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**Kefir Inhibits Proliferation and Induces Apoptosis of
Epithelial Colorectal Adenocarcinoma Cells**

By

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

School of Arts and Sciences

August 2011



Thesis approval Form (Annex III)

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ACKNOWLEDGEMENTS

First and foremost, I am genuinely thankful for Dr. Sandra Rizk-Jamati. Throughout all this time she has shown me continuous support, encouragement, and dedication. I am sincerely grateful to have had worked under her supervision, because as a person, and as an advisor, Dr. Sandra was considerate and devoted. She always believed in me and she was fully committed to me and this project.

I would also like to convey my appreciation to the Dean of Arts and Sciences Dr. Fouad Hashwa, the Chairperson of the Biology department Dr. Costantine Daher, the faculty members of the Biology department Dr. Sima Tokajian, Dr. Roy Khalaf, Dr. Pierre Zalloua, Dr. Brigitte Wex and Dr. Ralph Abi-Habib, and to the committee members Dr. Mirvat El-Sibai and Dr. Roula Abdel Massih.

I thank Ms. Maya Farah and Mrs. Helena Bou Farah for their technical support.

Finally special thanks go to my family and friends without whom none of this would be possible.

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Stephany E. El-Hayek

ABSTRACT

Kefir is a fermented milk product, to which many health benefits have been attributed. These include anti-microbial, anti-inflammatory, immunomodulatory, and metabolic benefits. Most importantly, many studies have proven that kefir may have anti-cancerous potential. The current study aims at examining the effect of this probiotic on colorectal cancer cells, *in vitro*. Kefir's anti-cancer potential was tested on Caco-2 and HT-29, colorectal adenocarcinoma cell lines, through cytotoxicity, proliferation, and apoptotic assays. Kefir's cytotoxicity was assessed by determining the percentage viability of cells, using the Trypan Blue Exclusion method. After determining the IC₅₀, the effect of various concentrations of kefir on the proliferation of Caco-2 and HT-29 cells was determined. Using WST-1 cell proliferation assay, kefir was shown to be anti-proliferative to the cells in a dose and time dependent manner. Through flow cytometry, kefir was also shown to induce cell cycle arrest at the G1 phase, which was assumed to be an apoptotic effect. This was then confirmed by Cell Death Elisa Plus kit, which showed that apoptosis increased with increasing kefir concentration. Western Blot analysis demonstrated that kefir induces the overexpression of Bax, while repressing Bcl-2.

Keywords: Kefir, Colorectal Cancer, Apoptosis, Bax, Bcl-2

TABLE OF CONTENTS

List of figures	x
List of tables	xi
List of abbreviations	xii
1. Literature review	1-21
1.1. Kefir	1
1.1.1. Introduction	1
1.1.2. The origin of Kefir	1
1.2. Kefir grains	2
1.2.1. Microbiology of Kefir grains	2
1.2.2. Metabolic activity of the co-cultures	4
1.3. Preparation of Kefir	5
1.4. Characteristics of Kefir	6
1.5. Chemical composition of Kefir	7
1.6. The matrix	8
1.6.1. Kefiran	8
1.7. Health benefits of Kefir	9
1.7.1. Probiotics	9
1.7.2. Kefir: a complex probiotic	10
1.7.3. Nutritional and gastrointestinal benefits	11
1.7.4. Antimicrobial effect of Kefir	11
1.7.5. Immunostimulatory effects of Kefir	12
1.7.6. Kefir's antimutagenic effect	14
1.7.7. Kefir's anticancerous effect	14
1.8. Colorectal cancer	16
1.8.1. Introduction	16
1.8.2. Incidence and prevalence of CRC	16

1.8.3. Diagnosis and screening	18
1.8.4. Polymorphisms related CRC	18
1.8.5. Treatment of CRC	19
1.8.6. CRC and diet	20
1.9. The apoptotic pathway	21
1.10. Purpose of the study	21
2. Materials and methods	21-26
2.1. Cell lines and culture	22
2.2. Preparation of kefir cell-free Fraction and milk	22
2.3. Antibodies and reagents	22
2.4. Cytotoxicity: Trypan Blue Exclusion Method	23
2.5. Proliferation: Cell Proliferation Reagent (WST-1)	23
2.6. Cell Cycle Analysis: Flow Cytometry	24
2.7. Apoptosis: Cell Death Elisa Kit	24
2.8. Western Blot Analysis	25
2.9. Statistical Analysis	26
3. Results	27-42
3.1. Cytotoxicity of Kefir	27
3.2. Kefir treatment reduces proliferation of Caco-2 and HT-29 cells	32
3.3. Kefir induces cell cycle arrest at the G1 phase	37
3.4. Kefir has a proapoptotic effect on Caco-2 and HT-29 cells	39
3.5. Kefir induces apoptosis in a Bax/Bcl-2 dependent manner	42
4. Discussion	43-48
5. Conclusion	49
6. Bibliography	50-57

LIST OF FIGURES

Figure	Page
Figure 1: Scanning electron micrograph of a kefir grain	4
Figure 2: Simplified model of lactose metabolism in bacteria and yeast cultures of kefir grains	5
Figure 3: Structure of kefiran	9
Figure 4: Possible active ingredients for kefir's beneficial effects	14
Figure 5: Trends in colorectal cancer mortality rates for selected countries in males, 1985 through 2005	17
Figure 6: Drugs used for targeted therapy for CRC	19
Figure 7: Targeted therapy for CRC.	20
Figure 8A: Viability of Caco-2 cells after 24 hours of treatment with different concentrations of milk and kefir.	28
Figure 8B: Viability of Caco-2 cells after 48 hours of treatment with different concentrations of milk and kefir.	28
Figure 8C: Viability of Caco-2 cells after 72 hours of treatment with different concentrations of milk and kefir.	29
Figure 9A: Viability of HT-29 cells after 24 hours of treatment with different concentrations of milk and kefir.	30
Figure 9B: Viability of HT-29 cells after 48 hours of treatment with different concentrations of milk and kefir.	30
Figure 9C: Viability of HT-29 cells after 72 hours of treatment with different concentrations of milk and kefir.	31
Figure 10A: Proliferation of HT-29 cells after 24 hours of treatment with different concentrations of milk and kefir.	33
Figure 10B: Proliferation of HT-29 cells after 48 hours of treatment with different concentrations of milk and kefir.	33
Figure 10C: Proliferation of HT-29 cells after 72 hours of treatment with different concentrations of milk and kefir.	34

Figure 11A: Proliferation of HT-29 cells after 24 hours of treatment with different concentrations of milk and kefir.	35
Figure 11B: Proliferation of HT-29 cells after 48 hours of treatment with different concentrations of milk and kefir	35
Figure 11C: Proliferation of HT-29 cells after 72 hours of treatment with different concentrations of milk and kefir.	36
Figure 12: Cell cycle analysis of milk and kefir treated cells.	38
Figure 13A: Induction of apoptosis in Caco-2 cells 24 hours after kefir treatment	40
Figure 13B: Induction of apoptosis in Caco-2 cells 48 hours after kefir treatment	40
Figure 14A: Induction of apoptosis in HT-29 cells 24 hours after kefir treatment	41
Figure 14B: Induction of apoptosis in Caco-2 cells 48 hours after kefir treatment	41
Figure 15: Western Blot analysis for Bax and Bcl-2	42
Figure 16: Possible pathway for kefir's proapoptotic role	46

LIST OF TABLES

Table	Page
Table 1: Characteristics of kefir	7
Table 2: Genetic alterations associated with current colon cancer therapies	18

LIST OF ABBREVIATIONS

DNA: Deoxyribonucleic acid

PCR: polymerase chain reaction

LDL: Low-density lipoprotein

HDL: High-density lipoprotein

Ig A: Immunoglobulin A

IL-4: Interleukin-4

IL-6: Interleukin-6

IL-10: Interleukin-10

TNF- α : Tumor necrosis factor-alpha

IFN- γ : Interferon-gamma

IL-1- α : Interleukin-1 alpha

Ig E: Immunoglobulin E

IL-12: Interleukin-12

Th-1: T helper 1

Cd2: cluster of differentiation 2

STAT 4: Signal Transducer and Activator of Transcription 4

IFNR: Interferon receptor

H&E: hematoxylin and eosin

TUNEL: Terminal deoxynucleotidyl transferase dUTP nick end labeling

UV: Ultraviolet

ROS: reactive oxygen species

DTH: Delayed-type hypersensitivity

MCF-7: Michigan Cancer Foundation-7

HTLV-1: Human T-lymphotropic virus 1

CRC: Colorectal cancer

UGT1A1: UDP glucuronosyltransferase 1 family, polypeptide A1

XPD: xeroderma pigmentosum group D

XRCC-1: X-ray repair complementing defective repair in Chinese hamster cells 1

MGMT: Methylated-DNA-protein-cysteine methyltransferase

VEGF: Vascular endothelial growth factor

FC- γ -RIIa: Low affinity immunoglobulin gamma Fc region receptor II-a

GSTP-1: Glutathione S-transferase P 1

EGFR: Epidermal growth factor receptor

EGF: Epidermal Growth Factor

TGF- α : Transforming growth factor alpha

Ras: Rat sarcoma

PI3K: Phosphatidylinositol 3-kinase

PKC: Protein kinase C

VEGFR: Vascular endothelial growth factor receptor

mTOR: mammalian target of rapamycin

Src: Schmidt-Ruppin A-2

KSP: kinesin spindle protein

K-Ras: Kirsten rat sarcoma

DMEM: Dulbecco's Modified Eagle Medium

FBS: Fetal Bovine Serum

UHT: Ultra high temperature

Bax: Bcl-2-associated X protein

Bcl-2: B-cell lymphoma 2

HRP: horseradish peroxidase

PI: Propidium Iodide

PBS: Phosphate Buffered Saline

G0: Gap 0

G1: Gap 1

ELISA: Enzyme-linked immunosorbent assay

ABTS: 2, 2'-azino-di(3-ethylbenzthiazolin-sulfonate)

SDS-PAGE: Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

PVDF: Polyvinylidene fluoride

ECL: Enhanced Chemiluminescence

IC50: Inhibitory concentration 50

AMPK: AMP-activated protein kinase

p53: protein 53

PARP: Poly (ADP-ribose) polymerase

RT-PCR: Reverse Transcription Polymerase Chain Reaction

MMP: Matrix metalloproteinase

Chapter I

Literature Review

1.1 Kefir

1.1.1 Introduction

Kefir is a well-known fermented milk product, obtained by the fermentation of milk with kefir grains. It is highly consumed in many countries, mostly in Eastern Europe, but also in Asia and America (Adriana & Socaciu, 2008; Farnworth, 2005). Around the world, kefir is manufactured and marketed as a refreshing, slightly alcoholic beverage, under different names (Kephir, Kiaphur, Kefer, Kepi and Kippi) (Adriana & Socaciu, 2008; Angulo, Lopez, & Lema, 1993; Farnworth, 2005). The making of kefir dates back to earlier centuries, and since then, many health benefits have been attributed to it (Farnworth, 2005). In its modern, consumable form, kefir is a beverage of viscous nature. In content, it is slightly carbonated, alcoholic, and acidic to some extent (Adriana & Socaciu, 2008; Angulo, et al., 1993; Koroleva, 1988; Simova, et al., 2002). Nowadays, kefir is recognized as a complex probiotic, and is under study for many potential in-vivo and in-vitro positive health effects.

1.1.2 The origin of kefir

The initial discovery of kefir is said to have been accidental, where milk stored in bags made of animal skin, underwent natural and spontaneous fermentation (Farnworth, 2005; Guzel-Seydem, Seydem, Greene, Tas, & 2006). Kefir originated in the Caucasian mountains of the former USSR, and since then it has been associated with general well-being and good health (Adriana & Socaciu, 2008; Angulo, et al., 1993; Farnworth, 2005). The name is derived from the Turkish word “keif”, meaning “good-feeling” (Adriana & Socaciu, 2008; Powell, 2006). Kefir grains, which are used to make kefir, and which contain a mixture of various bacteria and

yeast species, were considered a sacred gift from the Prophet Mohammad to his people (Powell, 2006).

