culture

L. casei, L. rhamnosus, B. longum, and *B. lactis* were inoculated separately in De Man, Rogasa, Sharpe medium (MRS) and grown statically overnight to an optical density (OD) of 0.7-0.8. *B. thetaiotaomicron* was inoculated in Brain Heart Infusion (BHI) medium and grown statically for 48 h-72 h to an OD of 0.7-0.8. This OD corresponds to bacterial numbers of ~10^9 cfu/ml, as determined by plate counting using MRS plates for *Lactobacilli* sp. and *Bifidobacterium* sp. and BHI plates for *B. thetaiotaomicron*. The bacterial density is representative of gut flora density within the large intestine (10^9-10^{11} cfu/ml) (Rambaud et al., 2007).

CFS Preparation

Bacterial CFS were prepared by centrifugation at 4000 *x*g, for 10 min, at 4°C of log-phase cultures (OD~0.8) of *L. casei, L. rhamnosus, B. longum, B. lactis,* and *B. thetaiotaomicron* grown in their respective growth media, followed by filtration through a 0.2 μm membrane. Collected bacterial CFS were stored in single use aliquots at -80°C until needed.

Cell Invasion

The effect of *L. casei, L. rhamnosus, B. longum, B. lactis,* and *B. thetaiotaomicron* CFS on HCT-116 cell invasion was determined using Matrigel-coated Boyden chambers as described previously (Park et al., 2007). HCT-116 cells were serum starved for 48 h before seeding at a density of 1 X 10^5 cells per well in a 24-well plate. The upper chamber contained either *L. casei*, *L. rhamnosus*, *B. longum*, *B. lactis*, or *B. thetaiotaomicron* CFS at 25% v/v in serum free DMEM. The same volume of uninoculated bacterial growth medium (MRS or BHI) was used at 25% v/v in serum free DMEM as each bacteria's respective control. An 8-μM pore-sized filter separated the cells from a lower chamber containing 10% FBS and 20ng/ml of hepatic growth factor (HGF), which served as chemoattractants. After 24 h, non-invading cells were removed from the top of the Matrigel coated filter separating the upper and lower chambers with a cotton swab. Cells that invaded through the Matrigel to the bottom of the membrane were fixed with methanol prior to staining with Wright's Stain. Stained cells were examined microscopically and counted in 10 random fields of view. To account for any influence of the CFS on numbers/viability of HCT-116 cells, duplicate growth curves were performed for each experiment where cells were incubated with CFS or control for the duration of the experiment (24 h) and then counted using a haemocytometer. These values were used for normalization of the data obtained from all invasion assays.

MMP-9 Zymography

Effect of bacterial CFS on MMP-9 activity was assessed by gelatin zymography as described in Park et al. (2007). HCT-116 cells were seeded at a density of 4 X 10^6 cells per 150-mm dish. Cells were washed with phosphate buffered saline (PBS) twice

and treated with CFS from *L. casei*, *L. rhamnosus*, *B. longum*, or *B. lactis* at 25% v/v in serum free DMEM for 24 h. The same volume of uninoculated bacterial growth medium in serum free DMEM was used as control. MMP-9 activity was measured in conditioned media concentrated using a Centricon column with a 30 kDa molecular weight cut off (MWCO). Extracellular MMP-9 protein concentrations were determined using the Bradford assay. Equal amounts of protein (50 μg) from each treatment were separated using a 0.1% gelatin/10% non-denaturing polyacrylamide electrophoresis gel. Following electrophoresis, gels were washed with 2.5% Triton X-100 for 30 min at room temperature, zymogram developing buffer for 30 min at room temperature, and then incubated overnight in zymogram developing buffer at 37°C. Gels were then stained with 0.25% Coomassie blue dye R250 and destained with 30% methanol and 10% acetic acid until clear bands appeared against a dark background. GE ImageQuant TL software was used for quantitation of the bands.

MMP-9 Western Blot Analysis

The effect of bacterial CFS on MMP-9 protein levels was assessed by western blot as described in Park et al. (2007). HCT-116 cells were plated and treated as described previously in zymography, above. Following treatment, conditioned medium was concentrated and protein concentration was measured as described above. Equal amounts of protein (50 μg) from each treatment were separated using an 8% SDS-PAGE gel and transferred to nitrocellulose membranes. Membranes were blocked with 5% Blotto in TBST (1 X TBS with 0.5% Tween-20) for 1 h at room temperature, followed by overnight incubation with anti-MMP-9 antibody at a 1:2000 dilution in 5% Blotto in TBST, and a 1 hr incubation with corresponding secondary antibody (IgG-HRP goat-anti-

rabbit) at a dilution of 1:2000 in 5% Blotto in TBST. Immunoreactivity was detected using a chemiluminescent substrate kit. Total protein in each lane was quantified by ponceau S staining and used for normalization of the MMP-9 protein levels measured by Western Blot. Quantitation was performed by densitometry using the GE ImageQuant TL software.

CFS Size-fractionation

For preliminary investigation of the bioactive compound in the bacterial CFS, ultrafiltration of the CFS was carried out through a 3,000 MWCO centrifugal concentrator to obtain fractions of > 3 kDa and < 3 kDa. The > 3 kDa was further fractionated by sequential ultrafiltration through membranes of pore sizes 100 kDa, 50 kDa, 30 kDa, 10 kDa, and 3 kDa to yield fractions of molecular weight > 100 kDa, 50-100 kDa, 30-50 kDa, 10-30 kDa, and 3-10 kDa, respectively. Uninoculated bacterial growth medium (MRS or BHI) treated similarly to CFS was used as control. Fractionated bacterial and control CFS were stored at -80°C until needed in single use aliquots.

Statistical Analysis

Values shown are the mean ± SEM of three independent experiments unless otherwise indicated. Data were analyzed in Excel (2008, Version 12.2.5) using two-tailed t-tests comparing each CFS treatment with control. A P value of < 0.05 was considered significant.

RESULTS

Lactobacillus sp. *and Bifidobacterium* sp. *CFS decrease the invasion of HCT-116 cells in vitro*

The ability of probiotic CFS to decrease colon cancer cell invasion through Matrigel-coated Boyden chambers was determined using HCT-116 human colorectal carcinoma cells (Park et al., 2007). The effects of four probiotic strains; two *Lactobacilli* (*L. casei* and *L. rhamnosus*) and two *Bifidobacteria* (*B. longum* and *B. lactis*) were examined. All strains tested decreased invasion with respect to control. Treatment of HCT-116 cells with CFS isolated from *L. casei* and *L. rhamnosus* significantly decreased cell invasion to 27.95 % ± 4.25 and 49.02% ± 17.65 of control, respectively (Fig. 2.1. A). Cells treated with *B. longum* CFS and *B. lactis* CFS also displayed a significant decrease in cell invasion to 38.83% ± 16.85 and 32.8% ± 6.64 of control, respectively (Fig. 2.1. B). These data indicate that secreted bioactive factors during bacterial growth and metabolism present in CFS are decreasing cell invasion.

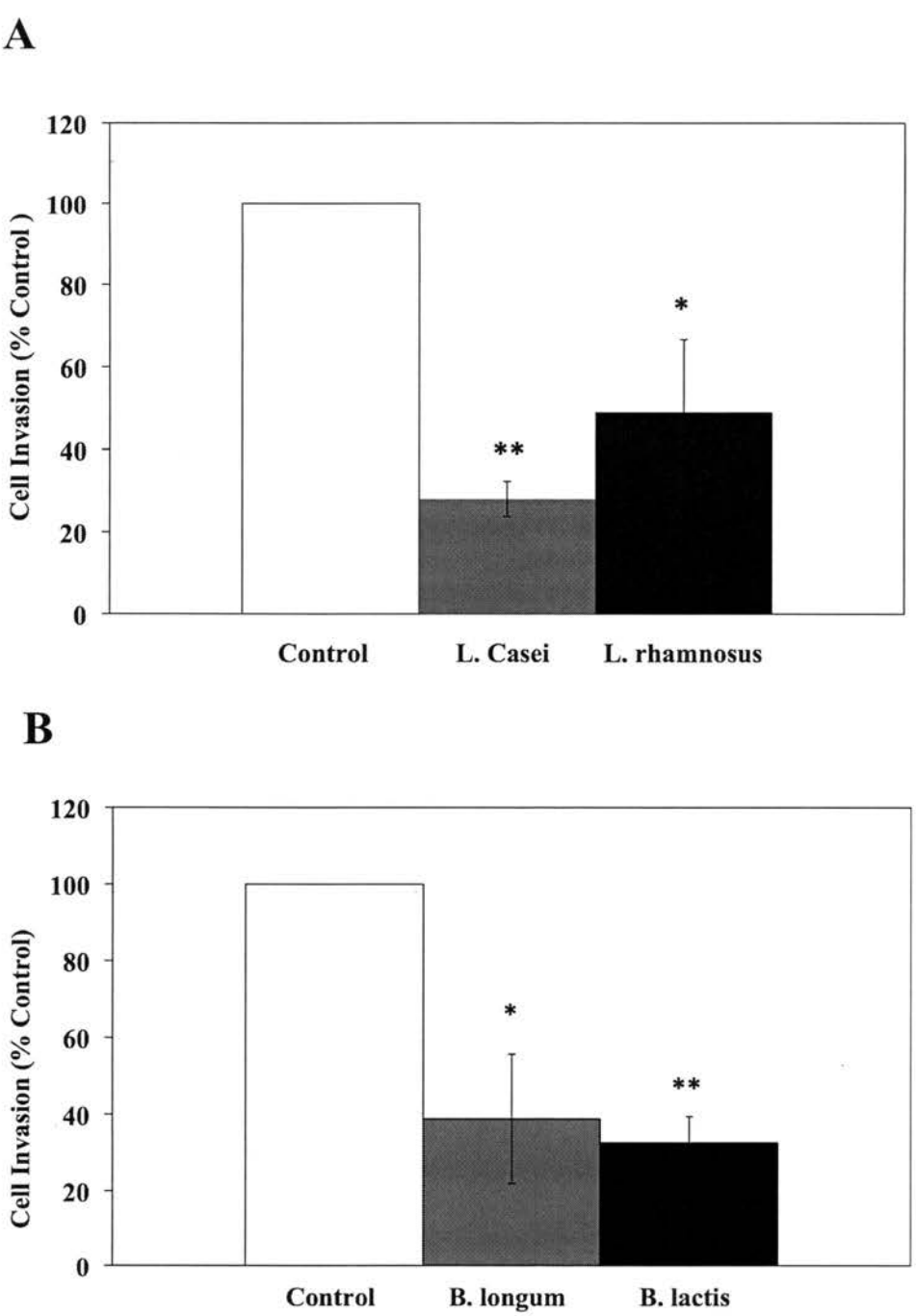

Figure 2.1. (A) *Lactobacillus* sp. and (B) *Bifidobacterium* sp. CFS decrease the invasion of HCT-116 cells *in vitro*. HCT-116 cells were serum starved for 48 h before seeding at a density of 1 x 10^5 cells/well into a 24-well Matrigel coated Boyden chamber. The cells were treated with probiotic CFS or control (uninoculated MRS broth) for 24 h and cell invasion was measured by counting the cells that migrated to the other side of the Matrigel coated membrane, as described in Methods. Cell invasion is expressed as percentage of control (uninoculated MRS broth) % invasion was then normalized to the effect of CFS on cell growth, as described in Methods. Data shown are mean ± SEM of n=3. *Significantly different from control ($P < 0.05$). **Significantly different from control ($P < 0.01$).

To demonstrate that the observed inhibition of cell invasion was specific to the probiotic strains tested in this study, the effect of a predominant commensal bacterium, *Bacteroides thetaiotaomicron*, on cell invasion was also investigated (Hooper and Gordon, 2001). HCT-116 cells were treated with *B. thetaiotaomicron* CFS at 25% v/v in serum free DMEM as described in Methods. *B. thetaiotaomicron* CFS treated cells did not exhibit a decrease in cell invasion, but rather a significant increase to 141.29% ± 13.66 of control (Fig. 2.2.). These data indicate that the observed decrease in cell invasion is exclusive to the probiotic strains tested and not gut commensal bacteria as represented by *B. thetaiotaomicron*.

