The Mechanism of Action of Kefir and its Effect on the Motility of Breast and Colorectal Cancer Cells

By
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A thesis submitted in partial fulfilment of the requirements for the degree of Master of Science in Molecular Biology

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The Mechanism of Action of Kefir and its Effect on the Motility of Breast and Colorectal Cancer Cell Lines

Nathalie M. El-Khoury

ABSTRACT

Kefir which is a fermented milk product originating from the Caucasus Mountains is gaining increasing popularity due to its attribution in preventing or curing many chronic diseases among them cancer. Previous studies have focused on the anti-tumoral activity of kefir *in vivo* yet not extensive studies have been performed to elucidate its mechanism of action. Till now kefir is known to modulate intestinal microbiota, modulate the activity of immune system cells, and suspected to induce apoptosis in cancer cells *in vivo*. Recent studies have focused on the direct effect of kefir cell-free fractions on cancer cells *in vitro*. Earlier work in our lab has shown that cell-free fractions of kefir have anti-proliferative and pro-apoptotic effect on Caco-2 and HT-29 colorectal adenocarcinoma cells. In this study, we aim to determine a possible mechanism of action of kefir and to study its effect on the motility of colorectal and breast cancer cell lines. Results from the reverse-transcription PCR experiment have shown that kefir decreases the expression of TGF-α and TGF-β1 in HT-29 cells in a dose dependent manner. An increase in the production of both cytokines is usually associated with neoplastic transformation. Western blot assay results confirmed the previously observed increase in apoptosis as it showed an increase in Bax:Bcl-2 expression upon kefir treatment. Also an increase in p53 independent-p21 expression was observed in HT-29 cells upon kefir treatment which would explain the anti-proliferative effect of kefir. Furthermore, results from the time-lapse movies, wound-healing, and invasion assays showed no effect on the motility of colorectal (Caco-2 and HT-29) as well as breast (MCF-7 and MB-MDA-231) cancer cells upon kefir treatment.

**Keywords:** Colorectal Cancer, Breast Cancer, Kefir, Proliferation, Apoptosis, Cell Motility, Cell Invasion.
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LIST OF ABBREVIATIONS

CRC: Colorectal cancer
5-FU: 5-Fluorouracil
VEGF: Vascular endothelial growth factors
EGFR: Epidermal growth factor receptor
DCIS: Ductal carcinoma in situ
LCIS: Lobular carcinoma in situ
DC: Ductal carcinoma
LC: Lobular carcinoma
Her-2: Human Epidermal Growth Factor Receptor 2
ER-positive: Estrogen receptor-positive
HR-positive: hormone-receptor-positive
TNBC: Triple negative breast cancer
CBE: Clinical breast examination
BSE: Breast self-examination
ACS: American Cancer Society
BRCA1: Breast cancer 1
BRCA2: Breast cancer type 2 susceptibility protein
MRI: Magnetic resonance imaging
Bcl-2: B-cell lymphoma 2
BH domain: Bcl-2 Homology domains
Bcl-xL: B-cell lymphoma-extra large
Bcl-w: Bcl-2-like protein 2
Bak: Bcl-2 antagonistic killer
Bax: Bcl-2–associated X protein
PUMA: Promoter upregulated modulator of apoptosis
Bid: Bcl-2 interacting domain death agonist
OMM: Outer mitochondrial membrane
SMAC: Second mitochondrial activator of caspases
dATP: Deoxyadenosine triphosphate
Apaf-1: Apoptosis protease-activating factor-1
P53: Protein 53
MDM-2: Mouse double minute 2 homolog
DNA: Deoxyribonucleic acid
pRB: Retinoblastoma protein
S phase: Synthesis phase
G1 Phase: Gap 1 phase
TGF-α: Transforming growth factor alpha
EGF: Epidermal growth factor
PI3K: Phosphoinositide 3-kinase
KRAS: Kirsten rat sarcoma viral oncogene homolog
BRAF: v-Raf murine sarcoma viral oncogene homolog B1
MEK: Mitogen-activated protein kinases
ERK: Extracellular signal-regulated kinases
STAT: Signal Transducer and Activator of Transcription
MMP: Matrix metalloproteinases
IL-8: Interleukin
TGF-β: Transforming growth factor beta
EMT: Epithelial to mesenchymal transition
MCF-7: Michigan Cancer Foundation-7
HTLV-1: Human T-lymphotropic virus 1
RT-PCR: Reverse Transcription Polymerase Chain Reaction
DMEM: Dulbecco's Modified Eagle Medium
FBS: Fetal Bovine Serum
ATCC: American Type Culture Collection
SDS-PAGE: Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
PVDF: Polyvinylidene fluoride
BSA: Bovine serum albumin
PBS: Phosphate buffered saline
ECL: Enhanced Chemiluminescence
RNA: Ribonucleic acid
cDNA: Complementary deoxyribonucleic acid

HEPES: Hydroxyethyl piperazineethanesulfonic acid

SEM: Standard error of the mean
Chapter I

Literature Review

1.1. Colorectal cancer

1.1.1. Incidence and statistics

In 2013, the American Cancer Society (ACS) ranked colorectal cancer (CRC) as the third leading cause of cancer-related mortalities in the United States in both men and women. In 1975, 60 new cases of colorectal cancer per 100,000 people were diagnosed every year in the United States, but this rate has declined by 2007 to approximately 45 new cases per 100,000 individuals. Moreover, the mortality rates decreased from 28 in 1975 to 17 by 2007 per 100,000 individuals (The National Cancer Institute, 2010). In 2013, it is estimated that 142,820 new cases of CRC will be diagnosed among which 50,830 death cases will arise (Siegel, Naishadhan, & Jemal, 2013).

1.1.2. Types

Among all CRC, 72% arise in the colon and the remaining 28% arise in the rectum (National Cancer Institute, 2010). Adenocarcinoma, the cancer caused by the mucus-secreting epithelial cells of the colon, account for more than 95% of CRC. Other less common tumors that can originate in the colon or rectum might include carcinoid tumors formed from neuroendocrine cells of the colon, lymphomas caused by immune system cells that reside in the colon, as well as sarcomas caused by different connective tissues including muscle and blood vessel cells. Therefore, when medical doctors refer to CRC they are most probably referring to adenocarcinomas (American Cancer Society, 2013). The sequence of events leading to the formation of CRC takes place over a period of 10-15 years and involves the accumulation of genetic mutations and chromosomal aberrations induced by several environmental factors as well as inherited genetic mutations (Hoff, Foerster, Vatn, Sauar, & Larsen, 1986; Hamilton, 1993; O’Brien, et al., 1993). The extended period of time leading to CRC allows sufficient time for chances to screen, properly interfere, and to potentially save the lives of more patients.
1.1.3. Screening and Diagnosis

By the time people are diagnosed with CRC, 25% of patients would have already developed metastasis. Among these patients with metastatic colorectal cancer, 50% will survive (Kufe, et al., 2003). This necessitates the need for tools and techniques to screen for and diagnose early stage cancer as well as adenomatous polyps.