1.2 Kefir grains

1.2.1. Microbiology of kefir grains

Kefir grains comprise a mutualistic symbiotic relationship between around 50 types of microorganisms, in a matrix of proteins and polysaccharides (Figure 1) (Abraham & De Antoni, 1999; Adriana & Socaciu, 2008; Angulo, et al., 1993; H. C. Chen, Wang, & Chen, 2008; Garrote, et al., 2004; Simova, et al., 2002). The symbiosis is extensively strong, where some studies showed that pure cultures of certain microorganisms in milk show reduced viability and/or low biochemical activity (Garrote, et al., 2004). The identification and isolation of the various types of bacteria and yeast found in kefir grains have been rendered difficult by the strong association between the many microorganisms. Proteomics, biochemical assays, and various molecular techniques (such as, PCR-denaturing gradient gel electrophoresis and 16S ribosomal DNA sequencing) have aided in unveiling the identity of many of these microorganisms (Adriana & Socaciu, 2008; H. C. Chen, et al., 2008; Garrote, et al., 2004; Simova, et al., 2002). Bacteria found in kefir grains include lactose-fermenting, acetic acid, mesophilic, thermophilic, lactobacilli, lactococci, streptococci, enterococci, leuconostocs, among other types of bacteria (Abraham & De Antoni, 1999; Adriana & Socaciu, 2008; Angulo, et al., 1993; H. C. Chen, et al., 2008; Garrote, et al., 2004; Simova, et al., 2002). Following is a list of most, but not all, bacterial species that constitute kefir grains: *Lactobacillus kefir*, *Lactobacillus delbrueckii*, *Lactobacillus kefiranofaciens*, *Lactobacillus rhamnosus*, *Lactobacillus kefirgranum*, *Lactobacillus casei*, *Lactobacillus parakefir*, *Lactobacilli paracasei*, *Lactobacillus brevis*, *Lactobacillus fructivorans*, *Lactobacillus bulgaricus*, *Lactobacillus plantarum*, *Lactobacillus hilgardii*, *Lactobacillus helveticus*, *Lactobacillus fermentum*, *Lactobacillus acidophilus*, *Lactobacillus viridescens*, *Lactococcus lactis subsp. lactis*, *Lactococcus lactis subsp. cremoris*, *Streptococcus thermophilus*, *Enterococcus durans*, *Leuconostoc sp.*, *Leuconostoc mesenteroides* (reported as *Leuconostoc kefir* by Ottogalli, Galli, Resmini, & Volonterio, 1973), *Acetobacter sp.*, *Acetobacter pasteurianus* (reported as *Acetobacter rancens* by Ottogalli et al. 1973), *Acetobacter aceti*, *Bacillus sp.*, *Micrococcus sp.*, *Bacillus subtilis*, and

Escherichia coli (Abraham & De Antoni, 1999; Adriana & Socaciu, 2008; Angulo, et al., 1993; H. C. Chen, et al., 2008; Garrote, et al., 2004; Simova, et al., 2002). Among all these, lactic acid bacteria comprise the majority of kefir grains bacterial species (Simova, et al., 2002). Among the Lactobacillus species, *L. bulgaricus* and *L. helveticus* are the dominant species (Simova, et al., 2002).

Kefir yeasts, on the other hand, are less well characterized than their prokaryotic partners in symbiosis (Adriana & Socaciu, 2008). They make up around 15% of the microorganisms in kefir grains (Simova, et al., 2002). Kefir grains host a diverse community that includes but is not limited to:

Kluyveromyces marxianus, *Candida friedrichii* (reported as *Saccharomyces lactis* by Ottogalli et al. 1973) and as *Kluyveromyces lactis* by Angulo et al. 1993), *Saccharomyces sp.*, *Candida pseudotropicalis*, *Saccharomyces cerevisiae*, *Candida tenuis*, (reported as *Saccharomyces carlbergensis* by Ottogalli et al. 1973), *Saccharomyces unisporus*, *Candida inconspicua*, *Saccharomyces exiguus*, *Candida maris* (reported as *Torolopsis holmii* by Iwasawa et al. 1982), *Saccharomyces turicensis*, *Candida lambica*, *Saccharomyces delbrueckii*, *Candida tannotelerans*, *Saccharomyces dairens*, *Candida valida*, *Torulasporea delbrueckii*, *Candida kefir*, *Brettanomyces anomalus*, *Candida holmii*, *Issatchenkia occidentalis*, *Yarrowia lipolytica* and *Pichia fermentans* (Abraham & De Antoni, 1999; Adriana & Socaciu, 2008; Angulo, et al., 1993; Marshall & Wendy, 1985; Simova, et al., 2002). Non-lactose fermenting yeasts make up the majority of the fungal species within kefir grains (Simova, et al., 2002). The diverse types of yeasts contribute to the symbiotic relationship through producing compounds that act as growth stimulants for the bacteria (Adriana & Socaciu, 2008) .

It is important to note, however, that the microflora of kefir grains from different sources exhibit some discrepancies in composition (Abraham & De Antoni, 1999; Angulo, et al., 1993; Farnworth, 2005). In addition, during the production of kefir, the microflora of the grains used for inoculation is seldom different from the microflora of the final fermented product. This further complicates the microbiological study of kefir, and renders comparison of experimental results obtained using different kefir grains difficult (Farnworth, 2005; Garrote, Abraham, & De Antoni, 2000; Golowczyc, Mobili, Garrote, Abraham, & De Antoni, 2007; Marshall & Wendy, 1985).

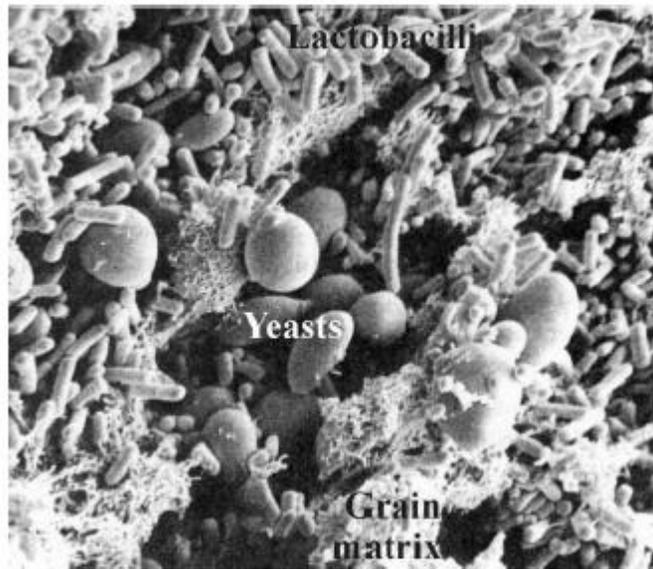


Figure1: Scanning electron micrograph of a kefir grain.
Source: Adriana & Socaciu, 2008

1.2.2 Metabolic activity of the co-cultures

Among the bacterial and yeast species found within kefir grains, there are some which utilize and/or ferment lactose, and those that depend on energy sources other than lactose (Adriana & Socaciu, 2008). Lactose-fermenting yeasts degrade lactose directly, converting it into ethanol and carbon dioxide. Lactic acid bacteria, which possess the hydrolyzing enzyme β -galactosidase, convert the disaccharide into galactose and glucose. The released monomers are the substrates on which the non-lactose fermenting yeasts depend for energy. These fungal species convert the monosaccharides into ethanol (Adriana & Socaciu, 2008; Farnworth, 2005). Ethanol, along with acetyldehyde, diacetyl, acetic acid, and carbon dioxide are all products resulting from yeast metabolic reactions (Figure 2) (Adriana & Socaciu, 2008). Each of these compounds contributes to the taste, aroma, and texture of kefir (Adriana & Socaciu, 2008; Farnworth, 2005; Simova, et al., 2002).

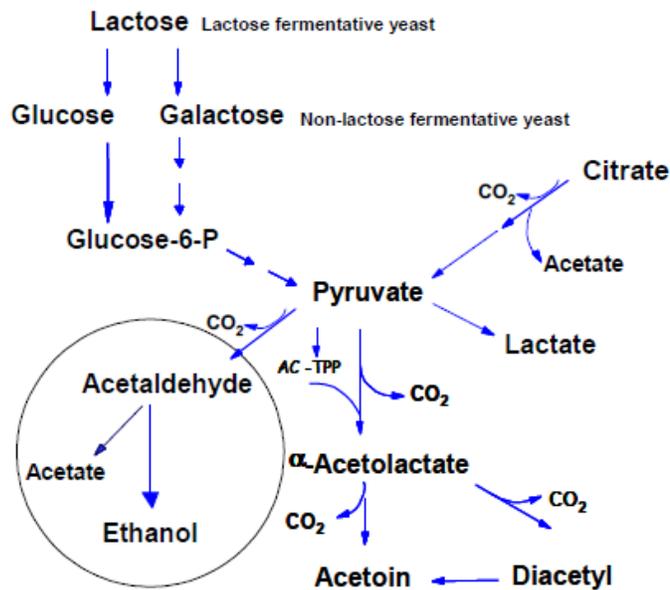


Figure 2: Simplified model of lactose metabolism in bacteria and yeast cultures of kefir grains.
Source: Adriana & Socaciu, 2008

1.3 Preparation of kefir

Traditionally obtained by the fermentation of cow milk, nowadays, kefir can commercially be made from several types of milk, such as goat or soymilk. Fermentation is achieved by inoculating pasteurized milk with kefir grains, which are small, white grains of irregular shape, ranging in size between 1 to 3 cm. They are insoluble in water and look like the florets of a cauliflower plant (Abraham & De Antoni, 1999; Adriana & Socaciu, 2008; Angulo, et al., 1993; Farnworth, 2005; Marshall & Wendy, 1985). During fermentation, which typically takes around 24 hours at 20-23°C, kefir grains undergo an approximate 25% increase in mass (Angulo, et al., 1993; Marshall & Wendy, 1985). Kefir grains can be reused for subsequent inoculation; their viability is only maintained through their continuous inoculation into fresh milk (Adriana & Socaciu, 2008; Farnworth, 2005; Marshall & Wendy, 1985; Simova, et al., 2002). At the beginning of the fermentation process, lactic acid streptococci bacteria multiply exponentially. This causes a reduction in the pH, which in turn favors the growth of lactobacilli, and inhibits the growth of the streptococci. Meanwhile, compounds produced by the yeasts in the mixture, allow for the further growth of aroma-producing heterofermentative streptococci. Towards the

end of the fermentation process, lactic acid bacterial growth expands as that of acetic acid bacteria and yeasts declines, which defines the composition of the fermented milk product (Koroleva, 1988; Marshall & Wendy, 1985; Simova, et al., 2002).

1.4. Characteristics of kefir

The complex and dynamic nature of the microbial population that makes up kefir grains is associated with many of kefir's physical and chemical properties (Abraham & De Antoni, 1999; Adriana & Socaciu, 2008; Angulo, et al., 1993; Marshall & Wendy, 1985). The pH, viscosity, taste, and chemical constitution of the final fermented product are all a function of the bacteria and yeast within the kefir grains that were used to ferment the milk (Table 1). These characteristics are also affected by the conditions used for the process of fermentation, which may include duration of fermentation, size of initial inoculums used, temperature, and agitation, as well as by the type of milk used (Abraham & De Antoni, 1999; Adriana & Socaciu, 2008; Angulo, et al., 1993; Marshall & Wendy, 1985; Simova, et al., 2002). Kefir is a refreshing, slightly alcoholic, carbonated, and acidic drink (Adriana & Socaciu, 2008; Angulo, et al., 1993; Koroleva, 1988; Marshall & Wendy, 1985; Simova, et al., 2002). In regards to taste and flavor, kefir has a yeasty mouth-feel and a slightly pungent and acidic taste. It is a viscous and creamy drink (Adriana & Socaciu, 2008; Koroleva, 1988; Marshall & Wendy, 1985; Simova, et al., 2002). The alcoholic content of kefir has been shown to vary according to preparation methods and other variables, but usually ranges between 0.01-2% (Koroleva, 1988; Powell, 2006; Simova, et al., 2002). With respect to acidity, the pH of kefir lies between 4.1-4.6 (Adriana & Socaciu, 2008; Farnworth, 2005; Powell, 2006). Ethanol, which also affects the taste, is produced primarily by yeast fermentation, and is what gives kefir its alcoholic nature. Carbon dioxide, although tasteless, brings out the tingling feel, characteristic of kefir. Diacetyl and acetyldehyde, intermediates in kefir grains metabolism, contribute to the flavor and viscosity of the fermented milk product. Acetic acid, another metabolic product, also affects taste and flavor (Adriana & Socaciu, 2008; Farnworth, 2005).