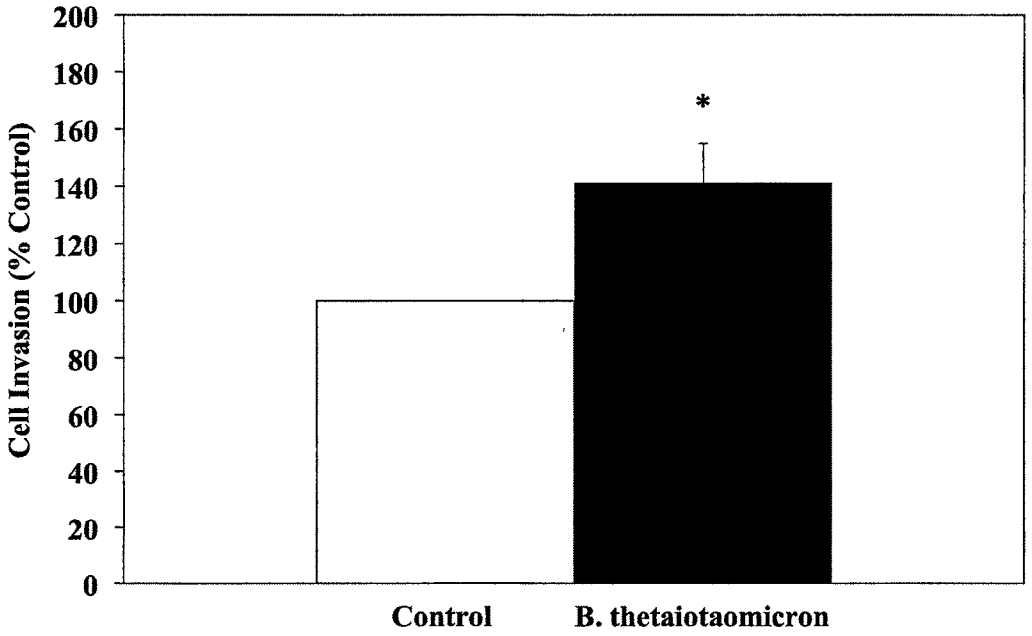

Figure 2.2. The commensal bacterium *Bacteroides thetaiotaomicron* CFS does not decrease cell invasion. HCT-116 cells were serum starved for 48 h before seeding at a density of 1 x 10^5 cells/well into a 24-well Matrigel coated Boyden chamber. The cells were treated with *B. thetaiotaomicron* CFS or control (uninoculated BHI broth) for 24 h and cell invasion was measured by counting the cells that migrated to the other side of the Matrigel coated membrane, as described in Methods. Cell invasion is expressed as percentage of control (uninoculated BHI broth), % invasion was then normalized to the effect of CFS on cell growth, as described in Methods. Data shown are mean ± SEM of n=5. *Significantly different from control ($P < 0.05$).

Lactobacillus* sp. *CFS decrease MMP-9 activity and protein levels

MMPs such as MMP-9 facilitate cell invasion by degrading the ECM during the metastatic process (Park et al., 2007). To determine whether probiotic CFS induced a decrease in cell invasion due to a decrease in MMP-9 activity, we analyzed MMP-9 activity by zymography. Both *Lactobacillus* species studies decreased MMP-9 enzyme activity. Treatment of HCT-116 cells with *L. casei* CFS tended to decrease enzyme activity (P= 0.08) to 89.63% ± 5.91 of control (Fig. 2.3. A.). Treatment of HCT-116 cells with *L. rhamnosus* CFS significantly decreased MMP-9 enzyme activity to 71.96% ± 1.37 of control (Fig. 2.3. A). Treatment of HCT-116 cells with *B. longum* and *B. lactis* CFS did not affect MMP-9 activity (Fig. 2.3. B). Taken together, these data indicate that the *Lactobacillus* sp. decrease cell invasion, at least in part, by influencing MMP-9 activity. The *Bifidobacterium* sp. could potentially be working via an alternative mechanism other than influencing MMP-9 activity to decrease cell invasion.

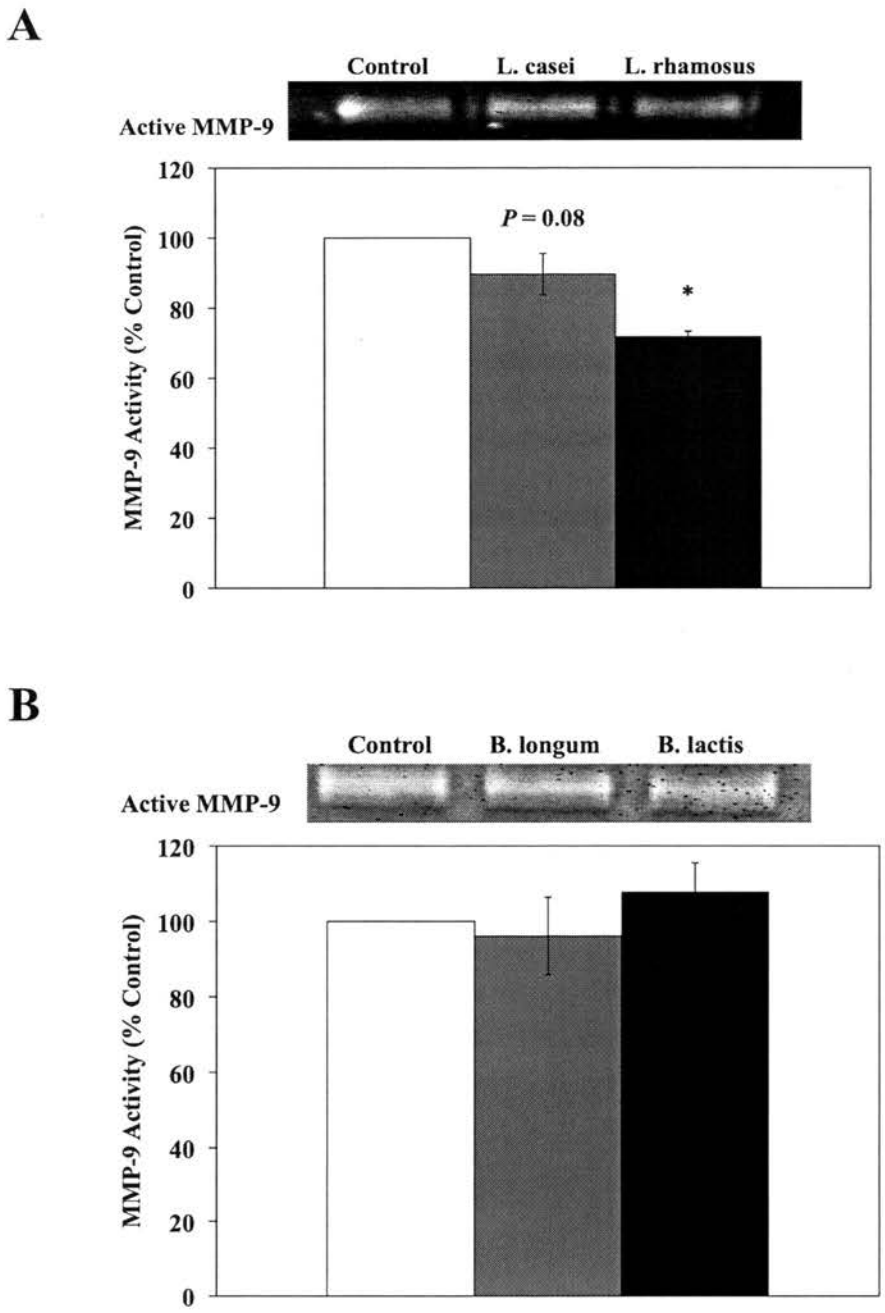

Figure 2.3. *Lactobacillus* CFS decrease MMP-9 activity. HCT-116 cells were treated with either *L. casei*, *L. rhamnosus*, *B. longum,* or *B. lactis* at 25% v/v in serum free DMEM. The same volume of uninnoculated bacterial growth medium (MRS Broth) in serum free DMEM was used as control. Conditioned media was collected and analyzed for enzyme activity, as described in Methods. MMP-9 activity was detected in conditioned media from cells treated with CFS from (A) *Lactobacillus* sp. and (B) *Bifidobacterium* sp. Data shown are mean ± SEM of n=3 (*Lactobacillus* sp.) and n=4 (*Bifidobacterium* sp.). *Significantly different from control ($P < 0.05$).

MMP-9 protein levels were analyzed via western blot (Park et al, 2007). Treatment of HCT-116 cells with *L. casei* CFS significantly decreased MMP-9 protein levels to 74.22% ± 6.72 of control (Fig. 7A). Treatment of HCT-116 cells with *L. rhamnosus* CFS significantly decreased MMP-9 protein levels to 61.38% ± 7.95 of control (Fig. 2.4. A). Treatment of HCT-116 cells with *B. longum* and *B. lactis* CFS did not affect MMP-9 protein levels (Fig. 2.4. B). Taken together these data indicate that the *Lactobacillus* sp. decrease cell invasion by decreasing MMP-9 protein levels, which translate to a decrease in MMP-9 activity. These data further validate that the effect of *Bifidobacteria* sp. on cell invasion is not mediated by an effect on MMP-9 protein levels or activity, but rather an alternative mechanism.

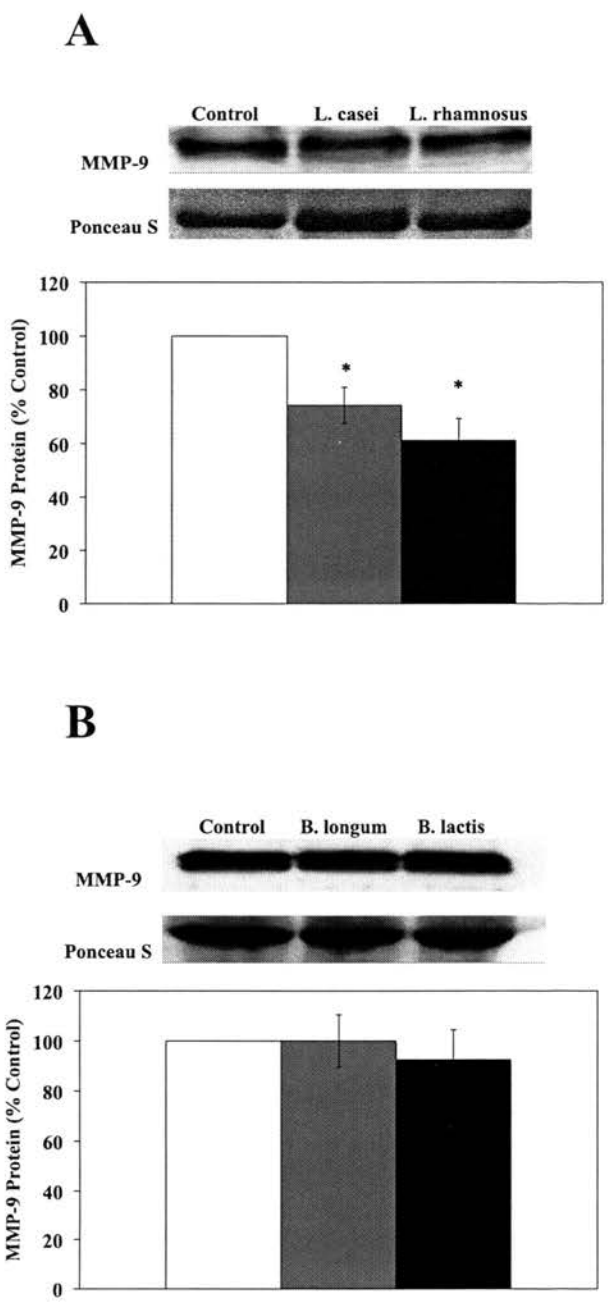

Figure 2.4. *Lactobacillus* CFS decrease MMP-9 protein levels. Effects of (A) *Lactobacillus* sp. and (B) *Bifidobacterium* sp. from CFS on MMP-9 protein levels. HCT-116 cells were treated with either *L. casei*, *L. rhamnosus, B. longum,* or *B. lactis* at 25% v/v in serum free DMEM. The same volume of uninoculated bacterial growth medium (MRS broth) in serum free DMEM was used as control. Proteins were collected from culture media as described in Methods. MMP-9 protein was detected in conditioned media and normalized using ponceau S as a loading control. Data shown are mean ± SEM of n=3 (*Lactobacillus* sp.) and n=5 (*Bifidobacterium* sp.). *Significantly different from control ($P < 0.05$).

Lactobacillus* sp. *CFS fractions decrease cell invasion via a bioactive compound of a molecular weight >3 kDa

For preliminary investigation of the nature of the secreted bacterial factor or metabolite responsible for a decrease in colon cancer cell invasion, *Lactobacillus* CFS were fractionated into a large molecular weight fraction > 3 kDa and a small molecular weight fraction < 3 kDa. *Lactobacillus* CFS fractions > 3 kDa and < 3 kDa collected from both species (*L. casei* and *L. rhamnosus*) were analyzed for their effect on colon cancer cell invasion using a Boyden Chamber Matrigel Invasion assay *in vitro*. Treatment of HCT-116 cells with the > 3 kDa fraction of *L. casei* and *L. rhamnosus* CFS resulted in a significant decrease in cell invasion to 29.97% ± 12.31 and 24.17% ± 10.15 of control, respectively (Fig. 2.5.). Fractions from *Lactobacillus* sp. < 3 kDa had no effect on cell invasion (Fig. 2.5.). These data indicate that the inhibitory compound present in the CFS is a large molecular weight bioactive molecule.