In 2008, the U.S. Preventive Services Task Force recommended that individuals between the ages of 50 and 75 years screen for colorectal cancer using three different screening methods to diminish the risk of death from CRC. The fecal occult blood testing is recommended to be performed annually. Two other tests are accompanied by a small risk of compilations, therefore sigmoidoscopy is advised every five years, and colonoscopy is recommended every ten years (Pignone & Sox, 2008).

1.1.4. Current cures

Surgical treatment remains the best option for the treatment of colorectal cancer but in a large number of cases solely it is not the ideal treatment since its outcome depends on the extent of the disease (Cunningham, et al., 2010). On the other hand, radiation therapy is not used as a standard treatment for CRC, yet it is used along with chemotherapy preoperatively (Latkauska, et al., 2010).

Significant successes have been achieved by chemotherapeutic drugs in the treatment of CRC to an extent that the survival of patients with CRC can now be extended by nearly two years (Wolpin & Mayer R, 2008). 5-Fluorouracil (5-FU) was one of the earliest and most commonly used drugs for the treatment of CRC (Sobrero, Guglielmi, Grossi, Puglisi, & Aschele, 2000). Later on, Oxaliplatin and Leucovorin have shown to enhance the survival of patients when added to 5-FU (Becouarn, et al., 1998). Irinotecan, an inhibitor of topoisomerase I, is able to enhance the overall survival of patients with metastatic CRC when administered with leucovorin and 5-FU (Fuchs, et al., 2007).

Recently, focus has been directed toward targeted therapy. For example, Bevacizumab, a monoclonal antibody that binds the Vascular Endothelial Growth Factors (VEGFs) which play a role in blood vessel formation, has shown to improve the survival
of patients when combined with chemotherapeutic drugs (Ferrara & Gerber, 2003). The epidermal growth factor receptor (EGFR) is also an important target in the treatment of colorectal cancer and the most effective antibodies used against it are cetuximab and panitumumab (Messersmith & Hidalgo, 2007). Yet the limitation of these targeted therapies lies in the fact that in rare cases tumors develop mutations in the intra-signaling pathways of EGFR rendering the treatment ineffective (Berg & Soreide, 2012).

1.2. Breast cancer

1.2.1. Incidence and statistics

In 2012, the American Cancer Society ranked breast cancer as the second deadliest cancer in women in the United States. Breast cancer rarely affects men; it is 100 times more common in women (American Cancer Society, 2012). Between 2005 and 2009 on average 124.3 cases of breast cancer were diagnosed per 100,000 women yearly (National Cancer Institute, 2013).

1.2.2. Types

In situ carcinomas of the breast are of two types, ductal carcinoma in situ (DCIS) or lobular carcinoma in situ (LCIS). These types of carcinomas can not invade surrounding tissues and do not metastasize. LCIS do not develop into invasive LC but may be a marker of an increased risk for invasive DC or LC development in any of the breasts (Simpson & Wilkinson, 2004). Yet, DCIS was shown to be a precursor to invasive DC. Invasive lesions have the ability to penetrate the basement membrane and spread into adjacent stroma in the breast and therefore have the potential to metastasize. Among invasive breast cancer 70 to 80% are invasive DC and almost 10% are invasive LC, the remaining are less common histologic types such as apocrine, tubular, medullary, mucinous, and papillary (Simpson & Wilkinson, 2004). A classification based on gene expression profiling done in 2000 by Perou et al. identified five subgroups of breast cancer they are basal epithelial-like group, Her-2 (Human Epidermal Growth Factor Receptor-2) overexpressing, normal breast like group, and ER positive luminal A and B. These subtypes have differential survival rates, prognosis, and response to different treatments. Also, from a clinical perspective, breast cancer is subdivided into three categories, the HR-positive (Hormone-
receptor positive) cells that express estrogen and progesterone receptors, Her-2 overexpressing cells, and the remaining are known as triple negative breast cancer (TNBC) since they lack any of these three receptors (Perou, et al., 2000; Sorlie, et al., 2001)

1.2.3. Screening and Diagnosis

In 2012, the American Cancer Society in 2012 recommended that women above the age of 40 do annual mammograms, an X-ray for the breast, in order to detect early breast cancer. It also recommends a clinical breast exam (CBE) to be performed every three years for women between the ages of 20 and late 30s and annually for women above the age of 40 by a health care professional. Breast self-examination (BSE) is also recommended by the ACS for woman above the age of 20. For women who are at a high risk (eg. having a BRCA1, Breast cancer 1, and BRCA2, Breast cancer type 2 susceptibility protein) an MRI (Magnetic resonance imaging) along with mammogram is recommended annually.

1.3.4. Current cures

Lumpectomy, the removal of the cancerous tissues, or mastectomy, the removal of the entire breast, are ideal for the treatment of early breast cancer, yet micro-metastatic cells may remain and cause recurrence (Hassan, Ansari, Spooner, & Hussain, 2010). Therefore, chemotherapy is regularly used as an adjuvant six weeks after surgery. The most commonly used combination used is fluorouracil, adriamycin, cyclophosphamide or fluorouracil, epirubicin, and cyclophosphamide. Also, postoperative locoregional radiation shows reduced risk of recurrence and mortality rates (Whelan, Julian, Wright, Jadad, & Levine, 2000).

Targeted therapy is widely used in the treatment of breast cancer. An important example is Trastuzumab (Herceptin), a monoclonal antibody that targets the Her-2 receptor, usually overexpressed in 20% of breast cancers, when added to chemotherapy decreased the recurrence of the disease by 53% (Romond, at al., 2005). Tamoxifen, an estrogen receptor antagonist in the breast, significantly decreases the death rate for ER-
positive (Estrogen receptor-positive) breast cancer, the most common type of breast cancer (EBCTCG, 2005). Megestrol acetate, a derivative of progesterone, is also used for the treatment of advanced breast cancer (Allegra, 1987). Ovarian ablation, oophorectomy, is also used following hormone therapy mainly to treat metastatic breast cancer in premenopausal women (Pater & Parulekar, 2003). TNBC cells which do not express the estrogen, progesterone, or the Her2 receptors on their plasma membrane do not respond well to targeted therapy is usually treated with chemotherapeutic drugs (Vaklavas & Forero-Torres, 2011).