Component	Kefir
pH	4.5±0.26
Lactic acid (g l ⁻¹)	8.18±0.61
Alcohol (%)	0.25±0.04
Carbon dioxide (g l ⁻¹)	0.85±0.15
Mesophilic streptococci (CFU ml ⁻¹)	8 (±4.58).10 ⁹
Thermophilic streptococci (CFU ml ⁻¹)	3 (±2,64).10 ⁶
Homofermentative lactobacilli (CFU ml ⁻¹)	8 (±2,64).10 ⁶
Yeasts (CFU ml ⁻¹)	2 (±2,64).10 ⁵
Viscosity (cst)	1.075±0,21
Aroma	strong, typically yeasty
Flavour	kefir-specific, refreshingly pungent
Texture	homogeneous, creamy, abundantly effervescent

Table 1: Characteristics of kefir
Source: Simova, et al., 2002

1.5 Chemical composition of kefir

Regarding chemical composition, several studies have suggested that kefir contains essential vitamins; however a consensus about the identity of these vitamins has yet to be reached, and the composition may differ according to preparation. Vitamins that may be found in kefir include: folic acid, biotin, B12, B1, B2, thiamine, and pyridoxine (Farnworth, 2005; Powell, 2006; Simova, et al., 2002). Compared to unfermented milk, kefir is a richer source of vitamins (Powell, 2006). On the other hand, the amino acid composition of kefir is known to include the essential amino acids Leucine, Lysine, and Valine, as well as the non-essential amino acids Alanine, Aspartic acid, and Serine. In addition to the previously mentioned acetyldehyde and diacetyl, kefir is also characterized by other compounds such as formic, succinic and propionic acids, as well as isoamyl alcohol, and acetone, which contribute to its unique taste (Farnworth, 2005; Powell, 2006). Lactic acid is the predominant type of organic acids in kefir (Farnworth, 2005; Powell, 2006; Simova, et al., 2002). Kefir also contains enzymes, such as proteinases and peptidases, produced by the microorganisms within kefir grains. These enzymes and their products, are necessary for proper metabolism and growth of the microorganisms, and are also assumed to be bioactive components related to kefir's beneficial effects (Abraham & De Antoni, 1999; Adriana & Socaciu, 2008).

1.6 The matrix

The various bacterial and fungal species, living in symbiosis within kefir grains, are suspended in a matrix which consists of proteins and several types of polysaccharides. These components are produced and secreted by the microorganisms themselves (Abraham & De Antoni, 1999; Angulo, et al., 1993; Farnworth, 2005; Kooiman, 1968; Lee, et al., 2007). About 34% - 47% of the dry weight of the kefir is made of proteins and polysaccharides respectively (Abraham & De Antoni, 1999). The different polysaccharides, produced by several bacterial species, especially lactic-acid bacteria, are cell-surface components. These are often lost to the extracellular milieu or secreted, hence they are termed exopolysaccharides (Farnworth, 2005).

1.6.1 Kefiran

The most studied exopolysaccharide in kefir, kefiran, makes up approximately 24% of the dry weight of kefir grains (Kooiman, 1968). Kefiran is a branched, water-soluble polymer that contains approximately equal amounts of D-glucose and D-galactose (Figure 3) (Abraham & De Antoni, 1999; Angulo, et al., 1993; Kooiman, 1968). Initially through experiments involving methylation and periodate-oxidation, the structure of kefiran was determined (Kooiman, 1968).

The linkages inside this glucogalactan may explain its relatively high resistance to enzymatic degradation (Kooiman, 1968). It is important to note that kefiran is one of several exopolysaccharides found in kefir grains, yet it is the most well known since many of kefir's health benefits may be attributed to it (Lee, et al., 2007; Powell, 2006). Kefiran is produced by bacteria, mainly lactobacilli, within the kefir grains (Abraham & De Antoni, 1999; Angulo, et al., 1993; Kooiman, 1968). *Lactobacillus brevis* is one of the major producers of kefiran (De Vuyst & Degeest, 1999).

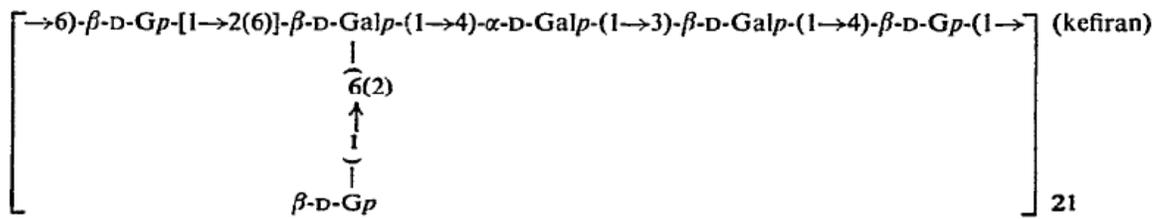


Figure 3: Structure of kefiran
 Source: Kooiman, 1968

1.7 Health benefits of kefir

Due to the health benefits that have been associated with and attributed to kefir consumption, it has become known as a complex probiotic.

1.7.1 Probiotics

A probiotic is defined as a consumable product, containing one or more type of microorganisms, which has health benefits. Kefir is distinguished from other probiotics, such as yoghurt, by having yeasts as well as bacteria, and by containing a large number of these microorganisms (Adriana & Socaciu, 2008; Angulo, et al., 1993; Farnworth, 2005). Most probiotics contain lactic acid bacteria, which along with many other bacterial and fungal species, are also found in kefir (Farnworth, 2008).

In general, probiotics are known to ameliorate health in three mechanisms (Oelschlaeger, 2010). First, probiotic foods may boost immunity, innate and/or acquired, which in turn enhances the body's ability to fight and prevent infectious and inflammatory diseases. The effect on the immune system may be through metabolites (ex peptides), cell wall components, or DNA (Farnworth, 2005; Hong, Chen, & Chen, 2010; Oelschlaeger, 2010; G. Vinderola, Perdigon, Duarte, Farnworth, & Matar, 2006). Second, the microorganisms inside the consumed foods may affect other microorganisms, commensal or pathogenic, in the host body. Thus, they may aid against infections and in maintaining a stable microflora. One way in which probiotics achieve this is by competing with pathogens for limited resources, such as iron. Moreover, they may secrete antimicrobial agents, such as bacteriocins, which are

produced by several types of Gram-positive and Gram-negative bacteria, and deconjugated bile acids, which have stronger antimicrobial agents than the host's bile salts. Probiotics may also possess anti-adhesive and anti-invasive effects against possible pathogens; however the mechanisms behind these effects are not completely understood. Third, probiotics may exert their effects by targeting microbial products or host products. For instance, probiotics may inhibit the production of bacterial and fungal toxins, which are the causative and active agents in many diseases and infections (Oelschlaeger, 2010).

1.7.2 Kefir: a complex probiotic

As one of the most complex consumed probiotics, kefir has been associated with many health benefits. Many studies have demonstrated that kefir has immunostimulatory and immunomodulatory effects, antimutagenic, as well as antitumorigenic effects (Cevikbas, et al., 1994; C. Chen, Chan, & Kubow, 2007; H. C. Chen, et al., 2008; de Moreno de Leblanc, Matar, Farnworth, & Perdigon, 2007; Guven & Gulmez, 2003; Hong, et al., 2010; Kubo, Odani, Nakamura, Tokumaru, & Matsuda, 1992; Lee, et al., 2007; Liu, Chen, & Lin, 2005; Maalouf, Baydoun, & Rizk, 2011; Matsuu, et al., 2003; Murofushi, Shiomi, & Aibara, 1983; Nagira, et al., 2002; Rizk, Maalouf, & Baydoun, 2009; Rodrigues, Carvalho, & Schneedorf, 2005; Shiomi, Sasaki, Murofushi, & Aibara, 1982; Thoreux & Schmucker, 2001; G. Vinderola, Perdigon, Duarte, Farnworth, et al., 2006). In addition, kefir has been shown to possess antibacterial and antifungal against many species (Cevikbas, et al., 1994; Garneau, Martin, & Vederas, 2002; Garrote, Abraham, & De Antoni, 2000; Golowczyc, Mobili, Garrote, Abraham, & De Antoni, 2007; Rodrigues, et al., 2005; Silva, Rodrigues, Filho, & Lima, 2009). On the other hand, gastrointestinal, metabolic and nutritional effects have also been attributed to kefir and kefir grains (de Vrese, Keller, & Barth, 1992; de Vrese, et al., 2001; Maeda, Zhu, Omura, Suzuki, & Kitamura, 2004; St-Onge, et al., 2002; Tamai, Yoshimitsu, Watanabe, Kuwabara, & Nagai, 1996; Urdaneta, et al., 2007).

These beneficial effects that kefir and kefir grains have shown may be attributed to bioactive ingredients within them. These include the exopolysaccharides, bacterial DNA, cell wall

components, as well as peptides (Hong, et al., 2010; Thoreux & Schmucker, 2001; G. Vinderola, Perdigon, Duarte, Farnworth, et al., 2006).

1.7.3 Nutritional and gastrointestinal benefits

Kefir, as discussed above, is a rich source of many organic and inorganic compounds that make it of high nutritional value (Farnworth, 2005) . Kefir has positive effects on the gastrointestinal tract as well. Various studies have proved that ingestion of kefir ameliorates lactose digestion in lactose intolerant people (de Vrese, et al., 1992; de Vrese, et al., 2001). De Vrese et al (2001) explains this property of kefir by the action of β -galactosidase enzyme of the microorganisms in kefir, the delayed intestinal transit that they induce, and the alteration of the intestinal milieu which in turn reduces symptoms of lactose intolerance (de Vrese, et al., 2001) . A study by Urdaneta et al (2007) aimed to examine the kefir's effect on metabolism and enzymatic activity. Their results showed that kefir enhances protein digestion and diminishes glycemic index (Urdaneta, et al., 2007). Reduction of blood glucose upon kefir administration was also noted by Maeda et al (2004).

With respect to cholesterol level, the action of kefir is still controversial. While several studies have shown that kefir leads to a reduction in cholesterol levels (Maeda, Zhu, Omura, Suzuki, & Kitamura, 2004; Tamai, et al., 1996), others have contradicted these results by claiming that kefir has no effect on total cholesterol, LDL cholesterol, HDL cholesterol, or triglyceride levels (St-Onge, et al., 2002).

1.7.4 Antimicrobial effect of kefir

Kefir's anti-microbial abilities have been proven by many studies. Garrote et al (2000) showed that milk inoculated with kefir grains had inhibitory effects on both Gram-positive and Gram-negative bacteria (Garrote, et al., 2000). Bacterial species against which kefir has been shown to act include *Staphylococcus aureus*, *Bacillus cereus*, *Salmonella typhi*, *Shigella sonnei*, *Listeria monocytogenes*, *Streptococcus pyogenes*, and *Escherichia coli* (Rodrigues, et al., 2005; Silva, et al., 2009). Moreover, kefir was shown to have a protective role against *Salmonella enteritidis* (Golowczyc, et al., 2007).

On the other hand, kefir also possesses anti-fungal properties, where several studies have demonstrated its inhibitory effects against *Candida albicans*, *Saccharomyces*, and other fungal species (Cevikbas, et al., 1994; Rodrigues, et al., 2005).

The mode of action for kefir's microbial growth inhibition is assumed to be due to products released by kefir grains' metabolic activity (Adriana & Socaciu, 2008). Compounds such as organic acids, hydrogen peroxide, acetaldehyde, diacetyl, carbon dioxide or bacteriocins, may act as anti-microbial agents (Adriana & Socaciu, 2008; Farnworth, 2005). For instance, hydrogen peroxide, produced by lactic acid bacteria, is known to inhibit the growth of *Pseudomonas spp* and *S. aureus*.

Lactic acid, a major constituent of kefir, has anti-microbial effects against *E. coli* and *B. cereus*, while carbon dioxide, produced by yeasts and homofermentative bacteria, inhibits obligate aerobes by replacing oxygen. On the other hand, acetaldehyde and diacetyl possess inhibiting effects on *S. aureus*, *E. coli*, *S. typhimurium*, and some yeasts. Bacteriocins, produced by lactic acid bacteria, can kill other microorganisms through cell membrane interactions (Adriana & Socaciu, 2008; Farnworth, 2005; Garneau, et al., 2002).