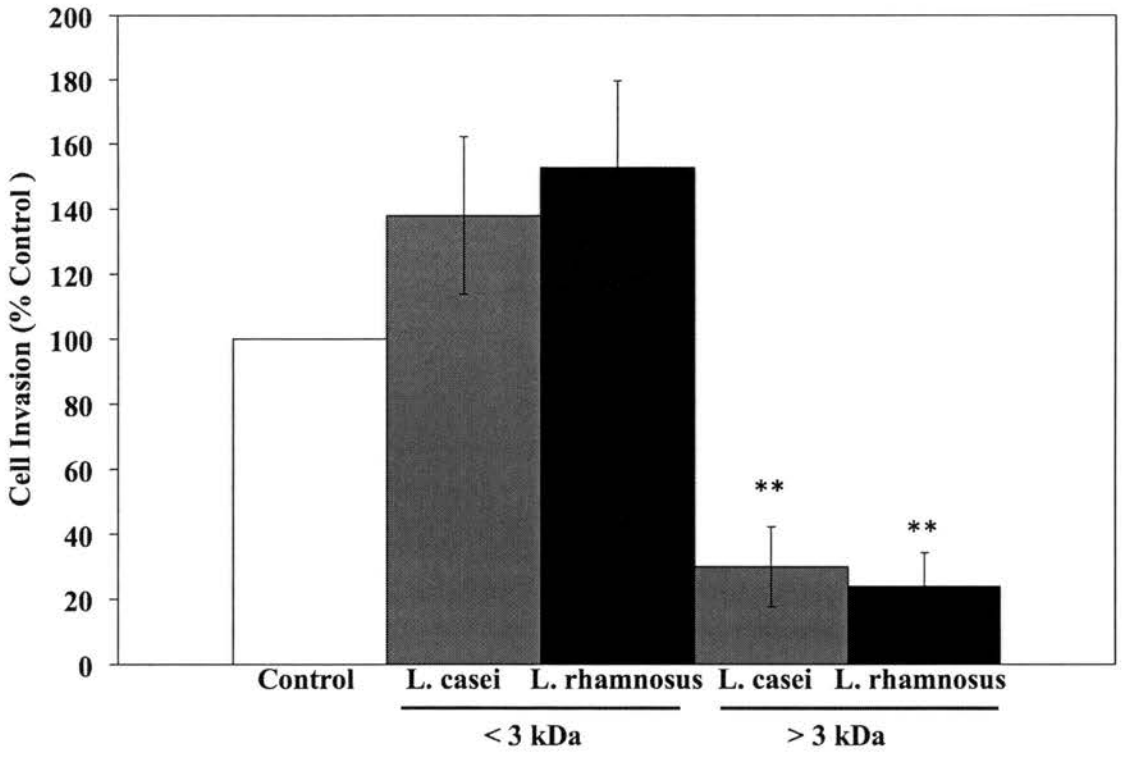

Figure 2.5. Inhibition of cell invasion by *Lactobacillus* sp. is mediated by a compound of molecular weight > 3 kDa. HCT-116 cells were serum starved for 48 h before seeding at a density of 1 x 10^5 cells/well into a 24-well Matrigel coated Boyden chamber. The cells were treated with fractionated CFS or control (uninoculated fractionated MRS broth) for 24 h and cell invasion was measured by counting the cells that migrated to the other side of the Matrigel coated membrane, as described in Methods. Cell invasion is expressed as percentage of control (respective uninoculated fractionated MRS broth), % invasion was then normalized to the effect of fractionated CFS on cell growth, as described in Methods. Data shown are mean ± SEM of n=4. **Significantly different from control ($P < 0.01$).

The decrease in cell invasion by Lactobacillus sp. ***CFS fractions is predominantly due to large molecular weight compounds of size >100 kDa and between 50-100 kDa***

To further characterize of the secreted bacterial factor or metabolite responsible for the decrease in colon cancer cell invasion, *Lactobacillus* CFS > 3 kDa were sub-fractionated into larger molecular weight fractions of > 100 kDa, 50-100 kDa, 30-50 kDa, 10-30 kDa, and 3-10 kDa. *Lactobacillus* CFS fractions > 100 kDa, 50-100 kDa, 30-50

kDa, 10-30 kDa, and 3-10 kDa were collected from both species (*L. casei* and *L. rhamnosus*) and their effect on colon cancer cell invasion was analyzed. Treatment of HCT-116 cells treated with the > 100 kDa fraction of *L. casei* and *L. rhamnosus* CFS resulted in a significant decrease in cell invasion to 45.97% ± 14.03 and 18.79% ± 3.65 of control, respectively (Fig. 2.6.). HCT-116 cells treated with the 50-100 kDa fraction of *L. casei* and *L. rhamnosus* CFS resulted in a significant decrease in cell invasion to 59.47% ± 23.82 and 35.71% ± 13.52 of control, respectively (Fig. 2.6.). Fractions from *Lactobacillus* sp. < 50 kDa had no effect on cell invasion. These data indicate that the bioactive compound found within the CFS could be a large molecular weight compound such as a nucleic acid, protein, or a polysaccharide.

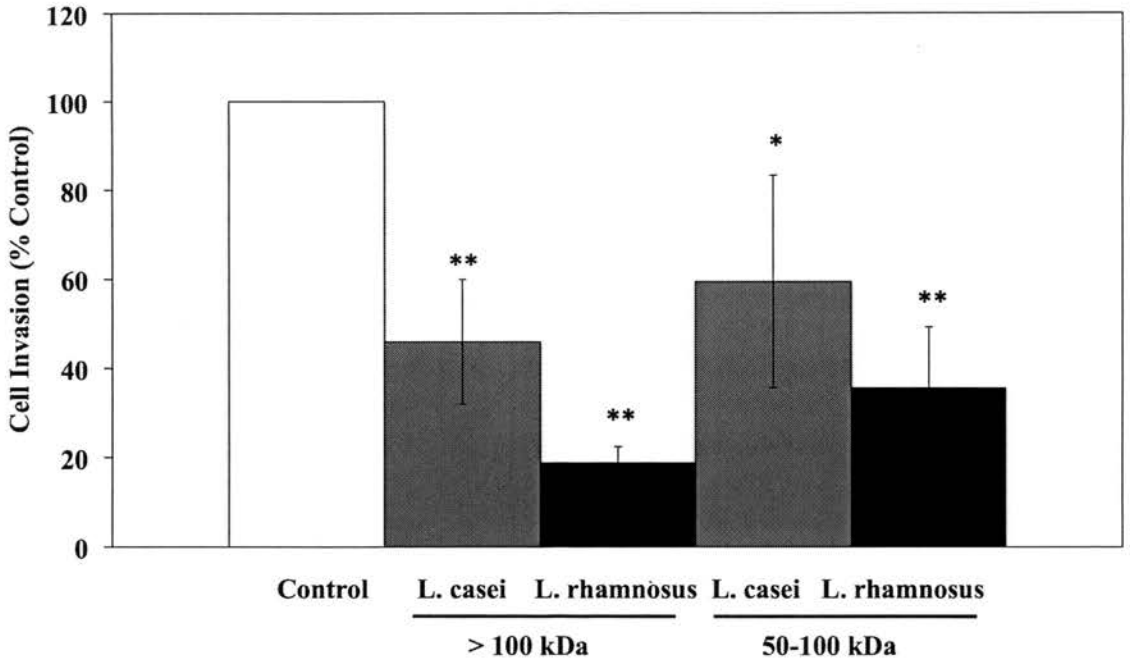

Figure 2.6. Inhibition of cell invasion by *Lactobacillus* sp. is mediated by secreted bacterial factors of molecular weight > 100 kDa and 50-100 kDa. HCT-116 cells were serum starved for 48 h before seeding at a density of 1 x 10^5 cells/well into a 24-well Matrigel coated Boyden chamber. The cells were treated with fractionated CFS or control (uninoculated fractionated MRS broth) for 24 h and cell invasion was measured by counting the cells that migrated to the other side of the matrigel coated membrane, as described in Methods. Cell invasion is expressed as percentage of control (respective uninoculated fractionated MRS broth), and normalized for cell growth, as described in Methods. Data shown are mean ± SEM of n=3 for *L. casei* fractions and n=4 for *L.rhamnosus* fractions. *Significantly different from control ($P < 0.05$). **Significantly different from control ($P < 0.01$).

DISCUSSION

Treatment with *Lactobacillus* sp. CFS showed a decrease in cell invasion with concomitant changes in MMP-9 activity. Decreases in cell invasion were also observed in the *Bifidobacterium* sp. however these effects were not mediated via MMP-9 activity. Fractionated *Lactobacillus* sp. CFS > 3 kDa, specifically > 100 kDa, and 50-100 kDa also demonstrated decreases in cell invasion.

This study demonstrates that secreted factors found in CFS collected from these LAB species decreases the invasion of the metastatic HCT-116 cell line *in vitro*. Furthermore, cell invasion was only observed with the probiotic bacteria tested in this study, and not with CFS from a non-probiotic commensal gut bacterium *B. thetaiotaomicron*. This suggests that the probiotic species tested secrete unique bioactive factors during their growth and metabolism that result in decreased cell invasion.

The activity of MMP-9, a matrix-metalloproteinase that has been positively correlated with increased tumor progression and colon cancer metastasis was examined (Hanahan and Weinberg, 2007; Park et al., 2007; Zucker and Vacirca, 2004). Decreased MMP-9 activity was observed in HCT-116 cells treated with the probiotic species *L. casei* and *L. rhamnosus*. Treatment of HCT-116 cells with the probiotic species *B. lactis* and *B. longum* had no effect on MMP-9 activity. Similar effects were also seen in MMP-9 protein levels. HCT-116 cells treated with *L. casei* and *L. rhamnosus* CFS exhibited decreased MMP-9 protein levels, whereas HCT-116 cells treated with *B. lactis* and *B. longum* did not show a decrease in MMP-9 protein levels. Although HCT-116 cell invasion decreased upon treatment with CFS from each species of probiotic bacteria, the observed results suggest that the effects of each bacterial sp. tested on cell invasion are not mediated solely by an effect on MMP-9 activity and protein levels. Thus, the cell invasion of HCT-116 cells treated with *B. lactis* and *B. longum* could be acting via an alternative mechanism. Members of the Rho family of small GTPases are involved in regulation of a variety of cellular processes that include, microfilament network, intercellular contact, and metastasis (Abecassis et al., 2003; Kamai et al., 2003). Over-expression of Rho has been linked to metastasis (Abecassis et al., 2003; Fritz et al., 1999;

Kamai et al., 2003). A potential mechanism by which *B. longum* and *B. lactis* may be mediating cell invasion could be through regulation of Rho proteins and their effect on metastasis.

To elucidate the bioactive factor responsible for inhibiting cell invasion, *Lactobacillus* sp. CFS were separated by molecular weight to a small biomolecule fraction (< 3 kDa) and one containing larger biomolecules (>3 kDa). The larger molecular weight fraction exhibited an inhibitory effect on cell invasion, fractions < 3 kDa had no effect on cell invasion. CFS > 3 kDa were further fractioned into molecular weight fractions of defined ranges, viz., > 100 kDa, 50-100 kDa, 30-50 kDa, 10-30 kDa, and 3-10 kDa. CFS fractions >100 kDa and 50-100 kDa exhibited a decrease in cell invasion, CFS < 50 kDa had no effect on cell invasion. The molecular weight of the inhibitory fractions suggests that the inhibitory bioactive compound may be a nucleic acid, protein, or a polysaccharide. *Lactobacilli* have been shown to secrete peptides and proteins that regulate intestinal epithelial cell survival and growth and such proteins could potentially be contributing to the decrease in cell invasion (Sanchez et al., 2009; Yan et al., 2007).

These secreted factors from probiotic CFS could potentially be used as chemopreventative treatments in colon cancer metastasis, however further studies are necessary to characterize these bioactive components.

III. CELL-FREE SUPERNATANTS FROM PROBIOTIC LACTOBACILLUS SP. INCREASE TIGHT JUNCTION PROTEIN, ZONULA OCCLUDEN-1

ABSTRACT

The promotional phase of colorectal tumor metastasis is mediated by permeability across the epithelial mucosa. Increased permeability arises from a weakening of the tight junctions, specifically Zona Occluden-1 (ZO-1). Increased epithelial permeability and downregulation of tight junctions has been associated with colorectal tumor metastasis. Probiotics have been shown to decrease intestinal permeability and improve epithelial barrier integrity by increasing ZO-1 protein levels. The objective of this study was to determine if probiotic CFS collected from *Lactobacillus* sp. and *Bifidobacterium* sp. could increase ZO-1 protein levels in the human colorectal carcinoma cell line HCT-116. *Lactobacillus* sp. CFS increased ZO-1 protein levels, whereas *Bifidobacterium* sp. had no effect on ZO-1 protein levels. In conclusion, *Lactobacillus* sp. CFS may exhibit a protective role in colon cancer by decreasing epithelial permeability via increased ZO-1 levels, as demonstrated in this study using a metastatic human colorectal cell line.