Despite the fact that significant improvements have been made in the treatment of early breast cancer, a major challenge in breast cancer remains in the treatment of metastatic cases (Hassan, Ansari, Spooner, & Hussain, 2010)

1.3. Signaling pathways in cancer

1.3.1. Bcl-2 family of apoptosis regulatory proteins

The Bcl-2 (B-cell lymphoma 2) protein family plays a crucial role in apoptosis through its anti-apoptotic as well as pro-apoptotic proteins. The expression of proteins in this family are dynamically balanced and alterations in their expression leads to either enhancement or prevention of cell death (Ola, Nawaz, & Ahsan, H, 2011). Members of this family possess Bcl-2 homology domains known as BH1, BH2, BH3, and BH4 which are necessary for the formation of heterodimeric binding among members of the Bcl-2 family (Danial & Korsmeyer, 2004). Antiapoptotic proteins of this family include Bcl-2, Bcl-xL, and Bcl-w. On the other hand, the pro-apoptotic include Bak and Bax or those that only possess the BH3 domain such as Puma (Promoter upregulated modulator of apoptosis) and Bid (Bcl-2 interacting domain death agonist) (Wang, Yin, Chao, Milliman, & Korsmeyer, 1996; Brunelle & Letai, 2009). In normal physiological conditions, Bak, a proapoptotic protein, is present in the outer mitochondrial membrane OMM in its inactive form while Bax is inactive in the cytosol (Youle & Strasser, 2008). Following stimulation by an apoptotic signal, Bax is translocated to the OMM and leads to the formation Bax/Bak homodimers that leads to the formation of pores in the OMM and causes the leakage of proteins from the intermembrane space into the cytosol such as Smac (Second
mitochondrial activator of caspases) and cytochrome c. Cytochrome c is then activated through its interaction with dATP and Apaf-1 (Apoptosis protease-activating factor-1) leading to the formation of apoptosome that causes the activation of caspases that cleavage various components required for the proper functioning of the cell (Yethon, et al. 2003; Wei, et al 2001). In the absence of apoptotic signals, antiapoptotic members of the bcl-2 prevent any leakage from the mitochondria by preventing the oligomerization of the proapoptotic members of the Bcl-2 family (Youle & Strasser, 2008). Therefore, the regulation of activity and expression of BH3-only proteins is critical in the induction of apoptosis. Generally, the proportion of pro-survival to pro-apoptotic proteins of the Bcl-2 family appears to regulate the sensitivity of the cell to the apoptotic signal (Ola, et al., 2011).

1.3.2. Roles of p53 and p21 in apoptosis

P53 (Protein 53) is a widely known transcription factor that localizes mostly in the nucleus and is able to induce the expression of several genes that lead to DNA damage repair, cell cycle arrest, and apoptosis (Oren & Rotter, 1999; Vousden & Lu, 2002). In normal physiology, the expression of p53 is maintained at a low level through its proteasomal degradation induced by the ubiquitin protein ligase MDM-2 (Mouse double minute 2 homolog) (Kubbutat, Jones, & Vousden, 1997). When DNA damage occurs, p53 becomes active by undergoing post-translational modifications which increase its stability and allows its accumulation in the nucleus (Prives & Hall, 1999). When active, p53 induces the expression of a set of genes involved in DNA damage repair and cell cycle arrest. After the DNA is repaired, the cell returns to its normal cell cycle. But when the DNA damage is severe and beyond repair, p53 would then induce apoptosis in the cell (El-Deiry, 2003). P53 exerts its effect through several target genes among them the cell cycle inhibitor p21. P21 binds to the cyclin-dependent kinase-2 and prevents it from phosphorylating the retinoblastoma protein pRB. The inactive pRB will bind transcription factors of the E2F protein and constrain it in an inactive form which will prevent the cell from proceeding to the S phase of the cell cycle and remain in the G1 phase. (Harper, Adami, Wei, Keyomars, & Elledge, 1993). However, p21 can also be induced in a p53-independent manner whether induced by DNA damage or not (Macleod, et al., 1995).
Another target of p53 is MDM-2 which is needed to negatively regulate the expression of p53 levels (Barak, Juven, & Haffner, 1993). P53 also induces its apoptotic effect by modulating the activity and expression of pro-apoptotic proteins of the Bcl-2 family such as Bax, PUMA, as well as others (Selvakumaran, et al., 1994; Yu, Zhang, Hwang, Kinzler, & Vogelstein, 2001).

### 1.3.3. TGF-alpha signaling pathway

Transforming growth factor alpha (TGF-α) is a member of the epidermal growth factor family EGF (Salomon, Brandt, Ciardiello, & Normanno, 1995). It shares a 40% homology in sequence with EGF and competitively competes for its receptor which is the Epidermal Growth Factor Receptor EGFR. EGFR belongs to the ErbB protein family of tyrosine kinases that play a significant role in cell survival, proliferation, motility, angiogenesis, and adhesion (Arteaga, 2003). Receptor activation triggers a downstream signaling pathways including the phosphoinositide 3-kinase (PI3K), the KRAS-BRAF-MEK-ERK pathway, the phospholipase C gamma, and the STAT signaling pathway (Yarden & Sliwkowski, 2001; Baselga & Albanell, 2002).

To maintain proper cell homeostasis, the expression of EGFR ligands is stringently regulated. In atypical cases, dysplasia could occur due to an increase in the expression of TGF-α or any other ligand belonging to the EGF family or due to a constitutively active mutation in the EGFR (Ji, et al., 2006). It has been reported that an elevation of EGFR expression is detected in 60 to 80% of cases of CRC (Goldstein & Armin, 2001). The higher its expression in several types of cancer the more aggressive the tumor would be and the poorer is its prognosis (Umekita, Ohi, Sagara, & Yoshida 2000). In human colon cancer, the level of TGF-α expression was observed to be four times more than that of normal mucosa (Liu, Woo, & Tsao, 1990). Similarly, an upregulation in the expression of TGF-α has been reported in many types of cancer compared to normal cells (Mellon, Cook, Chambers, & Neal, 1996).

Several in vitro studies have showed TGF-α significantly increase the invasive ability of several types of cancer cell lines and this effect could be abolished almost completely by the additions of antibodies targeting the EGF receptor (Hölting, Siperstein,
Clark, & Duh, 1995; Ueda, et al., 1998). In vivo studies showed that overexpression of TGF-α by colon cancer cells favors their progression and metastasis through the recruitment of cells that express VEGF, matrix metalloproteinases (MMPs), and interleukin-8 (IL-8), as well as by increasing metastasis to the lymphatic system (Sasaki, et al., 2008). These data provide sufficient evidence for the need to target the TGF-α/EGFR signaling pathway for therapeutic intervention.

### 1.3.4. TGF-beta signaling pathway

Transforming growth factors beta (TGF-β) are ligands that belong to the TGFβ superfamily. This superfamily includes cytokines such as activins, bone morphogenetic proteins (BMPs), inhibins, and the anti-Müllerian hormone (Schmierer & Hill, 2007). TGF-β signaling plays a role in many cellular responses such as cell cycle arrest, migration, invasion, Epithelial to mesenchymal (EMT) transition, as well as immune suppression (Derynck, & Miyazono, 2008). Until recently, TGF-β signaling pathway was a paradox since it could promote cell proliferation but can also inhibit its growth, it could lead to stem cell differentiation but also enhances its pluripotency, and it suppresses pre-malignant cells yet boosts metastatic ones. Current studies have shown that the response of a cell to the stimulation of TGF-β is dependent on three contextual determinants which are the epigenetic status of the cell, the proteins involved in the signaling transduction pathway, and the transcription factors present. Therefore, its effect varies among cell types and disease stage (Massagué, 2012).

In normal cells, TGF-β suppresses tumorigenesis by causing cell cycle arrest and apoptosis, yet in advanced cancer, where it is usually overexpressed, the cells develop mutation in the TGF-β receptor or internal proteins that allow them to avoid the cytostatic and apoptotic effect and can even use the increase TGF-β expression to induce their own proliferation (Massagué, 2008). Also this increased ligand production promotes invasion of the cancer cells, through EMT transition and increased angiogenesis, and causes the recruitments of cells involved in inflammation, all of which leads to disease progression and metastasis (Thiery, Acloque, Huang, & Nieto, 2009; Heldin, Vanlandewijck, & Moustakas, 2012). The effect of TGF-β is not only limited to the cancer cells but also has
potent effect on many immune system cells. It suppresses the proliferation of T cells and induces apoptosis in B cells (Ramesh, Wildey, & Howe, 2009).