1.7.5 Immunostimulatory effects of kefir

At the level of the immune system, many studies have shown that kefir may enhance immunity, both innate and acquired. Vinderola and colleagues tested the effect of oral administration of kefir, in solid fraction (including bacteria) and liquid supernatant fraction, in murine animal models. Previous works by these authors showed that kefir induced a dose-dependent release of IgA+, IL-4+, IL-6+ and IL-10+ cells in the small intestine of mice, in addition to enhancing the phagocytosis (C. G. Vinderola, et al., 2005). Both kefir fractions induced an upregulation of TNF α and IL-6 on peritoneal macrophages. On the other hand, in adherent cells from Peyer's patches, IL-1 α , IFN γ , TNF α , IL-6 and IL-10, were unregulated upon administration of solid kefir fraction by gavage (C. G. Vinderola, et al., 2005). The same lab demonstrated that this same immunomodulatory effect is also obtained when only the exopolysaccharide produced by *Lactobacillus kefirianofaciens* was orally administered into BALB/c mice (G. Vinderola, Perdigon, Duarte, Thangavel, et al., 2006). A study by Thoreux and Schmucker in 2001, in which 6 months and 26 months old rats were fed with kefir and then

injected with cholera toxin, aimed to determine the immunomodulatory effect of kefir in young and old rats. In the 6 months old rats, anticholera toxin IgA was upregulated as compared to their age-matched controls (not fed kefir). This upregulation, however, was not detected in the senescent rats. Total IgG titer was increased in both age groups compared to controls, while there was no significant difference in nonspecific total IgA titer between the controls and the kefir-fed rats (Thoreux & Schmucker, 2001).

Several studies have suggested anti-inflammatory properties of kefir. Rodrigues et al (2005) showed that kefir, as well as kefiran by itself, have an anti-inflammatory effect, inhibiting mediators of the inflammatory process. Using murine asthma models, Lee et al (2007) supported the claim of kefir's anti-inflammatory role. Intra-gastric administration of kefir caused a suppression of inflammatory reaction through a reduction in IgE and cytokine levels (Lee, et al., 2007).

An in-vitro anti-allergic effect of kefir was demonstrated by Chen et al (2008) and Hong et al (2010). The treatment of splenocytes and macrophages in vitro, with heat inactivated *Lactobacillus kefirianofaciens* and *Lactobacillus kefir* induced the release of IFN- γ and IL-12, cytokines involved in Th1 response (H. C. Chen, et al., 2008; Hong, et al., 2010). Furthermore, Hong et al (2010) tested the anti-allergic effects of kefir in vivo by oral administration of bacterial species from kefir grains, namely, *Lactobacillus kefirianofaciens* and *Lactobacillus kefir* into mice in which allergy was induced using ovalbumin. The bacteria- fed mice showed a decrease in production of IgE upon ovalbumin sensitization. Flow cytometry and microarray analysis suggest that this decrease could be due to the upregulation of certain genes such as *Cd2*, *Stat4*, and *Ifnr*, which are involved in inflammation and immune response. Hence, Hong et al (2010) have suggested one possible mechanism for the anti-allergic effects mediated by kefir bacteria. The exact mode of action, by which kefir functions in the immune system, is still not clearly discerned (Hong, et al., 2010). However, several studies have attributed kefir's immunomodulatory effects to the bacterial species within it. Some claim that the bacterial DNA could be altering the immune response, while others say that cell wall and peptidoglycan components could be the potential immunomodulins (Figure 4) (Hong, et al., 2010; Thoreux & Schmucker, 2001; G. Vinderola, Perdigon, Duarte, Thangavel, et al., 2006). Moreover, the immunomodulatory effects affiliated with kefir are not affected by the viability of the

microorganisms within the kefir, but rather, the same effects are obtained with nonviable bacteria (Hong, et al., 2010).

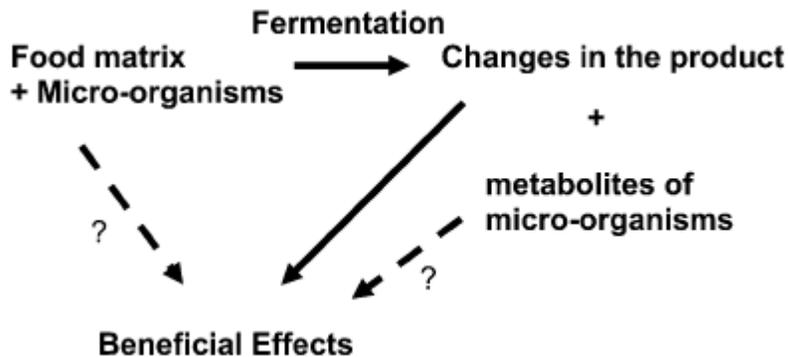


Figure 4: Possible active ingredients for kefir’s beneficial effects.
Source: Farnworth, 2008

1.7.6 Kefir’s antimutagenic effect

Kefir’s antimutagenic role was explored as well. Matssu et al (2003) aimed to study the protective role of kefir against radiation-induced intestinal damage and apoptosis. Rats that were fed kefir prior to radiation exposure were less susceptible to the induced damage, than the control rats, and showed less apoptotic activity. The level of apoptosis was determined first by H&E staining, TUNEL assay, and immunohistochemistry for caspase-3 (Matsuu, et al., 2003). Similarly, Nigara et al (2002) used HMV-1 and SK-MEL cells, human melanoma cell lines, and TIG-1 cells, human normal fibroblast cell lines, to verify if kefir has potential as an antimutagenic agent. Their results showed that, upon exposure to UV, cells treated with kefir were less damaged than untreated cells. The former underwent less morphology change, and kefir inhibited the increase in ROS (reactive oxygen species) induced by UV exposure. Moreover, in the kefir-treated cells the level of apoptosis detected was suppressed (Nagira, et al., 2002).

1.7.7 Kefir’s anticancerous effect

Kefir and kefir grains also hold antimutagenic and antitumor potential. Shiomi and colleagues were the first to test this potential. The effect of a polysaccharide extracted from kefir was examined in 1982, on mice with Sarcoma 180 and Ehrlich carcinoma. The administration of

the kefir polysaccharide was done orally on a group of mice and intraperitoneally on another. In both cases, tumor inhibition was detected (Shiomi, et al., 1982). In a study, Murofushi et al (1983) induced delayed-type hypersensitivity (DTH) in mice using picryl chloride. They then fed the mice with the same kefir polysaccharide used by Shiomi et al (1982). The oral administration of the kefir polysaccharide had induced a decrease in tumor growth (Murofushi, Shiomi, & Aibara, 1983). Similar results were obtained by Kubo et al (1992) who investigated kefir's antitumor activity against Ehrlich carcinoma. In another study, mice bearing sarcoma 180 tumor cells were fed kefir. Results showed decrease in tumor size as compared to kefir unfed mice. In addition, kefir administration had caused apoptotic tumor cell lysis (Liu, Chen, & Lin, 2005). In another in vivo study, the authors determined on the effect of kefir on Lewis Lung Carcinoma, and B16 melanoma infected mice respectively. Treatment with kefir inhibited metastasis progression as well as tumor growth (Furuwaka, Matsuoka, Takahashi, & Yamanaka, 2000). Kefir's antitumor role was further examined by Cevikbas et al (1994). In this study, mice with transplanted fusiform cell carcinomas were intraperitoneally injected with kefir. This treatment induced an inhibition of tumor necrosis (Cevikbas, et al., 1994). Mammary tumours were also experimented on with kefir. De Leblanc et al (2007) used 4T1, mammary tumor cells, and injected them into mice to induce breast tumor formation. Mice were then fed with kefir. The authors examined the rate of tumor formation on the kefir-fed mice compared to control mice, and kefir caused delayed tumor development, in a dose-dependent manner (de Moreno de Leblanc, et al., 2007).

On the other hand, an in-vitro study by Chen et al (2007) determined kefir's anti-tumorigenic and anti-proliferative potential on MCF-7 cells, a breast cancer cell line, and normal human mammary epithelial cells. Treatment was done in increasing concentrations, and for MCF-7 cells, inhibition of proliferation was dose-dependent. Kefir was also cytotoxic to the cancerous cells at certain doses, where viability decreased by at least 50%. The normal cells however, were not affected by the treatment. Cancer cells were also treated with unfermented milk, and the authors reported an increase in their proliferation (Chen, Chan, & Kubow, 2007). In 2009, Rizk et al (2009) examined the effect of cell-free fraction of kefir on proliferation and apoptosis in leukemic cell lines. Cytotoxicity, proliferation, and apoptosis assays were done on HuT-102 malignant T- lymphocytes (Rizk, et al., 2009). In another study in 2011, the same authors used CEM and Jurkat cells, HTLV-1-negative malignant T-lymphocytes, to assess

kefir's effect furthermore (Maalouf, Baydoun, & Rizk, 2011) . Both studies verified that treating these cells with cell-free fraction of kefir caused a decrease in proliferation and an increase in the level of apoptosis in a time and dose-dependent manner (Maalouf, et al., 2011; Rizk, et al., 2009).

1.8 Colorectal cancer

1.8.1 Introduction

Colorectal cancer (CRC) is the cancer of the large intestine. Most cases of CRC occur in the colon, while some originate in the rectum, and rarely in the anus. Obesity, sedentary lifestyle, smoking, and alcohol consumption are among the established risk factors of this type of cancer (Giovannucci, 2002). Other predisposing factors include age, gender, inflammatory bowel disease, and a family history of CRC (Aiello, et al., 2011).

1.8.2 Incidence and prevalence of CRC

CRC ranks as the 4th most prevalent type of cancer in males, and 3rd in females in the world. Its incidence however, varies greatly among different countries and regions (Figure 5) (Parkin, Bray, Ferlay, & Pisani, 2005). The highest incidence of this disease occurs in North America, Oceania, and eastern Europe, and the lowest incidence in Asia, Africa, and South America (Center, Jemal, Smith, & Ward, 2009). CRC is associated with high rates of morbidity and mortality, and a high economic burden on health care worldwide (Gellad & Provenzale, 2010). In some countries, the incidence of CRC is decreasing, yet in many others it is increasing (Center, et al., 2009; Gellad & Provenzale, 2010). The incidence of CRC is highest among people over 75 years of age (Laszlo, 2010).

In the United States, CRC is the 3rd most common type of cancer in both men and women. In 2009, statistics have shown that 75,590 and 71,380 cases of CRC were diagnosed among men and women respectively (Jemal, et al., 2009). As for mortality rates, in 2006, 20.5 males and 14.5 females out of every 100,000 died from CRC. The annual cost of CRC in the US averages between 4.5 to 9.6 billion dollars (American Cancer Society, 2010) . However, both mortality

rates and incidence of colorectal cancer are decreasing in the US, due to better diagnosis and screening, and improved treatment.

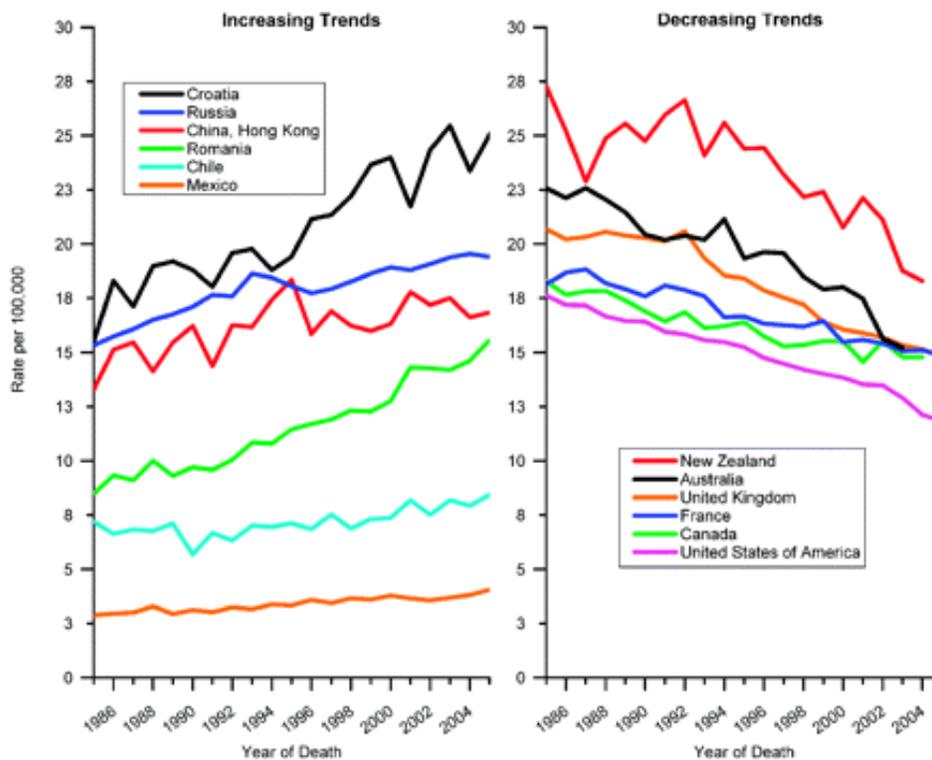


Figure 5: Trends in colorectal cancer mortality rates for selected countries in males, 1985 through 2005
Source: Center, et al., 2009

1.8.3 Diagnosis and screening

Several screening techniques are available for CRC, some of which allow early detection; while others are cancer prevention tools (Bretthauer, 2010; Center, et al., 2009; Gellad & Provenzale, 2010). The most used method of screening is colonoscopy, a form of cancer prevention tool, yet it still has to be evaluated in clinical trials (Bretthauer, 2010; Winawer, 2007). Fecal occult blood test is an early detection tool, which has the advantage of being cheap and non-invasive (Bretthauer, 2010; Center, et al., 2009; Gellad & Provenzale, 2010). However, it often results in false positives, and it only decreases CRC mortality by 16% (Bretthauer, 2010). Flexible sigmoidoscopy is a cancer prevention tool. The procedure it requires is costly and invasive, yet it has been shown to reduce mortality by 30% (Bretthauer, 2010; Center, et al., 2009; Gellad & Provenzale, 2010; Winawer, 2007). Other detection methods include stool DNA testing,

double contrast barium enema, computed tomographic colonography, and fecal immunochemical tests (Center, et al., 2009; Gellad & Provenzale, 2010).