INTRODUCTION

The epithelial lining of the large intestine is a "first line of defense" barrier that prevents the diffusion of solutes, macromolecules, and microorganisms between apical and basolateral domains (Laukoetter et al., 2008; Putaala et al., 2008; Turner, 2006). The epithelial lining also provides high-resistance intercellular seals that prevent paracellular diffusion between cells (Putaala et al., 2008; Turner, 2006; Yu and Yang, 2008). The individual epithelial cells (enterocytes) are the main cell type that form this barrier and are regulated in large part by the apical intercellular junctions, referred to as the tight junctions (TJs) (Putaala et al., 2008; Turner, 2006; Yu and Yang, 2008). The TJs are the most critical paracellular seal, and are the rate-limiting step for paracellular transit (Turner, 2006). Permeability of the TJs characterizes the overall barrier function of an intact intestinal epithelium (Turner, 2006). TJs are comprised of both transmembrane and cytoplasmic plaque proteins that are complex lipoprotein structures that form fibrils that traverse the lateral plasma membrane to interact with proteins from the adjacent cell (Ewaschuk et al., 2008). The TJ transmembrane proteins include occludin, claudin, and junctional adhesion molecule (JAM); and the cytoplasmic plaque proteins include zona occludens ZO-1, ZO-2, and ZO-3, cingulin, and 7H6 (Yu and Yang, 2009). Disruption of epithelial barrier function has been seen in intestinal diseases such as Crohn's disease, irritable bowel disease (IBD) (both active and inactive), enteric infections, coeliac disease, and some autoimmune diseases, such as Type I diabetes (Mennigen et al., 2009; Ng et al., 2009; Turner, 2006). In individuals with IBD, redistribution and downregulation of several tight junction proteins has been observed (Mennigen et al., 2009). Epithelial tumor cells, particularly those that manifest high

metastatic potential have also been shown to exhibit loss of TJs; specifically ZO-1, ZO-2, and occludin were decreased during tumor formation and metastasis (Dhawan et al., 2005; Kaihara et al., 2003; Laukoetter et al., 2008; Tobioka et al., 2004).

The importance of the large intestinal microflora and its composition in physiological and pathophysiological processes within the human GI is becoming increasingly evident (Laukoetter et al., 2008). Interest within the public sector in the use of dietary supplements and functional foods containing probiotic bacteria for the maintenance of general GI health and the prevention or treatment of intestinal infections has grown over the years (Corr et al., 2009). Evidence from both *in vitro* and *in vivo* studies suggests that probiotics have a beneficial, strain-specific effect on the intestinal barrier (Laukoetter et al., 2008; Putaala et al., 2008; Corr et al., 2009). It has been hypothesized that probiotics preserve the epithelial barrier function (Mennigen et al., 2009). *In vitro* studies on epithelial monolayers showed that probiotics improve barrier function following *Escherichia coli* infection or incubation with proinflammatory cytokines (Czerucka et al., 2000; Mennigen et al., 2009). In addition, several studies have shown that probiotics preserve the intestinal epithelial barrier in several *in vivo* models (IL-10 knockout colitis or sepsis) by increased expression of ZO-1 as well as by attenuating the effects of inflammatory cytokines, TNF-α and IFN-γ (Ewaschuk et al., 2008, Mennigen et al., 2009; Miyauchi et al., 2009; Ng et al., 2009). Therefore the objective of the study is to determine the effects of probiotic cell free supernatants (CFS) on tight junction integrity *in vitro* using the HCT-116 cell line in the context of colon cancer metastasis.

MATERIALS

ZO-1 Western Blot

Primary anti-ZO-1 antibody (#SC2005, Santa Cruz Biotechnology, Santa Cruz, CA) and its corresponding secondary antibody, IgG-HRP goat-anti-rabbit (Millipore) were used for the detection of ZO-1 protein levels. Primary anti-β-actin antibody (#118K4827, Sigma, St. Louis, MO) and its corresponding secondary antibody, IgG-HRP goat-anti-mouse (Santa Cruz Biotechnology, Santa Cruz, CA) were used for the detection of β-actin. Pierce Horseradish Peroxidase Super Signal West Pico Chemiluminescent Substrate Kit (Rockford, IL) was used as a chemiluminescence for the analysis of ZO-1 protein levels.

Restore PLUS Western Blot Stripping Buffer (Thermo Scientific, Rockford, IL) was used to strip membrane of antibodies.

METHODS

CFS Preparation

Bacterial cell free supernatants (CFS) were prepared by centrifugation at 4000 *x*g, 10 min, 4°C of log-phase cultures (OD~0.8) of *L. casei,* and *L. rhamnosus* grown in their respective growth media, followed by filtration through a 0.2 μm membrane. Collected bacterial CFS were stored at -80°C until needed in single use aliquots.

ZO-1 Western Blot Analysis

To determine if the inhibition of cell invasion by probiotic CFS was mediated by alterations in epithelial permeability we assayed the effect of CFS on the level of the tight junction protein ZO-1 via western blot analysis. HCT-116 cells were seeded at a density

of 1 X 10^6 cells per 60-mm dish in DMEM. Cells were washed with PBS twice and treated with CFS from *L. casei*, *L. rhamnosus*, *B. longum*, or *B. lactis* at 25% v/v in DMEM for 24 h. The same volume of uninoculated bacterial growth medium was used at 25% v/v in DMEM. Following 24 h treatment with bacterial CFS, cell lysates were collected to measure intracellular ZO-1 levels. HCT-116 cells were lysed in urea extraction buffer (6M urea, 0.1% Triton X-100, 10 mM Tris buffer, 5 mM $MgCl_2$, 1 mM DTT, 5 mM EGTA, 150 mM NaCl, 0.2 mM PMSF). Protein was measured as before. Equal amounts of protein (50 μg) from each treatment were separated using an 8% SDS-PAGE gel with urea and transferred to nitrocellulose membranes. Membranes were blocked with 5% Blotto in TBST for 1 h at room temperature, followed by overnight incubation with anti-ZO-1 antibody at a 1:200 dilution in 5% Blotto in TBST and a 1 hr incubation with corresponding secondary antibody (IgG-HRP goat-anti-rabbit) at a dilution of 1:2000 in 5% Blotto in TBST. Membranes were then stripped using western blot stripping buffer, and probed for β-actin, with anti-β-actin antibody at a 1:5000 dilution in 5% Blotto in TBST. Membranes were then incubated with corresponding secondary antibody (IgG-HRP goat-anti-mouse) at a dilution of 1:2000 in 5% Blotto in TBST. Immunoreactivity was detected using a chemiluminescent substrate kit. ZO-1 levels were normalized to the levels of β-actin, and quantifed by densitometry using the GE ImageQuant TL software.

Statistical Analysis

Values shown are the average of the mean ± SEM of four independent experiments. Data were analyzed using two-tailed t-tests comparing each CFS treatment with control. A P value of < 0.05 was considered significant.

RESULTS

Lactobacillus sp. *CFS increase tight junction protein, ZO-1*

Tight junctions seal adjacent epithelial cells to control the passage of ions, water and other molecules between cells, and maintain epithelial cell polarity (Feldman et al., 2005; Soler et al., 1999). It has been demonstrated that TJ integrity is compromised in epithelial cancers, specifically in the promotional phase of colorectal cancer and metastasis (Commane et al., 2005; Dhawan et al., 2005; Feldman et al., 2005, Soler et al., 1999). To determine how probiotic CFS could potentially play a role in increasing tight junction integrity, ZO-1 protein levels were analyzed via western blot. Treatment of HCT-116 cells with *L. casei* CFS tended to increase ZO-1 protein levels (P= 0.06) to 134.06% ± 18.85 of control (Fig. 10). Treatment of HCT-116 cells with *L. rhamnosus* CFS significantly increased ZO-1 protein levels to 170.15% ± 30.37 of control (Fig. 3.1.). Treatment of HCT-116 cells with *B. longum* and *B. lactis* CFS had no effect on ZO-1 protein levels (Fig. 3.2.). These data indicate that treatment with probiotic *Lactobacillus* sp. CFS can improve tight junction integrity in metastatic human colon cancer cells, thus decreasing their metastatic potential.

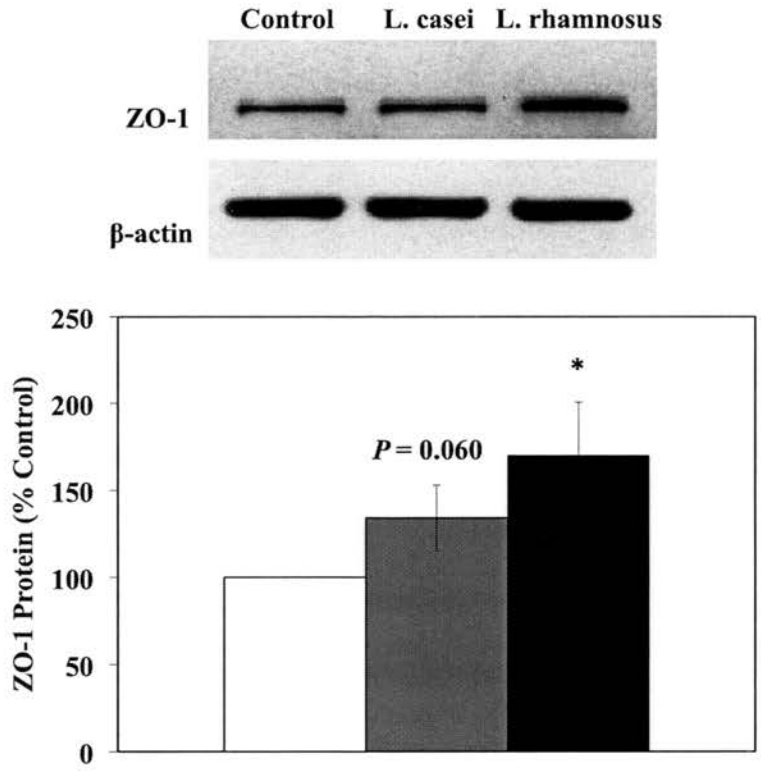

Figure 3.1. *Lactobacillus* CFS increase ZO-1 protein levels. HCT-116 cells were treated with either *L. casei* or *L. rhamnosus* at 25% v/v in DMEM. The same volume of uninoculated bacterial growth medium (MRS broth) in DMEM was used as control. Intracellular proteins were collected as described in Methods. ZO-1 protein levels were detected and normalized using β-actin as a loading control. Data shown are mean ± SEM of n=4. *Significantly different from control ($P < 0.05$).

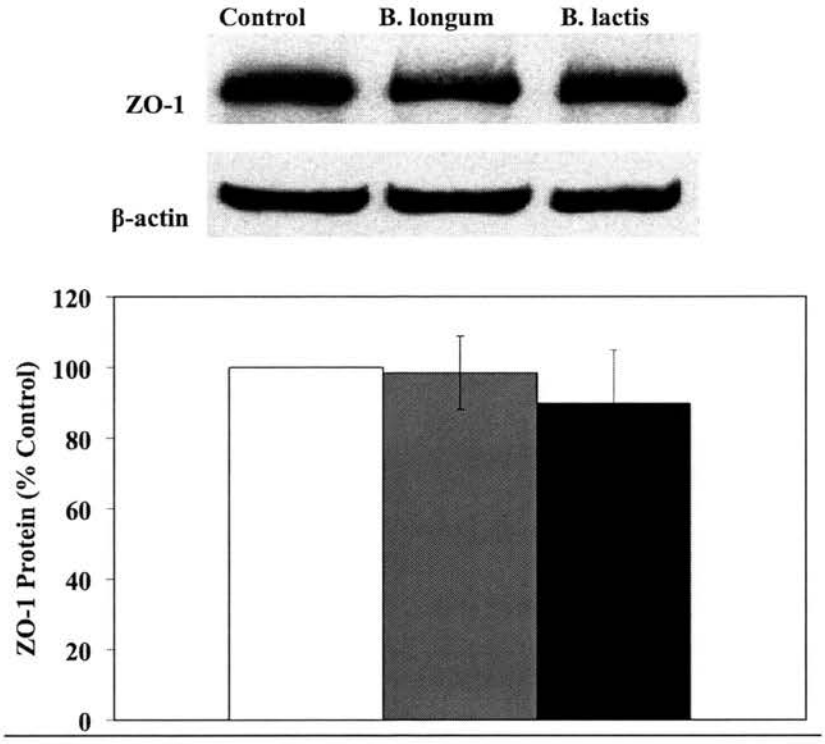

Figure 3.2. *Bifidobacterium* CFS have no effect on ZO-1 protein levels. HCT-116 cells were treated with either *B. longum* or *B. lactis* at 25% v/v in DMEM. The same volume of uninoculated bacterial growth medium (MRS broth) in DMEM was used as control. Intracellular proteins were collected as described in Methods. ZO-1 protein levels were detected and normalized using β-actin as a loading control. Data shown are mean ± SEM of n=4.