In colorectal cancer, TGF-β1 expression significantly increases with the advancement of cancer stage, invasive ability, and metastasis (Xiong, et al., 2002). TGF-β1 overexpression is not only observed in tissues of the CRC but also it is also overexpressed in the patient’s serum (Tsushima, et al. 1996). In CRC, TGF-β also affects the microenvironment by modulating, immune system cells, endothelial and stromal cells as well as others which consequently would increase cell invasiveness (Elliott & Blobe, 2005).

Drugs that target the TGF-β signaling pathway have been designed and some are currently in Phase III clinical trials for the treatment of cancer. These drugs include monoclonal antibodies against the TGF-β receptor, ligand competitive peptides, inhibitors of TGF-β kinase activity, inhibitors of gene expression, as well as anti-sense oligonucleotides, the latter which have proceeded the furthest in clinical trials (Akhurst & Hata, 2012). Some adverse side effects for these drugs have been seen in pre-clinical trials but most were absent in clinical trials. The only adverse effects observed in clinical trials were the development of non-malignant skin lesion which disappears upon the withdrawal from the drug (Morris, J. C. et al, 2008)

1.4. Motility, invasion, and metastasis of cancer cells

The ability of cells to migrate is essential for the development and homeostasis of a multicellular organism. Cell motility is required during embryonic development to allow cells to reach their destined positions to properly form tissues and organs. Also in normal physiology, cells migrate to injured tissues to repair wounds, vascular endothelial cells move to create new capillaries, and white blood cells relocate to sites of inflammation to fight foreign organisms. Yet, pathological forms of migration exist in cancer cells, where these cells acquire the ability to migrate which is otherwise absent in their normal counterparts (Lauffenburger & Horwitz, 1996; Sheetz, Felsenfeld, Galbraith, Choquet, 1999). Cell migration can be induced by either chemical or physical factors in the environment and comprises a complex process that involves the formation of a protrusion
at the leading side of the cell, followed by the creation of adhesions at the front edge, translocation of the cell, and then the retraction at the rear (Ridley, et al., 2003).

Many studies have confirmed the involvement of cell motility in metastasis and in 2010 alone more than a thousand published manuscripts have discussed this contribution. Whether it initially exists during diagnosis, or occurs during treatment or relapse, the dissemination of cancer cells from their primary site remains the primary cause of death in cancer patients (Palmer, Ashby, Lewis, & Zijlstra, 2011). Metastasis is a complex process not only limited to the ability of the cell to migrate but is dependent on the ability of the cell to invade neighboring tissues, intravasate nearby vasculature, survive in the blood or lymph, extravasate, and colonize at secondary sites (Fidler, 2003). A very important step in metastasis is invasion which is not only dependent on capabilities of the cells themselves but also on the surrounding microenvironment. Cancer cells reorganize their microenvironment by secreting growth factors, cytokines, and matrix metalloproteinases. These signals would alter the extra cellular matrix and affect neighboring stromal cells such as macrophages, endothelial cells, and fibroblasts (Egeblad & Werb, 2002). In turn, these cells would release growth factors and other cytokines to enhance the invasion and proliferation of cancer cells (Alexander & Friedl, 2012). The early steps in the invasion process involves the activation of signaling pathways that modulate the cytoskeleton of the invasive cells and modifies cell-matrix and cell-cell junction which is then followed by the active migration of the cancer cells into nearby microenvironments (Friedl & Wolf, 2003). Targeting any step in the metastatic process such as motility or invasion can limit the metastatic ability of cancer cells and retain them in their primary location.

1.5. Kefir

1.5.1. Probiotics

Recently because of the increasing need to find substitutes for conventional therapies, research has been directed toward the beneficial effects of probiotics and their potential role in the prevention and healing of various chronic diseases (Preidis et al. 2011; McNaught & MacFie, 2001). Probiotics have shown to exert beneficial effects on health and are now of great interest to the science community as well as to the food industry (Lopitz-Otsoa, Rementeria, Elguezabal, & Garaizar, J. 2006; Preidis et al. 2011).
A probiotic is either a single strain or a combination of different microorganisms claimed to positively affect the well-being of the host if ingested in adequate quantities (McNaught & Macfie, 2001). An example of probiotics is kefir, which is similar to yogurt in that it is a fermented milk beverage, but varies a lot in its constituents due to the fact that it produced by kefir grains (Lopitz-Otsoa et al. 2006).

1.5.2. Characteristics and composition

Kefir is an acidic drink containing small amounts of alcohol and is slightly carbonated (Lopitz-Otsoa et al. 2006). Kefir grains are a white, soft, gelatin-like mass composed of bacteria and yeast in a matrix of proteins, fat, and polysaccharides, with kefiran being the most important water-soluble polysaccharide (Lopitz-Otsoa et al. 2006). The yeasts and bacteria co-exist in a symbiotic relationship in the kefir grains and trying to culture different strains individually either inhibits their growth or decreases their biological activity which makes it difficult to examine the microbial population in the kefir grains (Lopitz-Otsoa et al. 2006). Kefir grains contain lactic acid bacteria (Lactobacillus delbruecki, Lactobacillus helveticus, Lactobacillus casei, Lactobacillus acidophilus, Lactobacillus brevis, etc.), yeasts (Saccharomyces sp., Candida, Torulopsis and Kluyveromyces), streptococci (Streptococcus salivarius), lactococci (Leuconostoc cremoris, Leuconostoc mesenteroides, Lactococcus lactis ssp. etc.) and infrequently acetic acid bacteria (Simova et al. 2005).

1.5.3. Preparation of kefir

The conventional method of producing kefir is by adding kefir grains to raw milk in a container and incubating them at room temperature for 24 hours. The fermented milk is filtered to retrieve the grains which would increase in biological mass, the grains can be used to inoculate a new batch of milk, and the process can be repeated continuously (Lopitz-Otsoa et al. 2006). To produce a new batch of kefir, it is essential to use kefir grains to inoculate the milk and not the previously produced kefir since the end product of the fermentation has a different microbiological profile from the kefir grains (Simova et al. 2002).
1.5.4. Origin of kefir

Kefir was produced for centuries in the Caucasus Mountains. Fresh milk from cows and goats were added to leather and goatskin sacs and allowed to ferment, then the fermented milk was removed and the sacs were replenished by fresh milk. After the continuous use of the same container, water-insoluble kefir grains started to form on the internal wall of the sac (Farnworth, 2005).