1.8.4 Polymorphisms related to CRC

Certain gene polymorphisms and mutations have been identified to be related to the development of CRC. The study of these polymorphisms is crucial in the treatment of CRC since they affect the choice of drugs to be used. Examples of these mutations include: UGT1A1*28, XPD (Lys751Gln), XRCC1 Arg399Gln, MGMT 2535G>T, VEGF C936T, Germline Fc- γ RIIa 131H/H, and GSTP1 Ile105Val (Table 2) (Aiello, et al., 2011).

Polymorphism	Effect of mutation	Anticancer agent(s)	Effect
XPD Lys751Gln heterozygosis and homozygosis	Influence of DNA repair capacity	Oxaliplatin/5FU	Worse response, shorter survival
UGT1A1*28	Reduced glucuronidation of SN38	Irinotecan	Predictive role in response to an irinotecan-containing regimen
GSTP1 Ile105Val homozygosis	Reduction of enzymatic activity	Oxaliplatin/5FU	Longer survival
XRCC1 Arg399Gln heterozygosis and homozygosis	Deficiency in DNA repair pathways	Oxaliplatin/5FU	Shorter survival
Fc- γ RIIa 131H/H/ Fc- γ RIIIa 158V/V	Higher affinity to human IgG2/ higher affinity to human IgG1	Cetuximab	Longer progression-free survival
Mutation of K-Ras	Constitutive activation of the Ras/Raf/MAPK pathway	Cetuximab	No response to cetuximab-based therapy
Thymidylate synthase *3/*3 genotype	Reduction of enzymatic activity	5FU	Better response to 5FU-based chemotherapy
MGMT 2535G>T	Reduction of enzymatic activity	Oxaliplatin	Longer progression-free survival
MTHFR 677 C/T MTHFR 1298 A/C	Altered enzymatic activity and hyperhomocysteinemia	FOLFOX regimen	Longer time to progression and overall survival

Table 2: Genetic alterations associated with current colon cancer therapies.

Source: Aiello, et al., 2011

1.8.5 Treatment of CRC

The drugs used in chemotherapy for metastatic CRC are showing improvement in their results, especially with the advent of targeted therapeutic agents. Such drugs can be given alone or in combination with the standard drugs (Aiello, et al., 2011; El Zouhairi, et al., 2011; Laszlo, 2010). 5-fluorouracil, the first chemotherapeutic drug to be developed for CRC, is still used nowadays, especially in combination with other targeted molecules. Other standard CRC drugs include Irinotecan, and Oxaliplatin (Aiello, et al., 2011; Laszlo, 2010). Anti-angiogenic agents are drugs that mainly target VEGF or VEGFR; include Bevacizumab and Aflibercept, among others (Figure 7) (Aiello, et al., 2011; El Zouhairi, et al., 2011). Vatalanib, Sunitinib, Vandetanib, Cediranib, and AMG 706 are direct inhibitors of VEGFR (El Zouhairi, et al., 2011). Bevacizumab is an anti-VEGF monoclonal antibody (Aiello, et al., 2011) . On the other hand, Cetuximab and Panitumumab are EGFR antagonists (Figure 6). They too are monoclonal antibodies (Laszlo, 2010). Novel therapeutic drugs, still under clinical trials, include mTOR inhibitors, PKC antagonists, and Src inhibitors, and KSP inhibitors (El Zouhairi, et al., 2011). However, the response post treatment for these mentioned drugs, is often variable, and dependent on the underlying polymorphism. The percentage of patients who show positive effects is also mostly, low (Aiello, et al., 2011; Laszlo, 2010). For instance, anti-EGFR drugs, fail in patients with a K-Ras mutation (Laszlo, 2010) On the other hand, many of these drugs have considerable side effects, which include gastrointestinal perforation, myocardial infarction, and hypertension (El Zouhairi, et al., 2011; Laszlo, 2010).

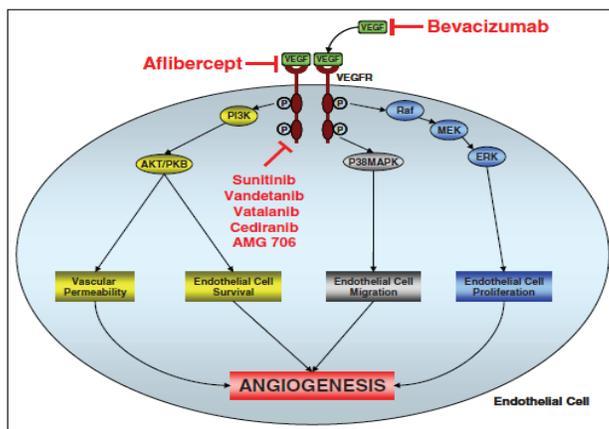


Figure 6: Drugs used for targeted therapy for CRC.
Source: El Zouhairi, et al., 2010

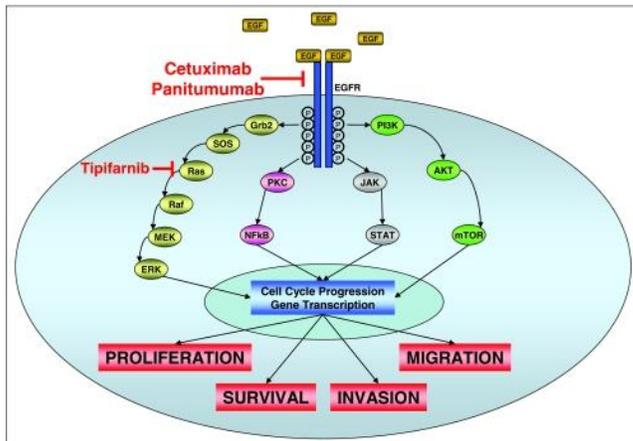


Figure 7: Targeted therapy for CRC.
Source: El Zouhairi, et al., 2010

1.8.6 CRC and diet

Diet is known to affect the occurrence and progression of many types of cancer in general, and CRC in particular (Pala, et al., 2011; Sung & Bae, 2010; Vrieling & Kampman, 2010). Certain dietary factors are claimed to be associated with the incidence recurrence, and survival of CRC. Increased body fat, high consumption of red meat, processed foods, and alcohol lead to higher risk of CRC (Vrieling & Kampman, 2010). On the other hand, consumption of certain foods, such as yoghurt, fruits, vegetables, cheese, and fish tend to lower this risk (Pala, et al., 2011; Vrieling & Kampman, 2010). However, *in-vivo* studies and trials about the correlation of CRC and such nutritional components are few. Firm data about dietary factors' role in CRC is yet to be established (Sung & Bae, 2010; Vrieling & Kampman, 2010).

There have been several *in-vitro* studies concerning dietary components and their anti-cancerous potential. These studies have shown that dietary compounds can act as anti-proliferative and pro-apoptotic agents on colorectal cancer cell lines *in vitro*. Such agents and compounds include osteopontin, fungal extracts, berry extracts, curcumin, and quercetin (Kim, et al., 2010; Lavi, Friesem, Geresh, Hadar, & Schwartz, 2006; Lavi, et al., 2010; Likui, Hong, Shuwen, Yuangang, & Yan, 2011; McDougall, Ross, Ikeji, & Stewart, 2008; Shehzad, Wahid, & Lee, 2010). These aimed to find if the compound or extract of interest could, someday, become a therapeutic target for CRC. One such compound, curcumin (diferuloylmethane), has

entered several clinical trials, and has completed phase I in some trials for its potential employment in CRC treatment. Various studies showed that curcumin was responsible for the overexpression of Bax and p53, and the under-expression of Bcl-2 (Shehzad, et al., 2010; Su, et al., 2006).

1.9 Apoptotic Pathway

Bcl-2 acts as a repressor of the apoptotic pathway, while Bax, counters this action, and thus promotes apoptosis. The ratio of Bax and Bcl-2 in a cell reflects the activation of the survival or apoptotic pathways (Oltvai, Milliman, & Korsmeyer, 1993). The apoptotic pathway, which involves the couple Bax and Bcl-2, begins with the activation of AMPK. The latter is known to control apoptosis in several cancers, including colorectal cancer (Oda, et al., 2000). Downstream of AMPK is p53, whose up-regulation in turn causes the up-regulation of the pro-apoptotic Bax and the downregulation of the anti-apoptotic Bcl-2 (Oda, et al., 2000; Prives & Hall, 1999). The disturbance in the normal balance between Bax and Bcl-2, in favor of Bax, triggers the activation of Caspase-3. This in turn causes the proteolytic cleavage of PARP, thus triggering apoptosis (Green & Amarante-Mendes, 1998).

1.10 Purpose of the study

After the established role of kefir as an anticancerous probiotic, the current study aims to investigate the effect of kefir-fermented milk on human colorectal adenocarcinoma cells in vitro in comparison to nonfermented milk.

Specifically, the present study revolved around three main objectives:

1. Determine the effect of kefir on cytotoxicity of Caco-2 and HT-29 cells.
2. Determine the effect of noncytotoxic kefir concentrations on apoptosis, proliferation, and cell cycle progression
3. Determine the mechanism by which kefir employs its anticancerous effect by studying expression of proapoptotic and anti-apoptotic proteins.

Chapter II

MATERIALS AND METHODS

2.1 Cell lines and 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.

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 0.45-µm filter and 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 UHT 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, and anti-Bcl2 antibodies were obtained from Santa Cruz Biotechnology.

Anti-mouse IgG HRP-conjugated secondary antibody was obtained from Promega.

Propidium Iodide (PI) was obtained from Sigma LifeScience.

2.4 Cytotoxicity: Trypan Blue Exclusion Method

HT-29 and Caco-2 cells were grown in 24 well plates (growth area: 2cm²) at a density of 2x10⁶ cells/ml. Cells were allowed to reach 50% confluency for 24 hours after seeding, and then they were treated with milk and kefir at the following concentrations (volume/volume): 5%, 10%, 15%, and 20%. Control wells were left untreated. After 24, 48, and 72 hours, the supernatant from each well was collected, cells were washed with PBS, and the PBS washes were added to the supernatant of each well. Cells were then trypsinised and collected separately from the well contents and PBS. 20µl from each collection tube was mixed with 20µl of Trypan Blue. 10µl of this mixture was placed in a counting chamber under the microscope, and the number of living and dead cells was recorded accordingly. For each well, two countings were done separately, PBS washes/well supernatant and trypsinised cells. Under the microscope, dead cells appear blue, since they are permeable to Trypan Blue, while viable cells exclude the stain and thus appear bright. The percentage of viable cells was reported.

2.5 Proliferation: Cell Proliferation Reagent (WST-1)

HT-29 and Caco-2 cells were seeded in 96 well plates (growth area: 0.6 cm²) at a concentration of 1x10⁶ cells/ml. After 24 hours of seeding, cells were treated with milk and kefir at the following concentrations (volume/volume): 5%, 10%, 15%, and 20%. Control wells were left untreated. For every milk and kefir concentration, a blank well was prepared, containing only media and the corresponding volume of kefir or milk. After 24, 48, and 72 hours, 10 µl of Cell Proliferation Reagent (WST-1; Roche, Germany) was added to each well. The plates were incubated at in a humidified incubator (37°C) in 95% air and 5% CO₂ for 1.5 hours. WST-1 is a tetrazolium salt that on contact with metabolically active cells is cleaved to produce formazan dye by mitochondrial dehydrogenases. Quantitation of formazan is done colorimetrically at 450 nm. The absorbance of the each blank well was subtracted from the corresponding sample

well. The results were normalized to the untreated controls, and the percent proliferation was reported.