DISCUSSION

The current study shows that probiotic bacteria increase ZO-1 protein levels in human colon cancer cells *in vitro.* Previous studies have demonstrated the ability of probiotic bacteria to improve tight junction integrity by decreasing gut permeability [increasing transepithelial resistance (TER)] and increasing ZO-1 protein levels *in vitro* and *in vivo* (Commane et al., 2005; Corr et al., 2009; Czerucka et al., 2000; Ewaschuk et al., 2008; Kindler et al., 2004; Mennigen et al., 2009; Montalto et al., 2003; Ng et al., 2009; Putaala et al., 2008). These studies however have primarily focused on chronic

inflammatory disorders and their effect on epithelial barrier function utilizing human epithelial Caco-2 cells (non-metastatic), human colon cancer T84 cells (low metastatic potential), human colon adenocarcinoma HT-29 cells (low metastatic potential), and murine models (Commane et al., 2005; Ewaschuk et al., 2008; Mennigen et al., 2009; Miyauchi et al., 2009). In addition, a previous study done by Klinder et al. (2004) examined the effects of probiotics in combination with prebiotics on TER and cell invasion using Caco-2 and HT-29 cell lines as *in vitro* models for colon cancer epithelial barrier integrity. Both these cell lines exhibit either none or low metastatic potential (Ishizu et al., 2007; Klinder et al., 2004). Furthermore, Ishizu et al. (2007) examined the potential of 10 human colon and stomach cancer cell lines (HT-29, WiDr, HCT-116, HCT-115, HCC-2998, MKN7, MKN28, MKN45, MKN74, and St-4) to establish metastasis in nude mice. HCT-116 cells consistently formed gross liver metastases when injected into the spleens of nude mice, whereas other cell lines produced little or no metastasis (Ishizu et al., 2007). The liver is the most common target organ for metastasis of cancers of the digestive system, Millikan et al. (1997) reported that up to 25% of patients with a primary colon tumor have synchronous hepatic metastasis and 50% develop metachronous metastasis.

Currently there are no studies purporting the effects of probiotics on tight junction integrity, specifically ZO-1, in the highly metastatic human colorectal carcinoma HCT-116 cell line. The main purpose of this study was to determine if probiotic CFS collected from *Lactobacillus* sp. and *Bifidobacterium* sp. could increase ZO-1 protein levels in HCT-116 metastatic human colorectal carcinoma cells *in vitro*. Our data demonstrate

that secreted factors present in *Lactobacillus* CFS increase ZO-1 protein levels; providing evidence to support anti-metastatic potential of the examined *Lactobacillus* sp. Although *Bifidobacteirum* had no effect on ZO-1 protein levels, further examination of *Bifidobacterium* sp. and their effect on epithelial barrier function is warranted. *Bifidobacterium* sp. could be potentially working through other tight junctions such as ZO-2, ZO-3, claudins, occludin, and junctional adhesion molecules (JAMS) (Gonzalez-Mariscal et al., 2000; Feldman et al., 2005; Furuse et al., 1994). A study done by Braniste et al. (2009) demonstrated an increase in occudin protein, JAMs, and decreased epithelial permeability, without any changes in ZO-1 protein levels. In contrast Ewaschuk et al. (2008) and Mennigen et al. (2009) demonstrated concomitant changes in occludin and ZO-1 protein levels with treatment of probiotic VSL#3. These studies demonstrate that up-regulation by various factors, such as solutes and nutrients can reinforce intestinal epithelial barrier function, without solely mediating only one aspect of tight junction integrity (Braniste et al., 2009; Ewaschuk et al., 2008; Mennigen et al., 2009).

These data demonstrate that probiotic CFS from *Lactobacillus* sp. can improve epithelial integrity within highly metastatic cells. Further research is needed to determine the bioactive compound contributing to these observed effects.

IV. GENERAL DISCUSSION

Probiotics have been the subject of a great deal of investigation regarding overall health and the human GI tract. In particular, much attention has focused on the large intestine (colon), the endogenous and exogenous microflora inhabitants, and their overall impact on diet and colon cancer. Evidence suggests that probiotics such as lactic acid bacteria (LAB) play a critical role in the development of colon cancer with several epidemiological studies establishing the effect LAB have on diet and the etiology of most large bowel cancers (Davis and Milner, 2009; Jong-Eun, 2007; Rafter, 2002; Rafter et al., 2007; Wollowski, et al., 2001). LAB provide several potential health and nutritional benefits that contribute chemopreventative effects on large bowel cancers via diverse mechanisms that include, the alteration of the GI microflora, enhancement of the host's immune response, antimutagenic effects, reduction of harmful bacteria, and antioxidative and antiproliferative activities (Burns and Rowland, 2000; Davis and Milner, 2009; Douglas and Sanders; 2008; Kim et al., 2007; Rastall, 2004). With research primarily focusing on probiotics and their role in prevention of colon cancer in combination with diet, little attention has focused on later detrimental and malignant stages of colon cancer, specifically metastasis.

Our study demonstrates how probiotic bacteria can regulate critical stages of metastasis in a species-specific manner, by investigating cell invasion and epithelial barrier integrity, utilizing the metastatic human colorectal carcinoma cell line HCT-116 *in vitro*. HCT-116 cells treated with both *Lactobacillus* sp. CFS and *Bifidobacterium* sp.

CFS decreased cell invasion *in vitro*. To confirm these effects were specific to the probiotic bacteria investigated in our study, a predominant commensal gut bacteria, *Bacteroides thetaiotaomicron* was also examined for its effects on cell invasion. HCT-116 cells treated with *B. thetatiotomicron* CFS did not decrease cell invasion.

MMP-9 activity and protein levels were examined to determine if a decrease in cell invasion was mediated by a decreased activity of MMP-9. HCT-116 cells treated with *Lactobacillus* sp. CFS decreased MMP-9 activity overall. Similar effects were observed in MMP-9 protein levels upon treatment with *Lactobacillus* sp. CFS. HCT-116 cells treated with *Bifidobacterium* sp. CFS had no effect on MMP-9 activity or protein levels.

Since both *Lactobacillus* sp. had an effect on cell invasion, MMP-9 activity, and MMP-9 protein levels, they were further investigated for the bioactive factors responsible for their anti-metastatic behavior. *Lactobacillus* sp. whole CFS were fractionated into a large molecular weight fraction (> 3 kDa) and a small molecular weight fraction (< 3 kDa). HCT-116 cells treated with the large molecular weight fraction decreased cell invasion, whereas the small molecular weight fraction had no effect on cell invasion.

The observed results suggested that the inhibitory bioactive compound could potentially be a nucleic acid, protein, large peptide, or a polysaccharide, therefore *Lactobacillus* sp. CFS fractions > 3 kDa were further fractionated into defined molecular weight fractions of a smaller range > 100 kDa, 50-100 kDa, 30-50 kDa, 10-30 kDa, and 3-10 kDa. *Lactobacillus* sp. CFS fractions >100 kDa and 50-100 kDa exhibited a decrease in cell invasion, whereas CFS < 50 kDa had no effect on cell invasion.

To determine the effects of probiotic CFS on epithelial integrity the tight junction protein, ZO-1 was examined. HCT-116 cells treated with *Lactobacillus* sp.whole CFS increased ZO-1 protein levels overall, whereas treatments with *Bifidobacterium* sp. whole CFS had no effect on ZO-1 protein levels. Our results not only confirm an increase in ZO-1 levels, but for the first time report an increase in ZO-1 protein levels within this metastatic human colon carcinoma cell line with treatments of *Lactobacillus* sp. probiotic bacteria. These data suggest another possible mechanism by which probiotic bacteria can have a prophylatic role in attenuating metastasis.

Our study demonstrates that metabolites (> 100 kDa and > 50 kDa) present in the *Lactobacillus* sp. CFS tested in this study contain anti-metastatic bioactive compounds that decrease cell invasion by decreasing MMP-9 activity and protein levels, and increasing the TJ protein ZO-1. A majority of literature has shown that the benefical bioactive compounds produced by *Lactobacillus* sp. are typically smaller molecular weight compounds (Chon et al., 2009; Lebeer et al., 2008; Ross et al., 2010; Tao et al., 2006; Vanderpool et al., 2008). In a study done by Yan et al. (2007), a different trend, similar to our findings, was observed, two novel isolated soluble proteins, p75 (75 kilodaltons) and p40 (40 kilodaltons), were produced by probiotic bacteria *Lactobacillus rhamnosus GG*, *Lactobacillus casei 334*, and *Lactobacillus casei 339*. Each of these purified proteins activated Akt in a dose dependent manner, inhibited cytokine-induced epithelial cell apoptosis, and reduced colon epithelial damage induced by TNF in HT-29 human colon carcinoma cells and young adult mouse colon cells (Yan et al., 2007). Our data showed a decrease in cell invasion with *Lactobacillus* sp. CFS fractions > 100 kDa and 50-100 kDa, it can be speculated based on our findings and previous literature that

our bioactive compounds could potentially be the proteins found in the Yan et al. (2007) study that are mediating cell invasion via the PI3K/Akt pathway.

The phosphatidylinositol 3-kinase (PI3K) pathway regulates various cellular processes, such as proliferation, growth, apoptosis, and cytoskeltal rearragement (Luo et al., 2003; Vivanco and Sawyers, 2002). Akt acts downstream of PI3K to regulate many similar biological processes as PI3K (Vivanco and Sawyers, 2002). Interestingly, it has been demonstrated that aberrant activation of the PI3K/Akt pathway has been widely implicated in many cancers (Luo et al., 2003; Palamarchuk et al., 2005; Vivanco and Sawyers, 2002). Therefore further investigation is needed to determine if our bioactive compounds present in the tested *Lactobacillus* CFS are potentially counteracting the affect of this pathway during cell invasion.

In addition to regulating Akt, p75 and p40 were also shown to attenuate hydrogen peroxide redistribution of occludin, ZO-1, E-cadherin, and β-catenin from intercellular junctions via a protein kinase C (PKC) dependent mechanism and ERK1/2 (Seth et al., 2008). Both PCK and ERK1/2 pathways increase stabilization of TJ during their activation (Lebeer et al., 2008; Seth et al., 2008). Since only whole CFS of *Lactobacillus* sp. were tested for increased levels of ZO-1 protein, further studies are needed to determine if *Lactobacillus* sp. CFS fractions (>100 kDa and 50-100 kDa) will show increases in ZO-1 protein levels, similar to our whole CFS tested. *Lactobacillus* sp. have been shown to influence many signaling pathways, however there is a general lack of knowledge of how *Lactobacillus* sp. effector molecules and their corresponding host receptors mediate these observed effects (Basuroy et al., 2003; Lebeer et al., 2008; Seth et al., 2008; Sheth et al., 2003; Yan et al., 2007). Therefore further mechanistic studies

are needed to determine how the *Lactobacillus* sp. tested in this study affect the pathways mentioned previously on overall cell invasion (Fig. 4.1.).

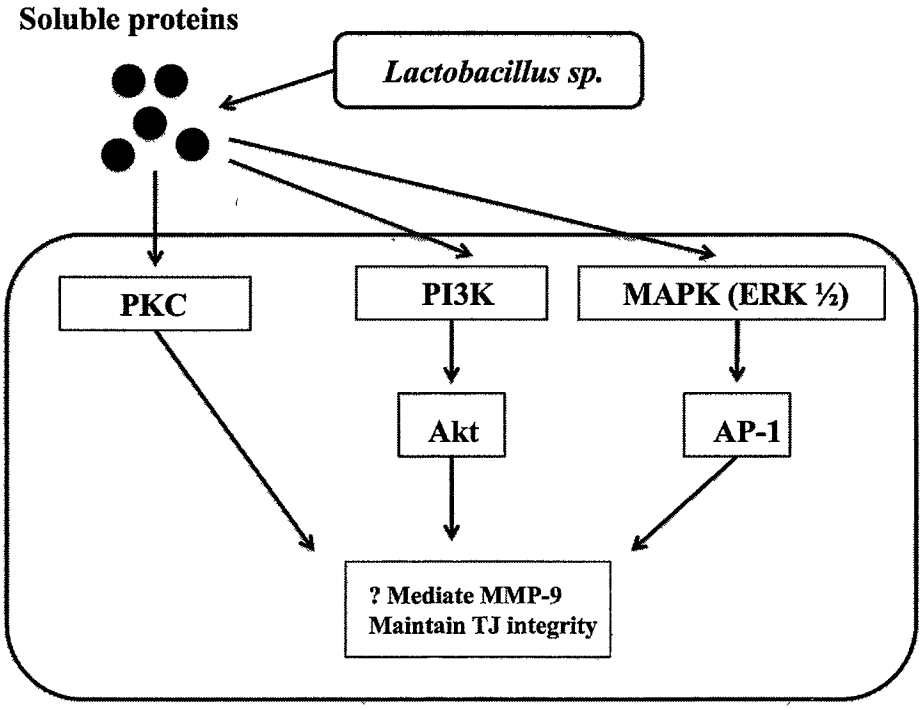

Figure 4.1. Pathways by which *Lactobacillus* sp. could potentially mediate colon cancer cell invasion (Leeber et al., 2008; Seth et al., 2008).

Our results also demonstrate how a common commensal bacteria, *B. thetaiotaomicron* did not decrease cell invasion, unlike its probiotic counterparts. Since each bacterial species was tested alone, further studies are necessary to determine how these commensal bacteria affect cell invasion when combined with a probiotic bacteria as well as how these bacteria behave *in vivo* when other physiological factors are present. It is important to note that probiotic bacteria have been shown *in vitro* and *in vivo* to attenuate the negative effects of many genera and species of microorganisms that inhabit the human gastrointestinal tract (Burns and Rowland, 2000; Fooks and Gibson, 2002;

Gibson and Roberfroid, 1995; Kim et al. 2007). Specifically, *Lactobacillus* sp. and *Bifidobacterium* sp. have been shown to have low activities of enzymes involved in carcinogen formation and metabolism in comparison to other major anaerobes in the gut such as *Bacteroides, Eubacteria,* and *Clostridia* (Burns and Rowland, 2000; Saito et al., 1992).