1.5.5. Health benefits of kefir

It has long been thought that kefir holds many healing powers for example the longevity of Bulgarian farmers has been assumed to be due their ingestion of kefir (Liu et al. 2002). Since the eighteenth century, many health benefits have been attributed to kefir including its ability to stimulate the immune system (Vinderola, Perdigón, Duarte, Farnworth, & Matar, 2006a), improve lactose digestion and tolerance in individuals with lactose intolerance (Hertzler & Clancy, 2003), decrease serum cholesterol levels (Liu et al. 2006), act as an antifungal and antibacterial agent against many pathogenic organisms (Rodrigues, Caputo, Carvalho, Evangelista, & Schneedorf, 2005), provide resistance against enteric and diarrheal diseases (Preidis et al. 2011), in addition to its anti-tumoral activity (Chen, Chan, & Kubow, 2007).

1.5.6. Anticancer effect

1.5.6.1. In vivo studies

The first publication to show an antitumoral activity of a water-soluble polysaccharide (KGF-C) separated from kefir grains was published in 1982 by Shiomi et al. Whether this polysaccharide was administered orally or peritoneally it prevented the growth of Ehrlich carcinoma or Sarcoma 180 in comparison to control mice although its mode of action was elusive (Murofushi, Shiomi, & Aibara, 1983; Shiomi, Sasaki, Murofushi, & Aibara, 1982). Later studies determined that KGF-C polysaccharide enhances the immune system by affecting T-cell and not B-cell action (Murofushi, Mizuguchi, Aibara, & Matuhasi, 1986). Additionally, studies have also shown that Lewis lung carcinoma and Ehrlich ascites carcinoma were both inhibited upon the oral administration of kefir (Furukawa, Matsuoka, & Yamanaka, 1990; Kubo, Odani,
Nakamura, Tokumaru, & Matsuda, 1992). Moreover, kefir produced from soy milk and cow’s milk considerably repressed tumor growth in mice injected with Sarcoma 180 as compared to unfermented milk and upon microscopic observations, it was suspected that the tumor size was decreased due to apoptosis (Liu, Wang, Lin, & Lin, 2002).

1.5.6.2 In vitro studies

In 2007, in vitro studies were performed on cell lines to determine whether kefir has any direct effect on cancer cells and its mode of action. Cell free kefir fractions exhibited an anti-proliferative effect on human mammary cancer cells (MCF-7) but did not affect normal human mammary epithelial cells (Chen et al. 2007). Similarly in vitro experiments using cell free kefir fractions showed anti-proliferative and pro-apoptotic effects on HTLV-1 (Human T-lymphotropic virus 1) positive and HTLV-1 negative T-lymphocytes but did not exert any effect on lymphocytes removed from the peripheral blood of healthy individuals (Rizk, Maalouf, & Baydoun, 2009; Maalouf, Baydoun, & Rizk, 2011). To better understand its mechanism of action, the levels of expression of transforming growth factors (TGFs) in the HTLV-1 negative cell lines CEM and Jurkat were determined using RT-PCR. When the CEM and Jurkat cell lines were treated with kefir, the levels of TGF-α which is a mitogen decreased whereas those of TGF-β which is usually a tumor suppressor in normal cells increased which is consistent with the observed anti-proliferative and pro-apoptotic effects of kefir. Moreover, in these cell lines the levels of MMP-2 and MMP-9 were evaluated using RT-PCR to examine the metastatic ability of the leukemic cell lines. No difference in the levels of MMP-2 was observed between control and treated cells whereas MMP-9 was not detected in either the control or the treated samples but this doesn’t rule out the effect of kefir on the metastatic ability of these cells (Maalouf et al. 2011).

Recent work in our lab showed that kefir has anti-proliferative and pro-apoptotic effects on Caco-2 and HT-29 colorectal cell lines (unpublished data). This finding is important since earlier studies showed that the prevention of carcinogenesis by kefir and other probiotics was either through inducing changes in the intestinal microbiota that
inhibit the growth of bacteria converting pro-carcinogens into carcinogens or by modulating the immune response (LeBlac & Perdigo, 2010).

1.5.6.3. Effect on metastasis

Among the few studies that examine the anti-tumoral activity of kefir, only one work discusses the anti-metastatic ability of kefir on cancer cells in vivo. A study in 2000 by Furukawa et al. showed that when the water-soluble polysaccharide fraction from kefir grains is administered orally either before or after tumor transplantation it inhibited the pulmonary metastasis of Lewis lung carcinoma. On the other hand, the water insoluble fraction of kefir grains was able to prevent metastasis in mice injected with the highly metastatic B16 melanoma cells compared to control mice (Furukawa, Matsuoka, Takahashi, & Yamanaka, 2000).

1.6. Purpose of the study

Until present the mechanism of action of kefir remains elusive, therefore the aim of this study is to determine a possible mechanism of action for the anti-proliferative and pro-apoptotic effect seen upon the treatment of colorectal cancer cell lines with cell-free fractions of kefir. Moreover, since its effect on the motility and metastasis of cancer cells has not been thoroughly studied, the purpose of this study also involves determining the effect of cell-free fractions of kefir on the motility and invasion of colorectal and breast cancer cell in vitro.
Chapter II

Materials and Methods

2.1. Cell line and cell culture

Two human colorectal adenocarcinoma cell lines, Caco-2 and HT-29, were cultured in DMEM medium supplemented with 10% FBS and 100U penicillin/streptomycin at 37°C and 5% CO₂ in a humidified chamber. Human breast cancer cell lines (MCF-7 and MDA-MB231) obtained from ATCC (American Type Culture Collection), were cultured in DMEM medium supplemented with 10% FBS and 100U penicillin/streptomycin at 37°C and 5% CO₂ in a humidified chamber.

2.2 Preparation of kefir cell-free fraction and milk

Sterilized, pasteurized skimmed milk (150 ml) was inoculated with kefir grains (50 g). Inoculated milk samples were incubated at 20°C for 24 hours in a sealed glass container. At the end of fermentation, the milk was strained to remove the kefir grains. The yeast and bacteria in the filtrate were removed by centrifugation (35,000 rpm for 10 minutes at 4°C). The supernatant was stored at -20°C until needed for treatment of cells. On the day of treatment, the kefir was thawed and then passed through a 0.22-μm filter (Millipore, Billerica, MA). This cell free fraction of fermented milk, also termed kefir, was applied directly to the cells in different volumes to establish the different concentrations required.

Sterilized, pasteurized skimmed milk was centrifuged (35,000 rpm for 10 minutes at 4°C). The supernatant was stored at -20°C until needed for treatment of cells. On the day of treatment, the milk was thawed and then passed through a 0.45-μm filter and a 0.22-μm filter (Millipore, Billerica, MA) successively. The filtrate was applied directly to the cells in different volumes to establish the different concentrations required.
2.3 Antibodies and reagents

Mouse monoclonal IgG anti-β-Actin, anti-Bax, anti-Bcl2, and anti-p53 antibodies and rabbit polyclonal anti-p21 were obtained from Santa Cruz Biotechnology. Anti-mouse and anti-rabbit IgG HRP-conjugated secondary antibodies were obtained from Promega.