2.6 Cell Cycle Analysis: Flow Cytometry

Caco-2 and HT-29 cells were seeded in 6 well plates (growth area: 9.5 cm²). When the plates reached full confluency, cells were treated with noncytotoxic kefir, and corresponding milk concentrations. Treatment was done for 24 hours, with 10 % milk and kefir, in addition to nontreated controls. At the end of the treatment period, cells were trypsinized and detached, then centrifuged at 1200 rpm at 5°C for five minutes. The pellet was washed and resuspended in 1 ml of ice-cold 1 × phosphate-buffered saline (PBS), and centrifuged again under the same conditions. The pellet was resuspended in 1 ml of ice-cold 1 × PBS, and ethanol was added to a final concentration of 70%. The fixed cells were left overnight at -20°C. The following day, cells were centrifuged and washed with PBS. The pellet was resuspended in 500 µl of 1x binding buffer (prepared from the 10X stock), and then 10µl of propidium iodide (PI) was added to each sample. The samples were incubated in the dark for 10 minutes.

Cells were analyzed using an Accuri C6 flow cytometer (Ann Arbor, MI USA), which indicated the distribution of the cells into their respective cell-cycle phases based on their DNA content. Sub-G0/G1 cells were less than 2n, G0/G1 cells were 2n, S/M phase cells were >2n. Cell DNA content was determined by CFlow[®] Software. An increase in cells in the pre-G phase is indicative of an increase in cell death. The percentage of cells in the sub-G0/G1 phase was compared to that of the control.

2.7 Apoptosis: Cell Death Elisa Plus Kit

To confirm the apoptotic effect of kefir on colorectal cancer cells, the Cell Death Elisa Plus kit was used. The non-cytotoxic concentrations of kefir were used to assess the effect of kefir and milk on apoptosis. Milk was also used as control. HT-29 and Caco-2 cells were grown in 96 well plates (growth area: 0.6 cm²) at a concentration of 1x10⁵ cells/ml. After 24 hours, cells were re-fed and then treated with milk and kefir at the following concentrations (volume/volume): 5%, 10%, 15%. Control wells were left untreated. After 24 and 48 hours of

treatment, cells were lysed with 200 μ l of the provided lysis buffer, and incubated for 30 minutes at room temperature. The plates were then centrifuged for 10 minutes at 200g. 20 μ l of the supernatant were placed in streptavidin-coated microtiter plates, followed by the addition of biotin-labeled anti-histone and peroxidase-conjugated anti-DNA antibodies. The anti-histone antibody, bound to the plate via biotin-streptavidin, also bound histones from released nucleosomes. The plate was then incubated at room temperature for 2 hours, after which ABTS (2, 2'-azino-di(3-ethylbenzthiazolin-sulfonate)) was added as a substrate for peroxidase enzyme. Enrichment factor was calculated as the recorded absorbance of each sample, divided by that of the untreated cells, according to manufactures' instructions.

2.8 Western Blot Analysis

Caco-2 cells were seeded in 6 well plates (growth area 9.5 cm²). When the plates reached full confluency, cells were treated with non-cytotoxic kefir, and corresponding milk concentrations. Treatment was done for 48 hours with 5% and 10% milk and kefir, in addition to nontreated controls. At the end of the treatment period, 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. Scraping was done on ice, and the resulting lysates were boiled for 5 minutes. Proteins were then quantified on NanoDrop (ND-1000 V 3.7.1) in order to achieve equal loading.

Protein samples were separated by SDS-PAGE on 15% (for β -Actin, Bax, and Bcl-2) gels and transferred to PVDF membranes overnight at 30V. The membranes were then blocked with 5% non-fat dry milk in PBS containing 0.1% Tween-20 for 1 hour at room temperature, with shaking. Membranes were then incubated with primary antibody at a concentration of 1:400 for 2 hours at room temperature, with gentle shaking. After the incubation with the primary antibody, the membranes were washed for one hour with shaking. After washing, membranes were 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 chemiluminescent reagent ECL (GE Healthcare). The results were obtained on an X-ray film (Agfa Healthcare).

2.9 Statistical analyses

All experiments were carried out in triplicates in 3 independent experiments. Results were reported as the mean value \pm standard deviation. Data were analyzed by one-way analysis of variance (ANOVA). The differences between the means of treated and control groups were tested for significance using Fisher's least significant differences at $P \leq 0.05$ (Fisher PLSD). An effect was considered significant when the value (\pm) of mean difference between groups exceeded Fisher PLSD in the one-factor ANOVA test.

Chapter III

RESULTS

3.1 Cytotoxicity of kefir

The assessment of cytotoxicity by Trypan Blue exclusion method demonstrated that kefir's IC50 for Caco-2 cells ranges between 11%, 10%, and 18% at 72, 48, and 24 hours respectively (Figures 8 A,B,C). For HT-29 cells, The IC50 was only reached at 48 and 72 hours of treatment (at the concentrations used), where it was determined to be 13% and 11% respectively (Figures 9 A, B, C). The arrows on the graphs indicate the IC50. At concentrations higher than the IC50, the viability of both cell lines decreased significantly ($p \leq 0.05$). Cytotoxicity levels were shown to be dose and time dependent, where maximum cytotoxicity ($\approx 100\%$) of kefir on Caco-2 cells was achieved with 20% kefir (volume per volume) 48 hours post treatment (Figure 8C). Similarly, for HT-29 cells, the maximum cytotoxicity ($\approx 100\%$) level was reached at 72 hours with 20% (Figure 9C).

The cytotoxicity of the milk (the same type of milk which was used to produce the kefir) was also assessed. The viability of both cell lines, 24, 48, and 72 hours after treatment with various milk concentrations, was not reduced, but rather, a slight increase in percentage viability was detected (Figures 8 A,B,C and 9 A,B,C).

Results are reported as percent of viable cells out of the total number of cells (dead and alive). The differences between kefir and milk were significant, as determined by statistical analysis. It is important to note that this method doesn't differentiate, among dead cells, between those that have undergone necrosis and those that have died through apoptosis.

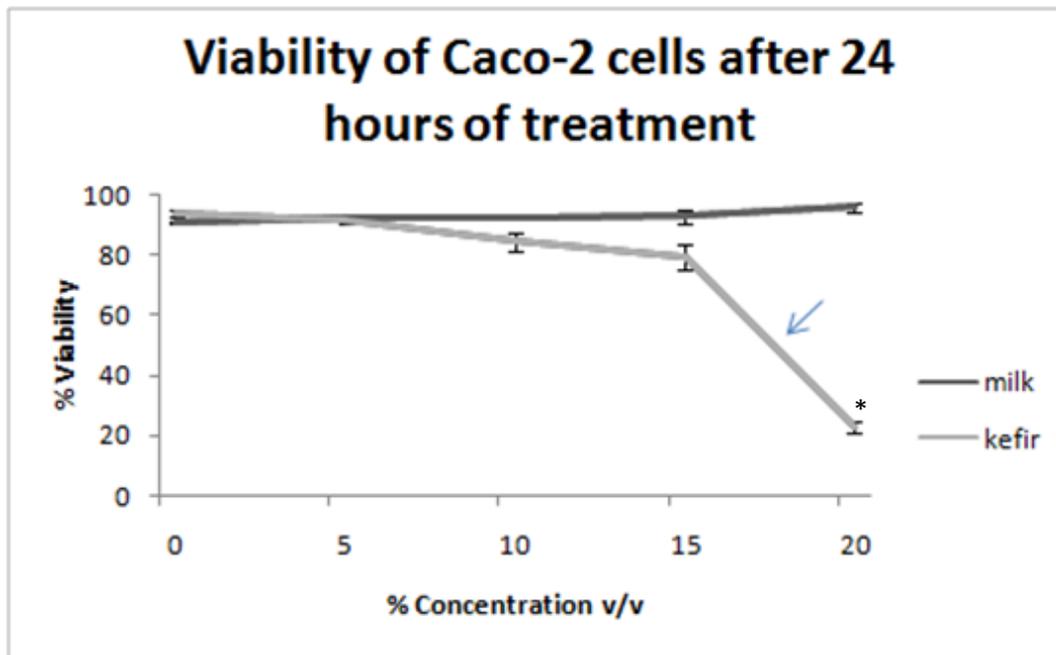


Figure 8A: Viability of Caco-2 cells after 24 hours of treatment. Cells were treated with kefir and milk to establish different concentrations. Cell viability was then determined using the Trypan Blue Exclusion method. The arrows on the graph indicate the IC50. The results were expressed as percent of total number of cells. Data is the mean \pm SEM. The asterix signifies $p < 0.05$.

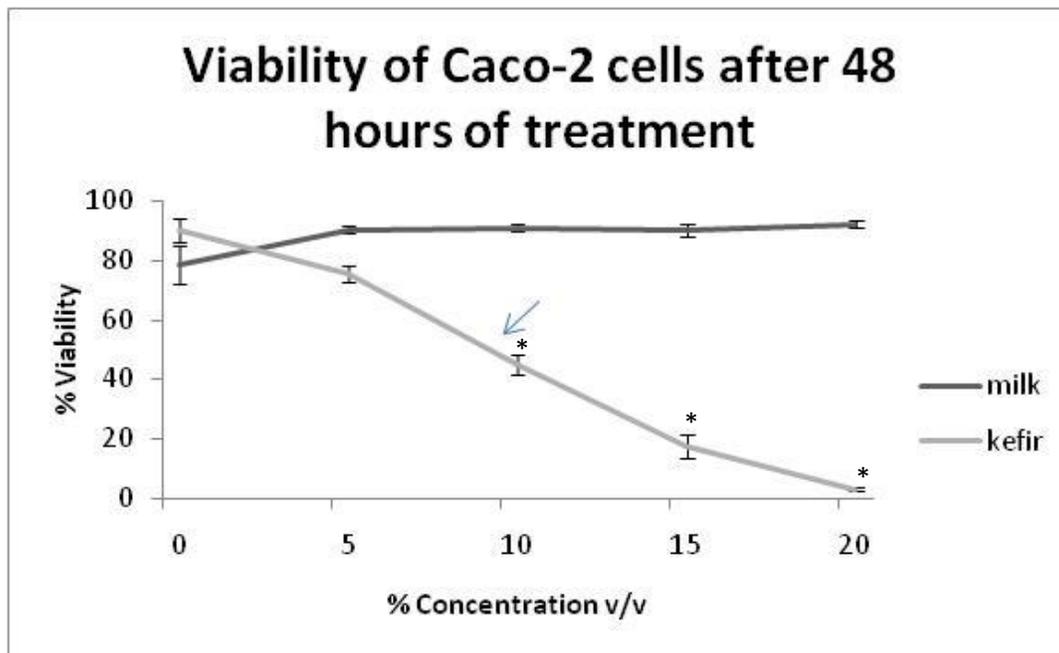


Figure 8B: Viability of Caco-2 cells after 48 hours of treatment. Cells were treated with kefir and milk to establish different concentrations. Cell viability was then determined using the Trypan blue exclusion method. The arrows on the graph indicate the IC50. The results were expressed as percent of total number of cells. Data is the mean \pm SEM. The asterix signifies $p < 0.05$.

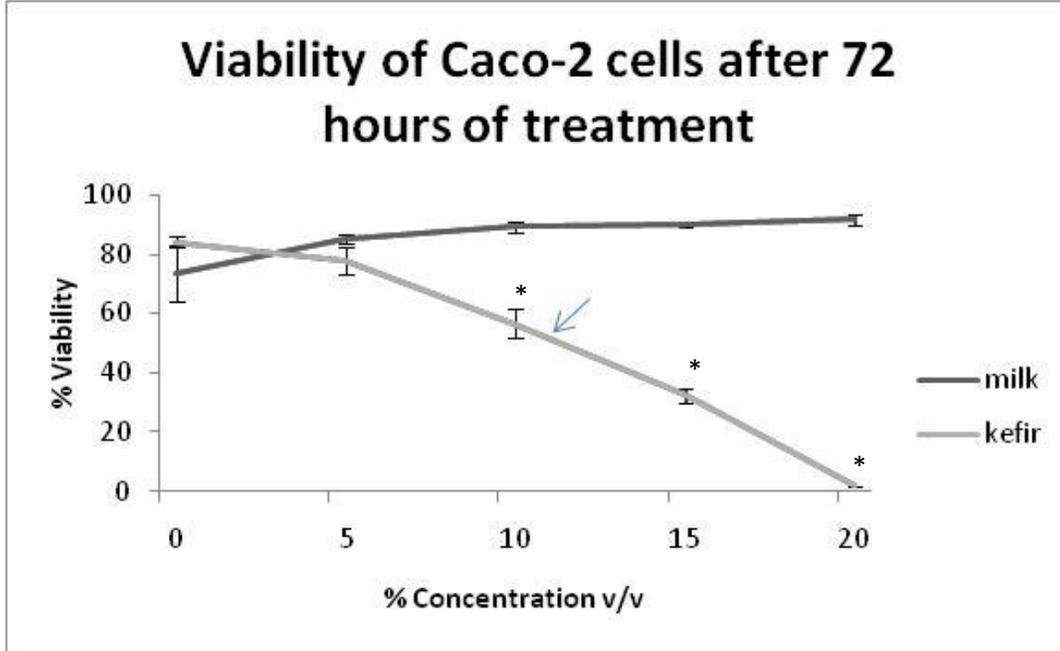


Figure 8C: Viability of Caco-2 cells after 72 hours of treatment. Cells were treated with kefir and milk to establish different concentrations. Cell viability was then determined using the Trypan Blue Exclusion method. The arrows on the graph indicate the IC50. The results were expressed as percent of total number of cells. Data is the mean \pm SEM. The asterix signifies $p < 0.05$.