The other probiotics tested in this study, *B longum* and *B. lactis* did attenuate cell invasion in HCT-116 cells *in vitro*, however these observed effects were not mediated by decreases in MMP-9 activity, MMP-9 protein levels, or increases in tight junction protein ZO-1; the mechanistic outcomes measured in our study. Thus *Bifidobacterium* sp. could be decreasing cell invasion via an alternative mechanism not investigated within our study. Some of these alternative mechanisms as described previously (chapter II and chapter III) include the regulation of Rho proteins and increases in other tight junction proteins (ZO-2, ZO-3, claudin, and occludin).

In addition to the regulation of MMP-9, other members of the MMP family, MMP-1, -7, and -13 have been shown to have increased activity or tissue levels associated with increased invasion and metastasis, and shortened survival with colon cancer (Egeblad and Werb, 2002; Leeman et al., 2003; Zucker and Vacirca, 2004). MMP-7 has been implicated in early-stage colorectal tumorigenesis as well as cell survival and new tumor formation (Leeman et al., 2003). MMP-1 has been identified as increasing cell migration, cell proliferation, and increased cancer susceptibility (Egeblad and Werb, 2002; Zucker and Vacirca, 2004). MMP-13 is expressed primarily by cancer cells at the invading edge of the tumor, its expression correlates with the capacity of tumor invasion (Ala-Aho et al., 2002). Investigating other MMPs and their roles in

colorectal cancer is another area in which future research is necessary. Probiotic CFS could mediate the up-regulaton or downregulation of these MMPs in metastatic cells.

Another possible mechanism could be the up-regulation of tissue inhibitor of MMP (TIMP). TIMPs function as endogenous protease inhibitors (Leeman et al., 2003). TIMPs can inhibit the activity of all MMPs; in addition, TIMPs can also bind to pro-MMP-9 to decrease its conversion to active MMP-9 (Leeman et al., 2003; Park et al., 2007). Furthermore, it has been demonstrated that an overexpression of TIMPs reduced experimental metastasis (Coussens et al., 2002). TIMPS were not investigated in our study, and thus could be a potential way that both *Lactobacillus* sp. and *Bifidobacterium* sp. CFS could potentially be mediating cell invasion.

The fatal characteristic of tumor cells is their ability to invade through the ECM to generate metastasis, therefore cancer research is leaning towards MMP inhibitors as a potential answer to tumor progression (Chen et al., 2009; Coussens et al., 2002; Overall and Lopez-Otin, 2002). MMPs are prime candidates because collectively they can degrade the ECM, MMPs are also known to be up-regulated in virtually all human and animal tumors as well as most tumor cells lines (Coussens et al., 2002; Hanahan and Weinberg, 2007; Zucker and Vacirca, 2004). Some current therapies being tested prior to their release into the pharmaceutical market are Marimastat, BMS-275291, Prinomastat, and Metastat (Coussens et al., 2002; Overall and Lopez-Otin, 2002). These potential anti-cancer drugs can mediate various mechanisms associated with MMP induction in human tumors (Overall and Lopez-Otin, 2002). MMPs however have a wide range of biological activities that have set limitations to these current therapies. Many early first generation MMP inhibitors had poor availability and thus a second generation was

immediately developed. Early phase I and phase II trials revealed that prolonged treatment with MMP inhibitors caused musculoskeletal pain and inflammation (Coussens et al., 2002; Overall and Lopez-Otin, 2002). This trend continued with other MMP inhibitors such as Prinomastat and Adamalysin (Coussens et al., 2002). Further studies revealed that some MMP inhibitors such as Tanomastat actually decreased survival time when compared to patients receiving placebo (Coussens et al., 2002). Clinical Phase II and Phase III studies have revealed that further research is still needed in understanding the overall function of MMPs in regards to cancer (Coussens et al., 2002; Overall and Lopez-Otin, 2002).

Probiotics can potentially aid in this realm of cancer research by providing bioactive compounds produced during normal growth and metabolism that can potentially target MMPs without the side effects associated with allopathic MMP inhibitor therapies. A new concept has evolved as probiotics have emerged as potential therapeutic targets in several clinical disorders (Shanahan, 2009). 'Mining' the microbiota for bioactives, novel drug therapies, and functional foods has led to the development of the term 'pharmabiotic' which encompases any material from the microbe to molecule that can be mined for health benefit from interactions within the alimentary tract (Kataria et al., 2009; Shanahan, 2009; Sleator and Hill, 2009). The understanding of the various bioactive molecules produced by probiotics is still an area of research that is not fully understood, and therefore leaves room for further in-depth investigation.

Our study primarily focused on the effects of probiotic CFS on cell invasion and epithelial barrier integrity by examining MMP-9 and ZO-1 protein levels *in vitro*.

Further studies are needed to understand how these bioactive compounds found within our CFS tested can affect colon cancer metastasis utilizing an *in vivo* model, as well as further investigating their effects when combined with other probiotic or commensal bacteria *in vitro* and *in vivo*. Transepithelial resistance (TER) was not examined in our study after measuring ZO-1 protein levels, TER could further validate the observed effects seen with increases in ZO-1 protein levels and therefore should be investigated. Although we did not study mechanistic pathways involved in the regulation of MMP-9, studies examining the roles of PI3K/Akt, PKC, and ERK1/2 pathways are possible areas of future research. Previous literature has shown that bioactive compounds produced by some *Lactobacillus* sp. can mediate mechanisms involved within these pathways and therefore could be potential avenues by which bioactive molecules found within our CFS could be mediating cell invasion and epithelial integrity (Seth et al., 2008; Yan et al., 2007).

In summary, our study demonstrates the ability of *Lactobacillus* sp. CFS to decrease cell invasion by decreasing MMP-9 activity and protein levels, and increasing ZO-1 protein. *Lactobacillus* sp. bacteria investigated in our study could be potentially mediating cell invasion by several mechanisms. It is therefore necessary to continue investigating how these bioactive factors play a role in colon cancer metastasis. Furthermore, there is tremendous potential in the use of probiotic bacterial derived bioactive compounds as novel therapeutic agents for the treatment of colon cancer metastasis. Probiotics not only can play a role in prevention and maintenance of overall GI health, but can also be used for the chemotherapeutic treatment of later detrimental stages of colon cancer metastasis

REFERENCES

1. Abecassis I, Olofsson B, Schmid M, Zalcman G, Karniguian A: RhoA induces MMP-9 expression at CD44 lamellipodial focal complexes and promotes HMEC-1 cell invasion. Exp Cell Res 2003; 291(2): 363-76.

2. Alessandro R, Di Bella MA, Flugy AM, et al.: Comparative study of T84 and T84SF human colon carcinoma cells: in vitro and in vivo ultrastructural and functional characterization of cell culture and metastasis. Virchows Arch 2006; 449(1): 48-61.

3. Asangani IA, Rasheed SA, Nikolova DA, et al.: MicroRNA-21 (miR-21) post-transcriptionally downregulates tumor suppressor Pdcd4 and stimulates invasion, intravasation and metastasis in colorectal cancer. Oncogene 2008; 27(15): 2128-36.

4. Astin: Why Patients Use Alternative Medicine. JAMA 1998; 279(19): 1548-1553.

5. Banerjee P, Merkel GJ, Bhunia AK: Lactobacillus delbrueckii ssp. bulgaricus B-30892 can inhibit cytotoxic effects and adhesion of pathogenic Clostridium difficile to Caco-2 cells. Gut Pathog 2009; 1(1): 8.

6. Basuroy S, Sheth P, Kuppuswamy D, Balasubramanian S, Ray RM, Rao RK: Expression of kinase-inactive c-Src delays oxidative stress-induced disassembly and accelerates calcium-mediated reassembly of tight junctions in the Caco-2 cell monolayer. J Biol Chem 2003; 278(14): 11916-24.

7. Bishop AL, Hall A: Rho GTPases and their effector proteins. Biochem J 2000; 348 Pt 2: 241-55.

8. Bitomsky N, Bohm M, Klempnauer KH: Transformation suppressor protein Pdcd4 interferes with JNK-mediated phosphorylation of c-Jun and recruitment of the coactivator p300 by c-Jun. Oncogene 2004; 23(45): 7484-93.

9. Bohle AS, Kalthoff H: Molecular mechanisms of tumor metastasis and angiogenesis. Langenbecks Arch Surg 1999; 384(2): 133-40.

10. Braniste V, Leveque M, Buisson-Brenac C, Bueno L, Fioramonti J, Houdeau E: Oestradiol decreases colonic permeability through oestrogen receptor beta-mediated up-regulation of occludin and junctional adhesion molecule-A in epithelial cells. J Physiol 2009; 587(Pt 13): 3317-2

11. Burns AJ, Rowland IR: Anti-carcinogenicity of probiotics and prebiotics. Curr Issues Intest Microbiol 2000; 1(1): 13-24.

12. Cassileth BR DG: Complementary and Alternative Therapies for Cancer. The Oncologist 2004; 9: 80-89.

13. Castaneda FE WB, Vijay-Kumar M, Patel NR, Roser S, Kolachala VL, Rojas M, Wang L, Oprea G, Garg P, Gewirtz AT, Roman J, Merlin D, Sitaraman S: Targeted Deletion of Metalloproteinase 9 Attenuates Experimental Colitis in Mice: Central Role of Epithelial-Derived MMP. Gastroenterology 2005; 129: 1991-2008.

14. Chen YJ, Chang WM, Liu YW, et al.: A small-molecule metastasis inhibitor, norcantharidin, downregulates matrix metalloproteinase-9 expression by inhibiting Sp1 transcriptional activity in colorectal cancer cells. Chem Biol Interact 2009; 181(3): 440-6.

15. Chon H CB, Jeong G, Lee E, Lee S: Suppression of proinflammatory cytokine production by specific metabolites of Lactobacillus plantarum 10hk2 via inhibiting NF-kB and p38 MAPK expressions. Comparative Immunology, Microbiology and Infectious Diseases 2009: 9.

16. Clevers: At the Crossroads of Inflamation and Cancer. Cell 2004; 118: 671-674.

17. Cole CB, Fuller R, Mallet AK, Rowland IR: The influence of the host on expression of intestinal microbial enzyme activities involved in metabolism of foreign compounds. J Appl Bacteriol 1985; 59(6): 549-53.

18. Commane D, Hughes R, Shortt C, Rowland I: The potential mechanisms involved in the anti-carcinogenic action of probiotics. Mutat Res 2005; 591(1-2): 276-89.

19. Commane DM, Shortt CT, Silvi S, Cresci A, Hughes RM, Rowland IR: Effects of fermentation products of pro- and prebiotics on trans-epithelial electrical resistance in an in vitro model of the colon. Nutr Cancer 2005; 51(1): 102-9.

20. Corr SC, Hill C, Gahan CG: Understanding the mechanisms by which probiotics inhibit gastrointestinal pathogens. Adv Food Nutr Res 2009; 56: 1-15.

21. Corthesy B, Gaskins HR, Mercenier A: Cross-talk between probiotic bacteria and the host immune system. J Nutr 2007; 137(3 Suppl 2): 781S-90S.

22. Coussens LM, Fingleton B, Matrisian LM: Matrix metalloproteinase inhibitors and cancer: trials and tribulations. Science 2002; 295(5564): 2387-92.

23. Czerucka D, Dahan S, Mograbi B, Rossi B, Rampal P: Saccharomyces boulardii preserves the barrier function and modulates the signal transduction pathway induced in enteropathogenic Escherichia coli-infected T84 cells. Infect Immun 2000; 68(10): 5998-6004.

24. Davis CD, Milner JA: Gastrointestinal microflora, food components and colon cancer prevention. J Nutr Biochem 2009; 20(10): 743-52.

25. del Peso L, Hernandez-Alcoceba R, Embade N, et al.: Rho proteins induce metastatic properties in vivo. Oncogene 1997; 15(25): 3047-57.

26. Demers MJ, Thibodeau S, Noel D, et al.: Intestinal epithelial cancer cell anoikis resistance: EGFR-mediated sustained activation of Src overrides Fak-dependent signaling to MEK/Erk and/or PI3-K/Akt-1. J Cell Biochem 2009; 107(4): 639-54.

27. Dhawan P, Singh AB, Deane NG, et al.: Claudin-1 regulates cellular transformation and metastatic behavior in colon cancer. J Clin Invest 2005; 115(7): 1765-76.

28. Dicks LM, Silvester M, Lawson PA, Collins MD: Lactobacillus fornicalis sp. nov., isolated from the posterior fornix of the human vagina. Int J Syst Evol Microbiol 2000; 50 Pt 3: 1253-8.