2.4. Western blotting

Cell lysates were prepared by scraping the cells in a sample buffer consisted of 4% SDS, 10% β-mercaptoethanol, 20% glycerol, 0.004% bromophenol blue, and 0.125 M Tris-HCl at a pH of 6.8. The resulting lysates were boiled for 5 minutes. Protein samples were separated by SDS-PAGE on 10% (for Actin and P53) or 12% (for Bax, Bcl-2, and P21) gels and transferred to PVDF membranes overnight at 30V. The membranes were then blocked with 5% BSA (Bovine Serum Albumin) in PBS containing 0.1% Tween-20 for 1 hour at room temperature and incubated with primary antibody at a concentration of 1:1000 for 2 hours at room temperature. After the incubation with the primary antibody, the membranes were washed and incubated with secondary antibody at a concentration of 1:1000 for 1 hour at room temperature. The membranes were then washed, and the bands visualized by treating the membranes with western blotting enhanced chemiluminescent reagent ECL (GE Healthcare). The results were obtained on an X-ray film (Agfa Healthcare). The levels of protein expression were compared by densitometry using the ImageJ software.

2.5. Reverse-transcription PCR

Cells were grown in 6-well plate at density of 1x10^6 cells/ml. After 24hrs, cells were treated with 0, 5, and 10% cell free fractions of kefir. 24hrs later, total RNA was extracted using RNeasy extraction kit (Qiagen) according to manufacturer’s instruction. Reverse transcriptase polymerase chain reaction (RT-PCR) was used to amplify RNA of β-actin, TGF-α and TGF-β. 2μg of RNA was converted to cDNA using the OneStep RT-PCR kit (Qiagen) as described by the manufacturer. All gene-specific primers designed to detect cDNA were obtained from Sigma-Aldrich. Primers for β-actin have the following sequences: Forward: 5’-
ATGAAGATCCTGACCGAGCGT-3’, Reverse: 5’-AACGCAGCTCAGTAACAGTCG-3’. Primers for TGF-α have a forward sequence of: 5’-ATGTTGTCCCTGCAAGTCC -3’ and a reverse sequence of: 5’-ACTATGGAGAGGGTGCTTT -3’. Primers for TGF-β Primers have a forward sequence of: 5’-GAAGTCACCCGCGTGCTAATGG -3’ and a reverse sequence of: 3’- GGATGTAAACCTCGGACCTGTGTG -5’. All primers were used at a final concentration of 0.6 µM. Primers were added to 5X Qiagen OneStep RT-PCR buffer providing a final concentration of 2.5 mM MgCl₂ in the reaction mix. A final concentration of 400µM of each dNTP was added along with 2.0 µL/reaction of enzyme mix. Final mastermix volume was adjusted to 50 µL using RNase-free water. Thermal cycler conditions, for both reverse transcription and PCR, was programmed as follows: reverse transcription at 50 °C for 30 min, initial PCR activation step at 95°C for 15 min, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at: 52 °C for β-actin, 48 °C for TGF-α and 50 °C for TGF-β1 for 1min, and extension at 72°C for 1min followed by a final extension step at 72 °C for 10min. From the PCR product, 10µL were run on 0.8% agarose gel stained with ethidium bromide at 100V for 30min. The resulting bands were visualized under UV light and photographed. β-actin was used as a loading control.

2.6. Wound Healing

Cells were grown to confluence on culture plates and a wound was made in the monolayer with a sterile pipette tip. After wounding, the cells were washed twice with PBS to remove debris and new medium was added. Phase-contrast images of the wounded area were taken at 0 and 24 hours after wounding. Wound widths were measured at 11 different points for each wound, and the average rate of wound closure was calculated in μm/hr using ImageJ software.

2.7. Motility assay

For motility analysis, images of cells moving randomly in serum were collected every 60 seconds for 2 hours using a 20X objective. During imaging, the temperature was controlled using a Nikon heating stage which was set at 37 °C. The
medium was buffered using HEPES and overlayed with mineral oil. The speed of cell movement was quantified using the ROI tracker plugin in the ImageJ software, which was used to calculate the total distance travelled by individual cells. The speed was then calculated by dividing this distance by the time (120 minutes) and reported in \( \mu \text{m/min} \). The speed of at least 10 cells for each condition was calculated.

2.8. Invasion assay

Cells were grown in 6-well plate. After 24 hrs, cells were treated with 0, 5, and 10% cell free fractions of kefir for another 24 hrs. Invasion assay was performed following treatment period using the collagen-based invasion assay (Millipore) according to manufacturer’s instructions. Briefly, 24hrs prior to assay, cells were starved with serum free medium. Cells were harvested, centrifuged and then resuspended in quenching medium (without serum). Cells were then brought to a concentration of 1x10^6 cells/ml. In the meantime, inserts were prewarmed with 300\( \mu \text{l} \) of serum free medium for 30min at room temperature. After rehydration, 250\( \mu \text{l} \) of media was removed from inserts and 250\( \mu \text{l} \) of cell suspension was added. Inserts were then placed in a 24-well plate, and 500\( \mu \text{l} \) of complete media (with 10% serum) was added to the lower wells. Plates were incubated for 24hrs at 37\(^\circ\)C in a CO\(_2\) incubator. Following incubation period, inserts were stained for 20min at room temperature with 400\( \mu \text{l} \) of cell stain provided with the kit. Stain was then extracted with extraction buffer (also provided). 100\( \mu \text{l} \) of extracted stain was then transferred to a 96-well plate suitable for colorimetric measurement using a plate reader. Optical Density was then measured at 560\( \mu \text{m} \).

2.8. Statistical analysis

All the results reported represent average values from three independent experiments. All error estimates are given as ± SEM. The p-values were calculated by t-tests or chi-square tests depending on the experiment using the VassarStats: Website for Statistical Computation (http://vassarstats.net/).
Chapter III

RESULTS

3.1. Kefir reduces the expression of TGF-α and TGF-β in HT-29 cells:

In order to determine a possible mechanism for the anti-proliferative effect of kefir observed in colorectal cancer cell lines, the expression of TGF-α and TGF-β1 was assessed at the mRNA level using RT-PCR in HT-29 cells. The results showed a decrease in the expression of both cytokines in a dose dependent manner upon kefir treating HT-29 cells for 24hrs (Figure 1A). Yet, the expression of both cytokines did not change in milk treated cells (Figure 1B).

Figure 1. Expression of TGF-α and TGF-β1 using reverse-transcription PCR in kefir and milk treated HT-29 cells. Figure 1A The expression of both cytokines in 0, 5, and 10% (v:v) kefir treated cells. Figure 1B The expression of both cytokines in 0, 5, and 10% (v:v) kefir treated cells.
3.2. Bax:Bcl-2 is increased in HT-29 cells

To determine a possible mechanism of action for the pro-apoptotic effect of kefir on colorectal cancer cells, the expression of Bax and Bcl-2 was assessed at the protein level using western blot analysis. We observed an increase in the Bax:Bcl-2 ratio upon kefir treatment while a slight decrease in this ratio was observed upon milk treatment.