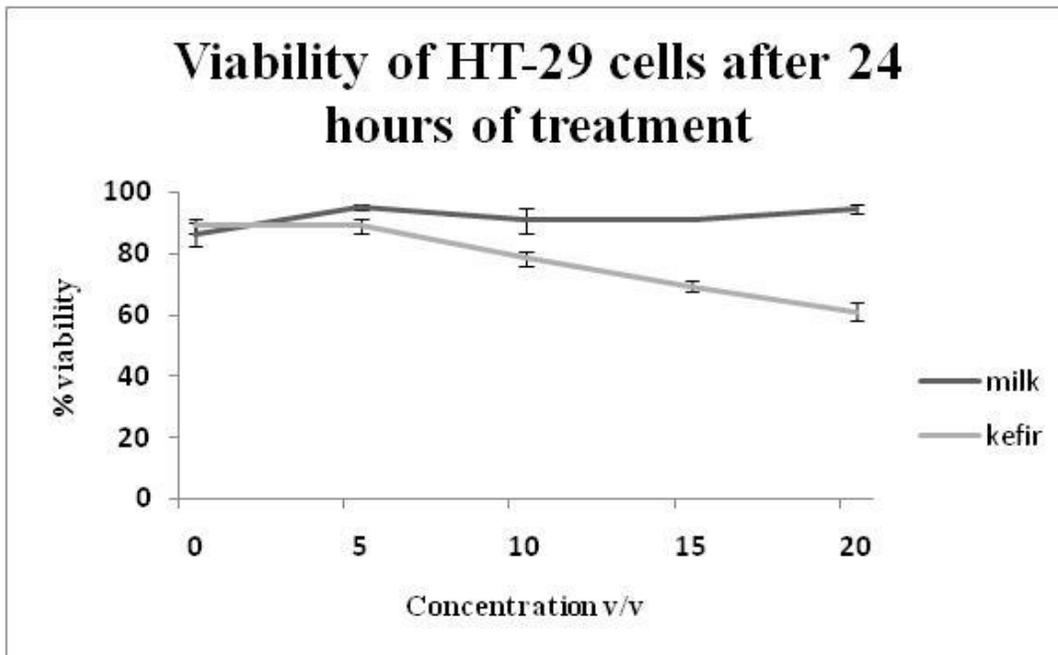


Figure 9A: Viability of HT-29 cells after 24 hours of treatment. Cells were treated with kefir and milk to establish different concentrations. Cell viability was then determined using the Trypan Blue Exclusion method. The results were expressed as percent of total number of cells. Data is the mean \pm SEM.

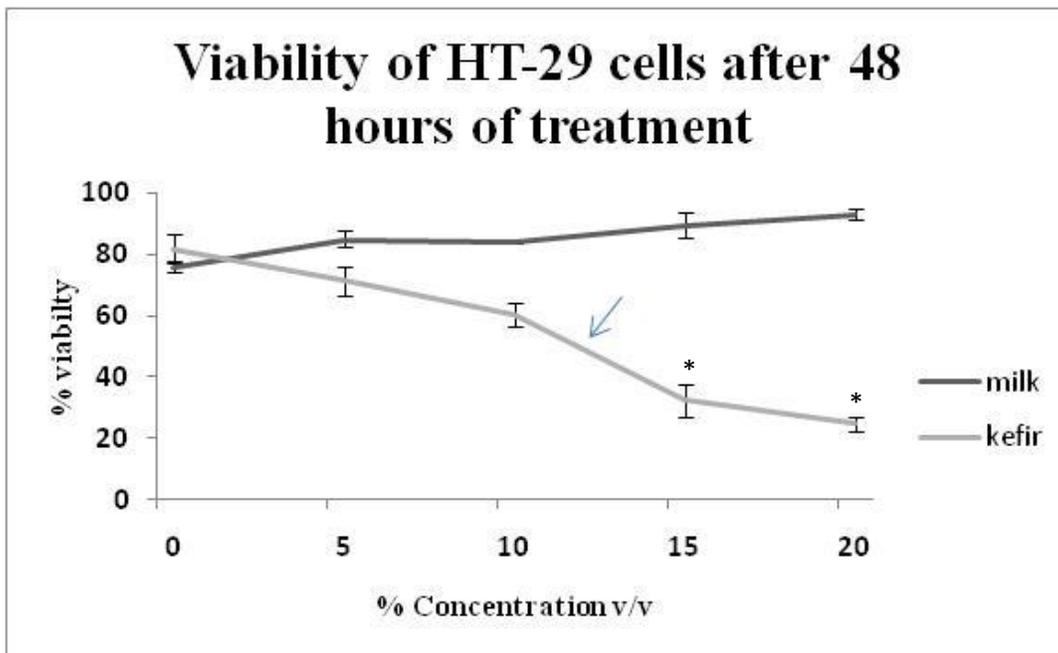


Figure 9B: Viability of HT-29 cells after 48 hours of treatment. Cells were treated with kefir and milk to establish different concentrations. Cell viability was then determined using the Trypan Blue Exclusion method. The arrows on the graph indicate the IC50. The results were expressed as percent of total number of cells. Data is the mean \pm SEM. The asterix signifies $p < 0.05$.

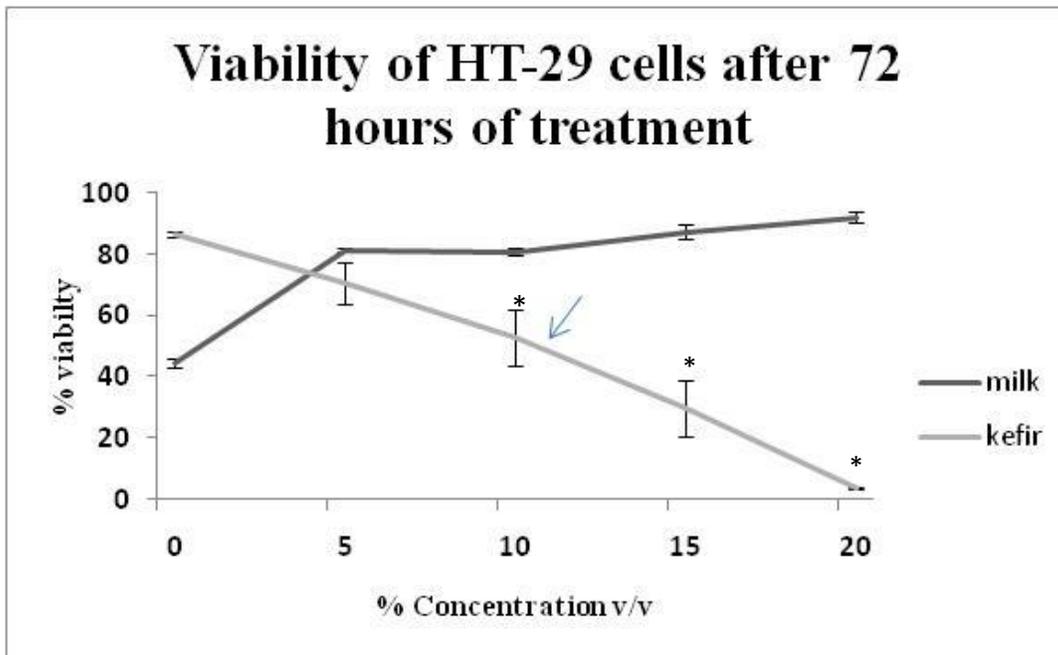


Figure 9C: Viability of HT-29 cells after 72 hours of treatment. Cells were treated with kefir and milk to establish different concentrations. Cell viability was then determined using the Trypan Blue Exclusion method. The arrows on the graph indicate the IC50. The results were expressed as percent of total number of cells. Data is the mean \pm SEM. The asterix signifies $p < 0.05$.

3.2 Kefir treatment reduces proliferation of Caco-2 and HT-29 cells.

The same concentrations of milk and kefir, which were used for the cytotoxicity assay (0%, 5%, 10%, 15%, 20% volume per volume) were applied on cells to assess their effect on proliferation. Results have shown that kefir significantly inhibited the proliferation of HT-29 and Caco-2 cells ($p \leq 0.05$). The extent of this anti-proliferative effect was greater when the cells were treated with higher kefir concentrations and for longer periods of treatment (Figure 10 A, B, C and figure 11 A, B, C). As compared to untreated Caco-2 cells, the percent decrease in proliferation reached a maximum of 97%, 125%, and 132%, at 24, 48, and 72 hours after treatment with the highest dose of kefir, respectively. At the kefir concentrations where the IC₅₀ was calculated in the Trypan Blue Exclusion Method, the percent decrease was 95% at 24 hours, 94% at 48 hours, and 97% at 72 hours (Figures 10 A, B, C).

HT-29 cells as well showed significant inhibition of proliferation upon kefir treatment, even though the effect was slightly less than that exhibited by the Caco-2 cells. The maximum percent decrease achieved with 20% kefir treatment was 79%, 89%, and 96% at 24, 48, and 72 hours after treatment, respectively (Figures 11 A, B, C). At the concentrations corresponding to the IC₅₀, the percent decrease was calculated to be 60% at 48 hours, and 38% at 72 hours.

As controls, cells were also treated with milk, at the same concentrations as kefir. All cells treated with milk showed a significant increase in proliferation, compared to the untreated cells. In both cell lines, the percent proliferation reached as high as 500%. The change in proliferation, designated by the activity of mitochondrial dehydrogenases, although significant, was not consistent. The response was not always dose- and time-dependent. However, in none of the milk-treated cells was the percent proliferation lower than that of the untreated cells. The differences between kefir and milk treatment were significant, as determined by statistical analysis.

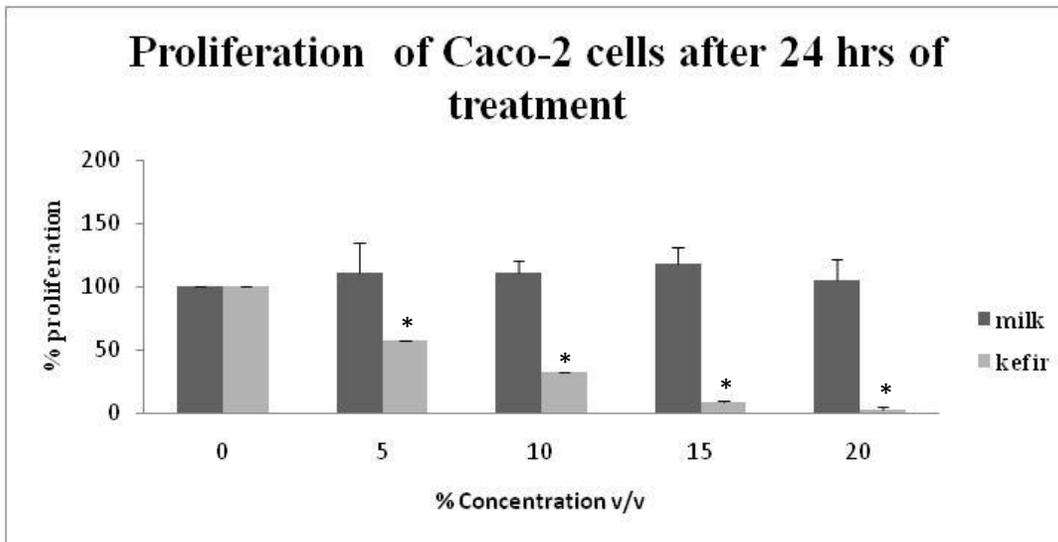


Figure 10A: Proliferation of Caco-2 cells after 24 hours of treatment with different concentrations of milk and kefir. Cells were treated with kefir and milk to establish different concentrations. Absorbance was measured at 450 nm after two hours of incubation with WST-1 cell proliferation reagent. Results were normalized to the untreated cells. Data is the mean \pm SEM. The asterix signifies $p < 0.05$.