29. Dise RS, Frey MR, Whitehead RH, Polk DB: Epidermal growth factor stimulates Rac activation through Src and phosphatidylinositol 3-kinase to promote colonic epithelial cell migration. Am J Physiol Gastrointest Liver Physiol 2008; 294(1): G276-85.

30. Douglas LC, Sanders ME: Probiotics and prebiotics in dietetics practice. J Am Diet Assoc 2008; 108(3): 510-21.

31. Drouault S, Corthier G, Ehrlich SD, Renault P: Survival, physiology, and lysis of Lactococcus lactis in the digestive tract. Appl Environ Microbiol 1999; 65(11): 4881-6.

32. Egeblad M, Werb Z: New functions for the matrix metalloproteinases in cancer progression. Nat Rev Cancer 2002; 2(3): 161-74.

33. Etienne-Manneveill S HA: Rho GTPase in cell biology. Nature 2002; 420(12): 629-635.

34. Ewaschuk JB, Diaz H, Meddings L, et al.: Secreted bioactive factors from Bifidobacterium infantis enhance epithelial cell barrier function. Am J Physiol Gastrointest Liver Physiol 2008; 295(5): G1025-34.

35. Feldman GJ, Mullin JM, Ryan MP: Occludin: structure, function and regulation. Adv Drug Deliv Rev 2005; 57(6): 883-917.

36. Ferguson LR, Harris PJ: The dietary fibre debate: more food for thought. Lancet 2003; 361(9368): 1487-8.

37. Fidler: The pathogenesis of cancer metastasis: the 'seed and soil' hypothesis revisited. Nature Reviews 2002; 3: 1-6.

38. Flood DM, Weiss NS, Cook LS, Emerson JC, Schwartz SM, Potter JD: Colorectal cancer incidence in Asian migrants to the United States and their descendants. Cancer Causes Control 2000; 11(5): 403-11.

39. Fooks LJ, Gibson GR: Probiotics as modulators of the gut flora. Br J Nutr 2002; 88 Suppl 1: S39-49.

40. Fund WCR: Diet, nutrition and the prevention of cancer: a global perspective. Washington, DC: World Cancer Research/American Institute of Cancer Research, 1997.

41. Furuse M, Itoh M, Hirase T, Nagafuchi A, Yonemura S, Tsukita S: Direct association of occludin with ZO-1 and its possible involvement in the localization of occludin at tight junctions. J Cell Biol 1994; 127(6 Pt 1): 1617-26.

42. Genda T, Sakamoto M, Ichida T, et al.: Cell motility mediated by rho and Rho-associated protein kinase plays a critical role in intrahepatic metastasis of human hepatocellular carcinoma. Hepatology 1999; 30(4): 1027-36.

43. Gibson GR, Fuller R: Aspects of in vitro and in vivo research approaches directed toward identifying probiotics and prebiotics for human use. J Nutr 2000; 130(2S Suppl): 391S-395S.

44. Gibson GR, Roberfroid MB: Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics. J Nutr 1995; 125(6): 1401-12.

45. Gill CI, Rowland IR: Diet and cancer: assessing the risk. Br J Nutr 2002; 88 Suppl 1: S73-87.

46. Gill SR, Pop M, Deboy RT, et al.: Metagenomic analysis of the human distal gut microbiome. Science 2006; 312(5778): 1355-9.

47. Giraudo E, Inoue M, Hanahan D: An amino-bisphosphonate targets MMP-9-expressing macrophages and angiogenesis to impair cervical carcinogenesis. J Clin Invest 2004; 114(5): 623-33.

48. Gonzalez-Mariscal L BA, Avila-Flores A: MAGUK proteins: structure and role in the tight junction. Cell and Developmental Biology 2000; 11: 315-324.

49. Guarner F, Malagelada JR: Gut flora in health and disease. Lancet 2003; 361(9356): 512-9.

50. Guarner F PG, Corthier G, Salminen S, Koletzko B, Morelli L: Should yoghurt cultures be considered probiotic. British Journal of Nutrition 2005; 93: 783-786.

51. Guo W, Giancotti FG: Integrin signalling during tumour progression. Nat Rev Mol Cell Biol 2004; 5(10): 816-26.

52. Hanahan D, Weinberg RA: The hallmarks of cancer. Cell 2000; 100(1): 57-70.

53. Harris PJ, Ferguson LR: Dietary fibre: its composition and role in protection against colorectal cancer. Mutat Res 1993; 290(1): 97-110.

54. Hooper LV, Wong MH, Thelin A, Hansson L, Falk PG, Gordon JI: Molecular analysis of commensal host-microbial relationships in the intestine. Science 2001; 291(5505): 881-4.

55. Hurwitz HI FL, Hainsworth JD, Heim W, Berlin J, Holmgren E, Hambleton J, Novotny W, Kabbinavar F: Bevacizumab in Combination With Fluorouracil and Leucovorin: An Active Regimen for First-Line Metastatic Colorectal Cancer. Journal of Clinical Oncology 2005; 23(15): 3502-3508.

56. Ishizu K, Sunose N, Yamazaki K, et al.: Development and characterization of a model of liver metastasis using human colon cancer HCT-116 cells. Biol Pharm Bull 2007; 30(9): 1779-83.

57. Jemal A, Siegel R, Ward E, Hao Y, Xu J, Thun MJ: Cancer statistics, 2009. CA Cancer J Clin 2009; 59(4): 225-49.

58. Kaihara T, Kusaka T, Nishi M, et al.: Dedifferentiation and decreased expression of adhesion molecules, E-cadherin and ZO-1, in colorectal cancer are closely related to liver metastasis. J Exp Clin Cancer Res 2003; 22(1): 117-23.

59. Kamai T, Tsujii T, Arai K, et al.: Significant association of Rho/ROCK pathway with invasion and metastasis of bladder cancer. Clin Cancer Res 2003; 9(7): 2632-41.

60. Kataria J LN, Wynn JL, Neu J: Probiotic microbes: do they need to be alive to be beneficial. Nutrition Reviews 2009; 67(9): 546-550.

61. Kawai T, Akira S: TLR signaling. Cell Death Differ 2006; 13(5): 816-25.

62. Kawai T AS: TLR Signaling. Cell Death and Differentiation 2006; 13: 816-825.

63. Key T SA, Willett WC, Allen NE, Spence EA, Travis RC: Diet, nutrition and the prevention of cancer. Public Health Nutrition 2004; 7(1A).

64. Kiefer J, Beyer-Sehlmeyer G, Pool-Zobel BL: Mixtures of SCFA, composed according to physiologically available concentrations in the gut lumen, modulate histone acetylation in human HT29 colon cancer cells. Br J Nutr 2006; 96(5): 803-10.

65. Kim JE, Kim JY, Lee KW, Lee HJ: Cancer chemopreventive effects of lactic acid bacteria. J Microbiol Biotechnol 2007; 17(8): 1227-35.

66. Kim SO, Sheikh HI, Ha SD, Martins A, Reid G: G-CSF-mediated inhibition of JNK is a key mechanism for Lactobacillus rhamnosus-induced suppression of TNF production in macrophages. Cell Microbiol 2006; 8(12): 1958-71.

67. Kinross JM, von Roon AC, Holmes E, Darzi A, Nicholson JK: The human gut microbiome: implications for future health care. Curr Gastroenterol Rep 2008; 10(4): 396-403.

68. Klinder A GE, Hughes R, Jonkers N, Karlsson P, McGlyn H, Pistoli S, Tuohy K, Rafter J, Rowland I, Loo JV, Pool-Zobel BL: Gut Fermentation Products of Inulin-Derived Prebiotics Benefically Modulate Markers of Tumour Progression in Human Colon Tumour Cells. International Journal of Cancer Prevention 2004; 1(1): 19-32.

69. Kohler H, McCormick BA, Walker WA: Bacterial-enterocyte crosstalk: cellular mechanisms in health and disease. J Pediatr Gastroenterol Nutr 2003; 36(2): 175-85.

70. Kumar A, Collins HM, Scholefield JH, Watson SA: Increased type-IV collagenase (MMP-2 and MMP-9) activity following preoperative radiotherapy in rectal cancer. Br J Cancer 2000; 82(4): 960-5.

71. Langley RR, Fidler IJ: Tumor cell-organ microenvironment interactions in the pathogenesis of cancer metastasis. Endocr Rev 2007; 28(3): 297-321.

72. Laukoetter MG, Nava P, Nusrat A: Role of the intestinal barrier in inflammatory bowel disease. World J Gastroenterol 2008; 14(3): 401-7.

73. Lebeer S, Vanderleyden J, De Keersmaecker SC: Genes and molecules of lactobacilli supporting probiotic action. Microbiol Mol Biol Rev 2008; 72(4): 728-64, Table of Contents.

74. Leeman MF, Curran S, Murray GI: New insights into the roles of matrix metalloproteinases in colorectal cancer development and progression. J Pathol 2003; 201(4): 528-34.

75. Li CX, Poznansky MJ: Characterization of the ZO-1 protein in endothelial and other cell lines. J Cell Sci 1990; 97 (Pt 2): 231-7.

76. Li D, Mrsny RJ: Oncogenic Raf-1 disrupts epithelial tight junctions via downregulation of occludin. J Cell Biol 2000; 148(4): 791-800.

77. Liizumi M LW, Pai S, Furuta E, Watabe K: Drug development against metastasis-related genes and their pathways: A rationale for cancer therapy. Biochim Biophys Acta 2008; 1786(2): 87-104.

78. Loftus IM, Naylor AR, Bell PR, Thompson MM: Matrix metalloproteinases and atherosclerotic plaque instability. Br J Surg 2002; 89(6): 680-94.

79. Luo J, Manning BD, Cantley LC: Targeting the PI3K-Akt pathway in human cancer: rationale and promise. Cancer Cell 2003; 4(4): 257-62.

80. Madara JL: Regulation of the movement of solutes across tight junctions. Annu Rev Physiol 1998; 60: 143-59.

81. Mai V, Draganov PV: Recent advances and remaining gaps in our knowledge of associations between gut microbiota and human health. World J Gastroenterol 2009; 15(1): 81-5.

82. Mai V, Morris JG, Jr.: Colonic bacterial flora: changing understandings in the molecular age. J Nutr 2004; 134(2): 459-64.

83. Marteau P, Minekus M, Havenaar R, Huis in't Veld JH: Survival of lactic acid bacteria in a dynamic model of the stomach and small intestine: validation and the effects of bile. J Dairy Sci 1997; 80(6): 1031-7.

84. Martin FP, Wang Y, Sprenger N, et al.: Probiotic modulation of symbiotic gut microbial-host metabolic interactions in a humanized microbiome mouse model. Mol Syst Biol 2008; 4: 157.

85. Martin TA, Das T, Mansel RE, Jiang WG: Enhanced tight junction function in human breast cancer cells by antioxidant, selenium and polyunsaturated lipid. J Cell Biochem 2007; 101(1): 155-66.

86. McCawely L ML: Matrix metalloproteinases: multifunctional contributors to tumor progression. Mol Med Today 2000; 6: 149-156.

87. Menard S, Candalh C, Bambou JC, Terpend K, Cerf-Bensussan N, Heyman M: Lactic acid bacteria secrete metabolites retaining anti-inflammatory properties after intestinal transport. Gut 2004; 53(6): 821-8.

88. Mennigen R, Nolte K, Rijcken E, et al.: Probiotic mixture VSL#3 protects the epithelial barrier by maintaining tight junction protein expression and preventing apoptosis in a murine model of colitis. Am J Physiol Gastrointest Liver Physiol 2009; 296(5): G1140-9.

89. Miele ME, Robertson G, Lee JH, et al.: Metastasis suppressed, but tumorigenicity and local invasiveness unaffected, in the human melanoma cell line MelJuSo after introduction of human chromosomes 1 or 6. Mol Carcinog 1996; 15(4): 284-99.

90. Miyauchi E, Morita H, Tanabe S: Lactobacillus rhamnosus alleviates intestinal barrier dysfunction in part by increasing expression of zonula occludens-1 and myosin light-chain kinase in vivo. J Dairy Sci 2009; 92(6): 2400-8.

91. Montalto M, Maggiano N, Ricci R, et al.: Lactobacillus acidophilus protects tight junctions from aspirin damage in HT-29 cells. Digestion 2004; 69(4): 225-8.

92. Moore WE, Moore LH: Intestinal floras of populations that have a high risk of colon cancer. Appl Environ Microbiol 1995; 61(9): 3202-7.

93. Moorthy G, Murali MR, Devaraj SN: Protective role of lactobacilli in Shigella dysenteriae 1-induced diarrhea in rats. Nutrition 2007; 23(5): 424-33.

94. Naaber P, Smidt I, Stsepetova J, Brilene T, Annuk H, Mikelsaar M: Inhibition of Clostridium difficile strains by intestinal Lactobacillus species. J Med Microbiol 2004; 53(Pt 6): 551-4.

95. Ng SC, Hart AL, Kamm MA, Stagg AJ, Knight SC: Mechanisms of action of probiotics: recent advances. Inflamm Bowel Dis 2009; 15(2): 300-10.