![Western Blot analysis images for Bax and Bcl-2 in kefir treated cells](image1)

![Western Blot analysis images for Bax and Bcl-2 in milk treated cells](image2)

![Bar graph representing the fold increase in Bax:Bcl-2 expression in kefir and milk treated HT-29 cells relative to actin](image3)

Figure 2. Fold increase in Bax:Bcl-2 expression in kefir and milk treated HT-29 cells. Figure 2A Western Blot analysis images for Bax and Bcl-2 in kefir treated cells. Figure 2B Western Blot analysis images for Bax and Bcl-2 in milk treated cells. Figure 2C Bar graph representing the fold increase in Bax:Bcl-2 expression in kefir and milk treated HT-29 cells relative to actin.
3.3. P21 but not P53 levels are increased in HT-29 cells upon kefir treatment

To further elucidate the mechanism of action for the pro-apoptotic effect and anti-proliferative effect of kefir on colorectal cancer cells, the expression of P53 and P21 was assessed at the protein level using western blot analysis. The results showed no significant increase in the expression of P53 in kefir treated, yet p21 levels dramatically increased upon treating with 10% kefir. For milk treated cells, the expression of P53 and P21 does not vary between control and 10% treated cells.

Figure 3. The expression of P21 and P53 in kefir and milk treated HT-29. Figure 3A Western Blot analysis images for P21 and P53 in kefir treated cells. Figure 3B Western Blot analysis images for P21 and P53 in milk treated cells. Figure 3C Bar graph representing the fold increase in expression of P53 in kefir and milk treated cells relative to actin. Figure 3D Bar graph representing the fold increase in expression of P21 in kefir and milk treated cells relative to actin.
3.4. Kefir treatment does not affect the motility of HT-29 and Caco-2 cells

The effect of kefir on the metastatic ability of cancer cells was only assessed once by Furukawa et al. in 2000 and the study was done in vivo. Therefore, we wanted to determine whether there is any direct effect on the motility of cancer cells in vitro upon kefir treatment and we started by looking at the motility of colorectal cancer cell lines Caco-2 and HT-29 using wound healing analysis. We observed no difference in the migration ability of control and kefir treated cells after 24 hrs of treatment (Figure 4).

Figure 4. The migration ability of control and kefir-treated Caco-2 and HT-29 cells in vitro using wound healing analysis. Figure 4A Images from the wound healing assay measuring the motility of HT-29 cells. Figure 4B Quantitation of the migration rate of figure 4A. Figure 4C Images from the wound healing assay measuring the motility of Caco-2 cells. Figure 4D Quantitation of the migration rate of figure 4C. Data are the mean ± SEM from 3 experiments. The results were not significant with p>0.05
3.5. Kefir treatment does not affect the motility of MCF-7 and MDA-MB-231 cells

After observing no difference in the motility of CRC cells upon kefir treatment, we decided to explore whether kefir might induce an effect on the motility of other cancer types in vitro. Therefore we chose to look at MCF-7, breast cancer cells lines. We chose these cell lines in particular since the effect of cell-free fractions of kefir on MCF-7 has been previously established in 2007 by Chen et al. and it was also observed that kefir hindered the proliferative ability of these cells similar to our findings. And since we were looking at breast cancer cell lines, we also chose to explore the effect of kefir on another breast cancer cell line which is MDA-MB-231 cell line. In both of these cell lines, we observed a slight decrease in the migration ability between control and treated cells but the decrease was non-significant.
After observing no effect on the migration ability of MCF-7 and MDA-MB-231 using the wound healing assay, we decided to assess the effect of kefir on their migration using a different assay known as time-lapse movie. Consistent with our previous results, we observed a slight decrease in the migration ability of both cell lines upon kefir treatment, yet the decrease was also non-significant.

**Figure 6.** The migration ability of control and kefir-treated MCF-7 and MDA-MB-231 cells *in vitro* using time-lapse movies. Figure 6A Migration path of the MCF-7 cells randomly moving in serum complete media for 2 hrs. Figure 6B. Bar graphs measuring the migration rate for the path traveled by MCF-7 cells in figure 6A. Figure 6C Migration path of the MDA-MB-231 cells randomly moving in serum complete media for 2 hrs. Figure 6D. Bar graphs measuring the migration rate for the path traveled by MDA-MB-231 cells in figure 6C. Data are the mean ± SEM from 3 experiments. The results were not significant with p > 0.05
3.6. *In vitro* invasion of HT-29 cells is not affected by kefir treatment.

After looking at the motility of colorectal and breast cancer cell lines in two-dimensions and observing no effect upon kefir treatment, we decided to look at whether kefir has any effect on the invasive ability of the colorectal cancer cell line HT-29. Using the collagen-based invasion assay, we noticed no difference in the invasive ability of HT-29 cell lines between control and 10% kefir treated cells.

![Figure 7](image.png)

**Figure 7.** The invasive ability of control and kefir-treated HT-29 cells *in vitro*. Figure 7A Bar graph showing the absorbance of the stain retained by the invasive cells at 560nm which reflects the amount of invading cells. Figure 7B Representative images showing the collagen invading cells in purple which were seeded at a concentration of 1x10^6 cells/ml and allowed to migrate toward 10% FBS containing media. Data are the mean ± SEM from 3 experiments. The results were not significant with p> 0.05
Chapter IV

DISCUSSION

It was always assumed among the Bulgarian farmers that kefir held special healing powers, and they believed that their longevity was attributed to their ingestion of kefir (Liu et al. 2002). Although kefir has been made in the Caucasus Mountains for hundreds of years, it was kept in secrecy within tribes who held it as their legacy, and not until recently has kefir grains been distributed to different regions of the world (Farnworth, 2005). Since then many studies have been made to confirm whether kefir has any health benefits, and these studies showed that kefir is able to enhance the immune system, inhibit the growth of pathogenic bacteria and fungi, decrease serum cholesterol levels, and most importantly have antitumoral activity among other beneficial effects (Vinderola, et al., 2006a.; Liu et al. 2006; Rodrigues, et al. 2005; Chen, et al., 2007). Studies done in vivo showed that kefir is able to prevent the growth of tumors such as Sarcoma 180, Ehrlich carcinoma, as well Lewis lung carcinoma (Murofushi, et al., 1983; Shiomi, et al., 1982). Yet its mechanism of action remained elusive and some data showed that this effect could be indirectly induced by kefir through the modulation of the immune system (Murofushi, et al., 1986). Since 2007, in vitro studies testing the effect of cell free fraction on breast and T-lymphocyte cancer cell lines showed that kefir has anti-proliferative and pro-apoptotic effects on these cell lines (Chen et al. 2007; ). And similarly in 2009, a study performed by Rizk et al. showed the same effect on T-lymphocytes (Rizk, et al., 2009; Maalouf, et al., 2011). Our lab have recently showed that kefir have the same effects on Caco-2 and HT-29 colorectal cell lines. So we aimed at trying to determine its possible mechanism of action.

The expression of TGF-α and TGF-β1 at the mRNA level was first assessed to determine a possible explanation for the anti-proliferative effect of kefir. TGF-α is a known mitogen usually seen upregulated in many types of tumors especially CRC where its expression exceeds four times that of normal colorectal tissues. TGF-α expression was downregulated in HT-29 cells upon kefir treatment in a dose dependent manner with 5 and
10% kefir (v:v) (Ji, et al., 2006; Liu, et al., 1990) (Figure 1A). These results are consistent with previous results in our lab showing a decrease in the proliferation of HT-29 and consistent with a previously published paper by Maalouf et al. in 2011. Moreover, such observation is important knowing that TGF-α also plays a significant role in invasion and metastasis *in vivo* through the recruitment of cells expressing VEGF, interleukins, as well as MMPs, and when the TGF-α/EGFR receptor was targeted by antibodies, the invasive ability of CRC cells was abolished, (Sasaki, et al., 2008; Ueda, et al., 1998; Hölting, et al., 1995). This further shows the importance of targeting TGF-α not only to inhibit cancer growth but also its invasion and metastasis.