96. O'hara A ORP, Fanning A, O'Mahony C, MacSharry J, Lyons A, Bienenstock J, O'Mahony L, Shanahan F: Functional modulation of human intestinal epithelial cell responses by Bifidobacterium infantis and Lactobacillus salivarius. Immunology 2006; 118: 202-215.

97. O'Hara AM SF: The gut flora as a forgotten organ. EMBO reports 2006; 7: 688-683.

98. Ogimato SA, Fukuda K: World Cancer Research Fund/American Institute of Cancer Research. Japan Cancer Cause Control 2000; 11(1): 9-23.

99. Organization FaAOotUNaWH: Regulatory and clinical aspects of dairy probiotics. Food and Agriculture Organization of the United Nations and World Health Organization Expert Consultation Report. 2001.

100. Otte JM PD: Fuctional modulation of enterocytes by gram-positive and gram-negative microorganisms. American Journal Physiology Gastroenterology Liver Physiology 2004; 286: G613-G626.

101. Overall CM, Lopez-Otin C: Strategies for MMP inhibition in cancer: innovations for the post-trial era. Nat Rev Cancer 2002; 2(9): 657-72.

102. Palamarchuk A, Efanov A, Maximov V, Aqeilan RI, Croce CM, Pekarsky Y: Akt phosphorylates and regulates Pdcd4 tumor suppressor protein. Cancer Res 2005; 65(24): 11282-6.

103. Paltiel O AM, Peretz T, Cherny N, Kaduri L, Pfeffer RM, Wagner N, Soskolne V: Determinants of the Use of complementary Therapies by Patients with Cancer. Journal of Clinical Oncology 2001; 19(9): 2439-2448.

104. Park EY, Wilder ET, Lane MA: Retinol inhibits the invasion of retinoic acid-resistant colon cancer cells in vitro and decreases matrix metalloproteinase mRNA, protein, and activity levels. Nutr Cancer 2007; 57(1): 66-77.

105. Ponz de Leon M PA: Pathogenesis of colorectal cancer Digest Liver Dis 2000; 32: 807-821.

106. Pool-Zobel BL: Inulin-type fructans and reduction in colon cancer risk: review of experimental and human data. Br J Nutr 2005; 93 Suppl 1: S73-90.

107. Pool-Zobel BL, Sauer J: Overview of experimental data on reduction of colorectal cancer risk by inulin-type fructans. J Nutr 2007; 137(11 Suppl): 2580S-2584S.

108. Poste G, Fidler IJ: The pathogenesis of cancer metastasis. Nature 1980; 283(5743): 139-46.

109. Putaala H, Salusjarvi T, Nordstrom M, et al.: Effect of four probiotic strains and Escherichia coli O157:H7 on tight junction integrity and cyclo-oxygenase expression. Res Microbiol 2008; 159(9-10): 692-8.

110. Rabot S, Rafter J, Rijkers GT, Watzl B, Antoine JM: Guidance for substantiating the evidence for beneficial effects of probiotics: impact of probiotics on digestive system metabolism. J Nutr; 140(3): 677S-89S.

111. Rafter J: Lactic acid bacteria and cancer: mechanistic perspective. Br J Nutr 2002; 88 Suppl 1: S89-94.

112. Rafter J, Bennett M, Caderni G, et al.: Dietary synbiotics reduce cancer risk factors in polypectomized and colon cancer patients. Am J Clin Nutr 2007; 85(2): 488-96.

113. Rambaud JC BJ, Corthier B, Flourie B: Gut Microflora Digestive Physiology and Pathology. France: John Libbey Eurotext, 2007.

114. Rao RK PD, Seth A, Yan F: Probiotics the Good Neighbor: Guarding the Gut Mucosal Barrier. American Journal of Infectious Diseases 2009; 5(3): 188-192.

115. Rastall RA: Bacteria in the gut: friends and foes and how to alter the balance. J Nutr 2004; 134(8 Suppl): 2022S-2026S.

116. Reid G: The scientific basis for probiotic strains of Lactobacillus. Appl Environ Microbiol 1999; 65(9): 3763-6.

117. Resta-Lenert S BK: Live probiotics protect intestinal epithelial cells from the effects of infection with enteroinvasive Escherichia coli (EIEC). Gut 2003; 52: 988-997.

118. Rhodes JM, Simons M: The extracellular matrix and blood vessel formation: not just a scaffold. J Cell Mol Med 2007; 11(2): 176-205.

119. Richardson MA ST, Palmer JL, Greisinger A, Singletary SE: Complementary/Alternative Medicine Use in a Comprehensive Cancer Center and the Implications for Oncology. Journal of Clinical Oncology 2000; 18(13): 2505-2514.

120. Rooj AK, Kimura Y, Buddington RK: Metabolites produced by probiotic Lactobacilli rapidly increase glucose uptake by Caco-2 cells. BMC Microbiol; 10: 16.

121. Ross RP MS, Hill C, Fitzgerald GF, Stanton C: Specific metabolite production by gut microbiota as a basis for probiotic function. International Dairy Journal 2010; 20: 269-276.

122. Rowland IR, Rumney CJ, Coutts JT, Lievense LC: Effect of Bifidobacterium longum and inulin on gut bacterial metabolism and carcinogen-induced aberrant crypt foci in rats. Carcinogenesis 1998; 19(2): 281-5.

123. Saito Y TT, Rowland IR: Effects of Soybean oligosaccharides on the human gut microflora in in vitro culture. microbial Ecology Health Dis 1992; 5: 105-110.

124. Salmeron I FP, Charalampopoulos D, Pandiella S: Volatile compounds produced by the probiotic strain Lactobacillus plantarum NCIMB 8826 in cereal-based substrates. Food Chemistry 2009; 117: 265-271.

125. Sanchez B, Chaignepain S, Schmitter JM, Urdaci MC: A method for the identification of proteins secreted by lactic acid bacteria grown in complex media. FEMS Microbiol Lett 2009; 295(2): 226-9.

126. Scharlau D, Borowicki A, Habermann N, et al.: Mechanisms of primary cancer prevention by butyrate and other products formed during gut flora-mediated fermentation of dietary fibre. Mutat Res 2009; 682(1): 39-53.

127. Schmitz AA, Govek EE, Bottner B, Van Aelst L: Rho GTPases: signaling, migration, and invasion. Exp Cell Res 2000; 261(1): 1-12.

128. Segain JP, Harb J, Gregoire M, Meflah K, Menanteau J: Induction of fibroblast gelatinase B expression by direct contact with cell lines derived from primary tumor but not from metastases. Cancer Res 1996; 56(23): 5506-12.

129. Seth A, Yan F, Polk DB, Rao RK: Probiotics ameliorate the hydrogen peroxide-induced epithelial barrier disruption by a PKC- and MAP kinase-dependent mechanism. Am J Physiol Gastrointest Liver Physiol 2008; 294(4): G1060-9.

130. Shanahan F: Therapeutic implications of manipulating and mining the microbiota. J Physiol 2009; 587(Pt 17): 4175-9.

131. Sheth P, Basuroy S, Li C, Naren AP, Rao RK: Role of phosphatidylinositol 3-kinase in oxidative stress-induced disruption of tight junctions. J Biol Chem 2003; 278(49): 49239-45.

132. Slattery ML, Potter JD, Coates A, et al.: Plant foods and colon cancer: an assessment of specific foods and their related nutrients (United States). Cancer Causes Control 1997; 8(4): 575-90.

133. Sleator RD, Hill C: Rational design of improved pharmabiotics. J Biomed Biotechnol 2009; 2009: 275287.

134. Smalley KS, Brafford P, Haass NK, Brandner JM, Brown E, Herlyn M: Up-regulated expression of zonula occludens protein-1 in human melanoma associates with N-cadherin and contributes to invasion and adhesion. Am J Pathol 2005; 166(5): 1541-54.

135. Soler AP, Miller RD, Laughlin KV, Carp NZ, Klurfeld DM, Mullin JM: Increased tight junctional permeability is associated with the development of colon cancer. Carcinogenesis 1999; 20(8): 1425-31.

136. Stamatova I, Meurman JH, Kari K, Tervahartiala T, Sorsa T, Baltadjieva M: Safety issues of Lactobacillus bulgaricus with respect to human gelatinases in vitro. FEMS Immunol Med Microbiol 2007; 51(1): 194-200.

137. Tao Y, Drabik KA, Waypa TS, et al.: Soluble factors from Lactobacillus GG activate MAPKs and induce cytoprotective heat shock proteins in intestinal epithelial cells. Am J Physiol Cell Physiol 2006; 290(4): C1018-30.

138. Teitelbaum JE, Walker WA: Nutritional impact of pre- and probiotics as protective gastrointestinal organisms. Annu Rev Nutr 2002; 22: 107-38.

139. Tobioka H, Isomura H, Kokai Y, Tokunaga Y, Yamaguchi J, Sawada N: Occludin expression decreases with the progression of human endometrial carcinoma. Hum Pathol 2004; 35(2): 159-64.

140. Turnbaugh PJ, Ley RE, Hamady M, Fraser-Liggett CM, Knight R, Gordon JI: The human microbiome project. Nature 2007; 449(7164): 804-10.

141. Turner JR: Molecular basis of epithelial barrier regulation: from basic mechanisms to clinical application. Am J Pathol 2006; 169(6): 1901-9.

142. Ulisse S, Gionchetti P, D'Alo S, et al.: Expression of cytokines, inducible nitric oxide synthase, and matrix metalloproteinases in pouchitis: effects of probiotic treatment. Am J Gastroenterol 2001; 96(9): 2691-9.

143. Vanderpool C, Yan F, Polk DB: Mechanisms of probiotic action: Implications for therapeutic applications in inflammatory bowel diseases. Inflamm Bowel Dis 2008; 14(11): 1585-96.

144. Vinderola G, Matar C, Perdigon G: Role of intestinal epithelial cells in immune effects mediated by gram-positive probiotic bacteria: involvement of toll-like receptors. Clin Diagn Lab Immunol 2005; 12(9): 1075-84.

145. Vivanco I, Sawyers CL: The phosphatidylinositol 3-Kinase AKT pathway in human cancer. Nat Rev Cancer 2002; 2(7): 489-501.

146. Vogelstein B, Fearon ER, Hamilton SR, et al.: Genetic alterations during colorectal-tumor development. N Engl J Med 1988; 319(9): 525-32.

147. Waas ET, Lomme RM, DeGroot J, Wobbes T, Hendriks T: Tissue levels of active matrix metalloproteinase-2 and -9 in colorectal cancer. Br J Cancer 2002; 86(12): 1876-83.

148. Walther A JE, Swanton C, Midgley R, Tomlinson I, Kerr D: Genetic prognostic and predictive markers in colorectal cancer. Nature Reviews 2009; 9: 489-496.

149. Wang M VI, Kalthoff: Correlation between metastatic potential and variants from colorectal tumor cell line HT-29. World Journal of Gastroenterology 2003; 9(11): 2627-2631.

150. Weeks JC, Cook EF, O'Day SJ, et al.: Relationship between cancer patients' predictions of prognosis and their treatment preferences. JAMA 1998; 279(21): 1709-14.

151. Wirfalt E MD, Reedy J, Mitrou P, Flood A, Subar AF, Leitzmann M, Mouw T, Hollenbeck AR, Schatzkin A, Kipnis V. : Associations between food patterns defined by cluster analysis and colorectal cancer incidence in the NIH-AARP diet and health study. European Journal of Clinical Nutrition 2009; 63: 707-717.

152. Wollowski I, Rechkemmer G, Pool-Zobel BL: Protective role of probiotics and prebiotics in colon cancer. Am J Clin Nutr 2001; 73(2 Suppl): 451S-455S.

153. Yan F, Cao H, Cover TL, Whitehead R, Washington MK, Polk DB: Soluble proteins produced by probiotic bacteria regulate intestinal epithelial cell survival and growth. Gastroenterology 2007; 132(2): 562-75.

154. Yang HS, Matthews CP, Clair T, et al.: Tumorigenesis suppressor Pdcd4 down-regulates mitogen-activated protein kinase kinase kinase kinase 1 expression to suppress colon carcinoma cell invasion. Mol Cell Biol 2006; 26(4): 1297-306.

155. Yoon SO, Park SJ, Yun CH, Chung AS: Roles of matrix metalloproteinases in tumor metastasis and angiogenesis. J Biochem Mol Biol 2003; 36(1): 128-37.

156. Yu Q, Stamenkovic I: Cell surface-localized matrix metalloproteinase-9 proteolytically activates TGF-beta and promotes tumor invasion and angiogenesis. Genes Dev 2000; 14(2): 163-76.

157. Yu QH, Yang Q: Diversity of tight junctions (TJs) between gastrointestinal epithelial cells and their function in maintaining the mucosal barrier. Cell Biol Int 2009; 33(1): 78-82.

158. Zeng ZS, Huang Y, Cohen AM, Guillem JG: Prediction of colorectal cancer relapse and survival via tissue RNA levels of matrix metalloproteinase-9. J Clin Oncol 1996; 14(12): 3133-40.

159. Zucker S, Vacirca J: Role of matrix metalloproteinases (MMPs) in colorectal cancer. Cancer Metastasis Rev 2004; 23(1-2): 101-17.

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