A downregulation in the expression level of TGF-β1 was noticed in HT-29 cells upon kefir treatment in a dose dependent manner (Figure 1A). These data were perplexing at the beginning since they contradicted a previously published paper by Maalouf at el. in 2011 on HTLV-negative T-lymphocytes that showed an upregulation in TGF-β1 upon kefir treatment. TGF-β1 has long been assumed to act as a tumor suppressor yet many studies have shown data conflicting with its known role. Therefore recent studies found explanations for its dual role and demonstrated that the response of a cell to TGF-β1 is context dependent and is affected by the epigenetic status of the cell, the proteins present in the signaling pathways, and by transcription factors (Massagué, 2012). In normal cells it is a tumor suppressor but in many cancers its overexpression is exploited by the cells and used as a mitogen (Massagué, 2008). In CRC TGF-β1 expression significantly increases with increased invasion and metastasis, therefore it could be an important target to limit its growth (Xiong, et al., 2002). Not only does it affect growth but TGF-β1 suppresses immune system cells in the microenvironment, recruits cells required for the invasion of CRC cells, induces EMT transition, and stimulates angiogenesis (Thiery, et al., 2012; Ramesh, et al., 2009). Therefore our data might be of great significance especially that drugs targeting the TGF-β signaling pathway especially through inhibiting the expression of TGF-β have now reached phase III clinical trials and the only observed side effect in clinical trials is the formation of non-malignant skin lesions that disappear after the withdrawal from the treatment (Akhurst & Hata, 2012; Morris, J. C. et al, 2008). The decrease of both cytokines was only observed in kefir and not milk treated cells which
confirms that it is either a single or a combination of end products from the fermentation which are inducing the effect and not components originally found in milk (Figure 1).

To determine a possible explanation for the pro-apoptotic effect of kefir on CRC cell lines, we looked at the expression of Bax:Bcl-2 at the protein level using western blot analysis. Both of these proteins belong to the Bcl-2 family of protein where Bax acts as a pro-apoptotic protein whereas Bcl-2 acts as an anti-apoptotic protein (Wang, et al., 2009). It is generally considered that an increase in proportion of pro- to anti-apoptotic proteins signals a cell to undergo apoptosis (Ola, et al., 2011). In our findings, we noticed an upregulation in Bax:Bcl-2 expression in HT-29 cells consistent with the observed increase in apoptosis previously confirmed by our lab through the Cell-death ELISA based kit. For the milk treated cells, a decrease in Bax:Bcl-2 ratio was observed which is also in accordance with the previously proven data in our lab showing a decrease in apoptosis and increase in proliferation in milk treated HT-29 cells (Figure 2).

To further elucidate the mechanism of action of kefir, the expression levels of P53 and P21 proteins were assessed. P53 is known to induce cell cycle arrest and DNA repair and might also cause apoptosis in extremely damaged cells whereas P21, a target of P53, is a known cell cycle inhibitor that locks cells in the G1 phase (Oren & Rotter, 1999; Harper, et al., 1993). For milk treated cells a slight decrease in P21 levels was observed. Treating cells with 5 and 10% kefir (v:v) led to a very slight increase in P53 levels but a dramatic increase in P21 levels when treated with 10% kefir (v:v) (Figure 3). This increase in P21 levels might explain the cell cycle arrest observed at the G1 phase previously in our lab through cell cycle analysis by flow cytometry. Also our data suggest that P21 induction is P53-independent (Macleod, et al., 1995). Unpublished data in our lab also showed that kefir induce apoptosis in leukemic T-lymphocyte cell lines in a p-53 independent manner.

Since metastasis is the main cause of death in cancer patients, and since the effect of kefir on metastasis has only been studied once before by Furukawa et al. in 2000, we wanted to assess the effect of cell-free fractions of kefir on the motility of CRC cancer cell
lines *in vitro* (Palmer, et al., 2011). Using wound-healing analysis, no difference in motility between the control and 10% kefir treated HT-29 and Caco-2 CRC cells was observed (Figure 4), yet these data contradict the previously published study in 2000 which showed that kefir inhibited metastasis of Lewis lung carcinoma and B16 melanoma.

The effect of cell free fractions of kefir were then assessed on weakly metastatic MCF-7 and highly metastatic MDA-MB-231 breast cancer cell lines. Using both time-lapse movies and wound-healing assays to look at the motility of these two cells lines, we noticed a slight decrease in the motility of MCF-7 and MDA-MB-231 yet the decrease was non-significant (Figure 5 & 6). Yet metastasis does not depends only on the motility of the cells but also involves invasion of the microenvironment, intravasation into blood or lymphatic vessels, survival in the blood or lymph, extravasion, and colonization at secondary sites (Fidler, 2003). Therefore, to determine whether kefir might be modulating the invasive ability of the cells as a mechanism for inhibiting metastasis, a collagen-based in vitro assay was performed. A decrease in the invasive ability of HT-29 cells was expected since a downregulation in the expression of TGF-α and TGF-β1 was observed upon kefir treatment, and knowing that they both play an important role in enhancing the invasive ability of cancer cells (Hölting, et al., 1995; Xiong, et al., 2002). Yet after performing the collagen-based invasion assay *in vitro* and no difference in the invasive ability between control and 10% treated cells was noticed (Figure 7). Thus an explanation for the contradiction observed between the motility and invasion data *in vitro* and that of the published article in 2000 could be explained by several hypotheses. One explanation could be that kefir is acting indirectly to inhibit metastasis *in vivo* by modulating the immune system. Another hypothesis could be that kefir might be intervening in other processes involved in metastasis beside motility and invasion. A third possibility is that components found in kefir might be modified in our body by enzymes leading to the formation of active compounds which are able to inhibit metastasis *in vivo*.
Chapter V

CONCLUSION

In this study we were the first to elucidate a potential mechanism of action for kefir’s effect on colorectal cancer in vitro as well as its effect on the motility and invasion. The downregulation in the expression of TGF-α and TGF-β1 explain the decrease in the proliferation of HT-29 cells in vitro. Also the observed overexpression of p21 which was seen to be p53-independent could be the reason behind the cell cycle arrest at the G1 phase previously seen upon kefir treatment. Moreover, we have seen that the expression of Bax to Bcl-2 at the protein level increases upon kefir treatment consistent with the increase in apoptosis induced by kefir. On the other hand, the effect of kefir on the motility and invasion of colorectal and breast cancer cell lines was insignificant, but this does not rule out the fact that kefir does have an effect on the metastatic ability of cancer cells. Kefir’s effect on metastasis could be by modulating other steps in the metastatic process or that its effect is only observed in vivo. Future plans are to confirm the effect of kefir on the growth of colorectal cancer in vivo and also to assess its effect on the metastasis of this cancer through the intrasplenic injection of HT-29 that causes liver metastasis.


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