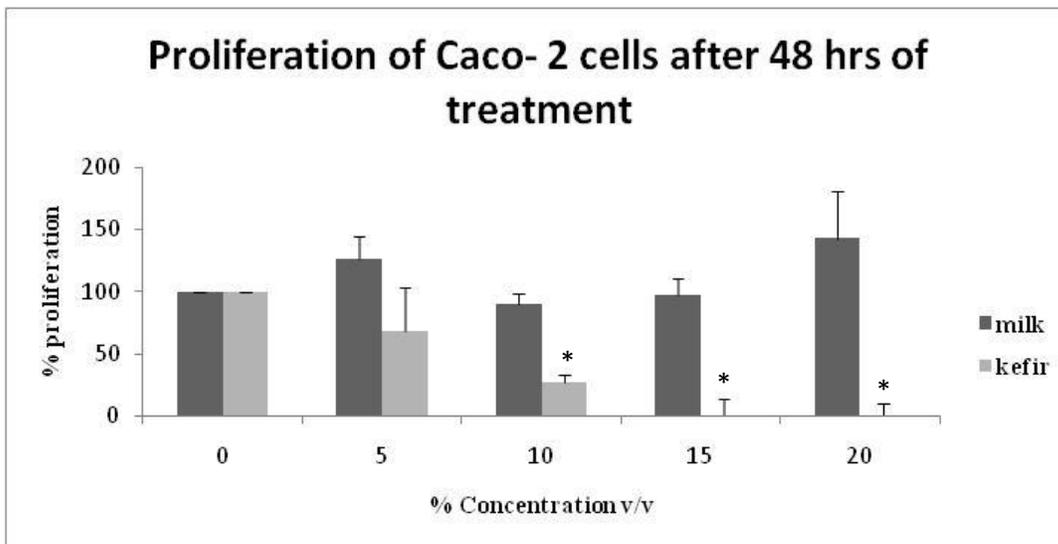


Figure 10B: Proliferation of Caco-2 cells after 48 hours of treatment with different concentrations of milk and kefir. Cells were treated with kefir and milk to establish different concentrations. Absorbance was measured at 450 nm after two hours of incubation with WST-1 cell proliferation reagent. Results were normalized to the untreated cells. Data is the mean \pm SEM. The asterix signifies $p < 0.05$.

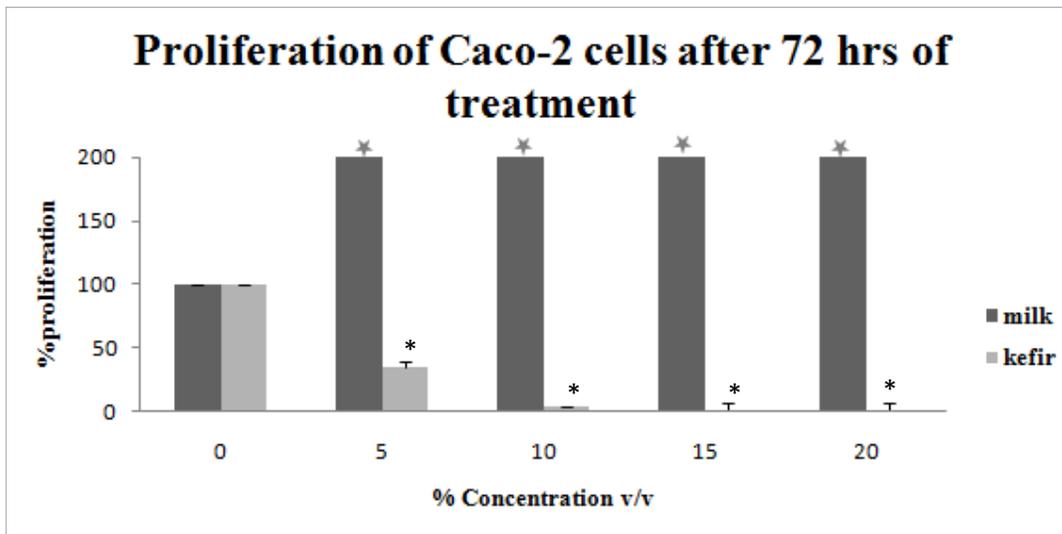


Figure 10C: Proliferation of Caco-2 cells after 72 hours of treatment with different concentrations of milk and kefir. Cells were treated with kefir and milk to establish different concentrations. Absorbance was measured at 450 nm after two hours of incubation with WST-1 cell proliferation reagent. Results were normalized to the untreated cells. The stars above the bars indicate a percent proliferation higher than 200%. Data is the mean \pm SEM. The asterix signifies $p < 0.05$.

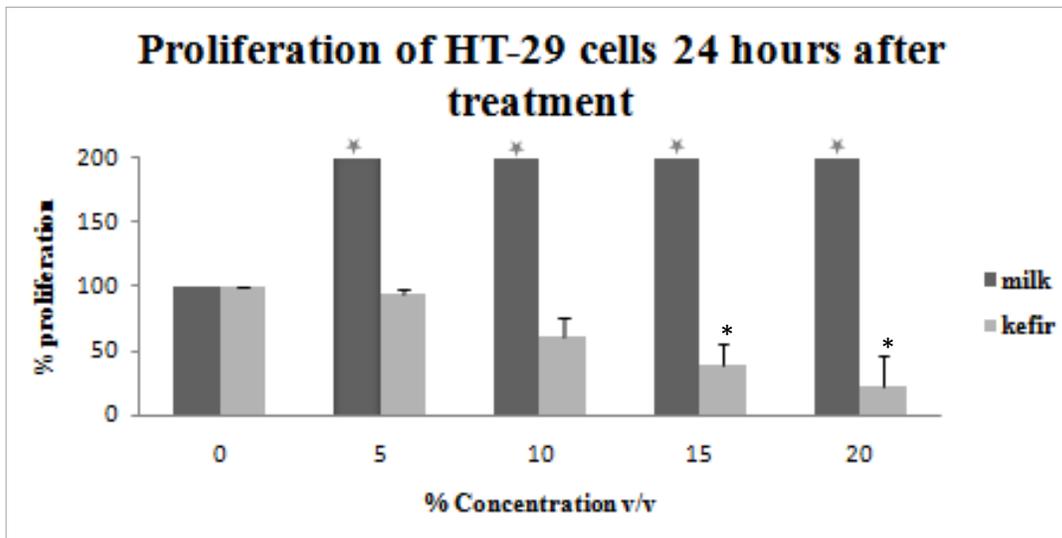


Figure 11A: Proliferation of HT-29 cells after 24 hours of treatment with different concentrations of milk and kefir. Cells were treated with kefir and milk to establish different concentrations. Absorbance was measured at 450 nm after two hours of incubation with WST-1 cell proliferation reagent. Results were normalized to the untreated cells. The stars above the bars indicate a percent proliferation higher than 200%. Data is the mean \pm SEM. The asterix signifies $p < 0.05$.

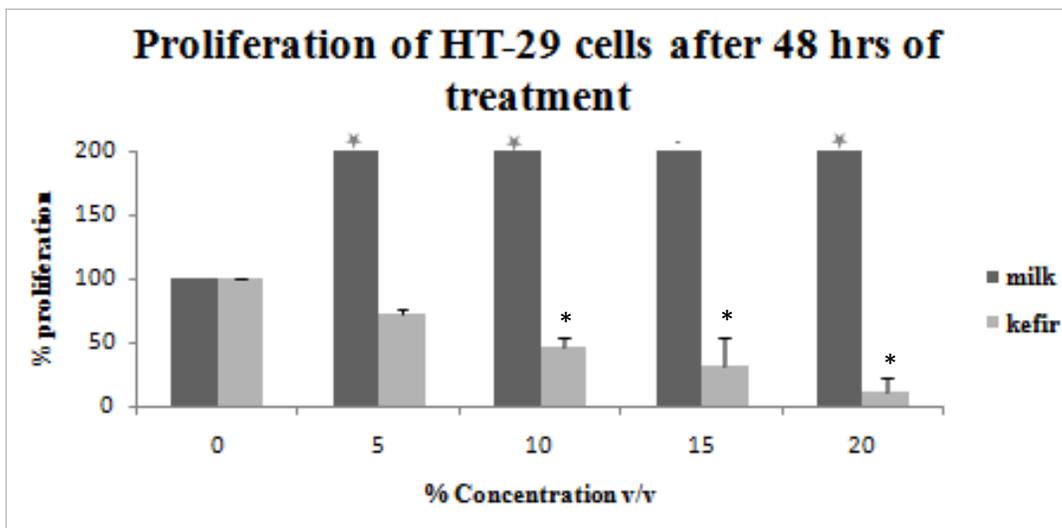


Figure 11B: Proliferation of HT-29 cells after 48 hours of treatment with different concentrations of milk and kefir. Cells were treated with kefir and milk to establish different concentrations. Absorbance was measured at 450 nm after two hours of incubation with WST-1 cell proliferation reagent. Results were normalized to the untreated cells. The stars above the bars indicate a percent proliferation higher than 200%. Data is the mean \pm SEM. The asterix signifies $p < 0.05$.

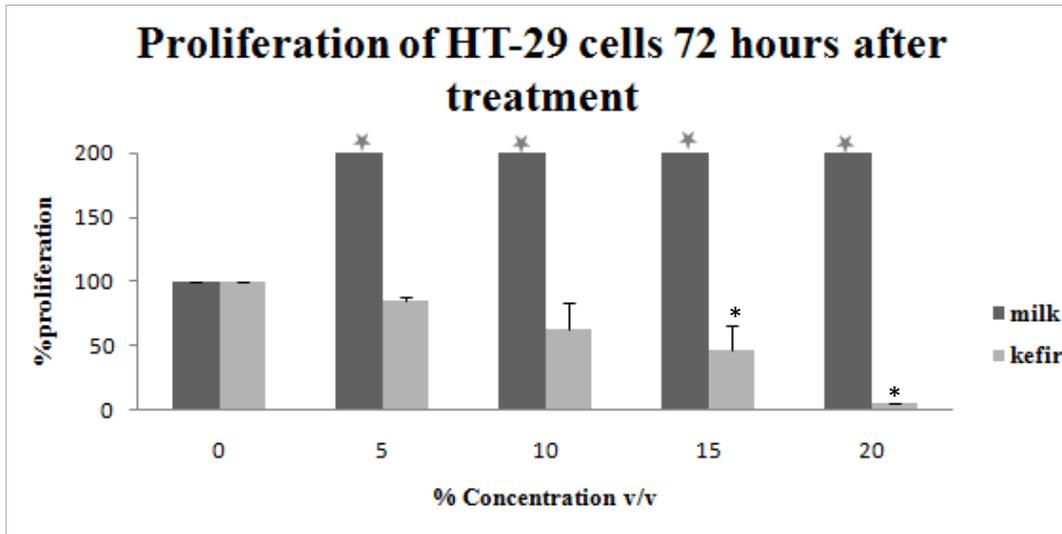


Figure 11C: Proliferation of HT-29 cells after 72 hours of treatment with different concentrations of milk and kefir. Cells were treated with kefir and milk to establish different concentrations. Absorbance was measured at 450 nm after two hours of incubation with WST-1 cell proliferation reagent. Results were normalized to the untreated cells. The stars above the bars indicate a percent proliferation higher than 200%. Data is the mean \pm SEM. The asterisk signifies $p < 0.05$.

3.3 Kefir induces cell cycle arrest at the G1 phase.

After verifying that kefir was inhibiting cell proliferation in colorectal cancer cells, we aimed to evaluate whether this effect was through cell cycle arrest. Caco-2 and HT-29 cells were treated with 10% kefir and nonfermented milk for 24 hours and stained then with PI. Cell cycle analysis was done through flow cytometry. Through the analysis of their DNA content, cells were assigned to their respective phases: sub-G0/G1 cells were less than 2n, G0/G1 cells were 2n, S/M phase cells were >2n. Treating the cells with kefir induced a shift in the cell cycle distribution, where the sub-G0/G1 DNA content was significantly higher in kefir-treated cells, compared to the nontreated controls. Correspondingly, the population of cells in S/M phase was reduced.

In Caco-2 cells, the sub-G0/G1 population increased from 14 to 45.2 % as a result of 10% kefir treatment, while the S/M phase cells decreased from 15.5 to 3.6% (Figure 12A & C). On the other hand, when these cells were treated with 10% milk, a slight increase in sub-G0/G1 population from 14 to 16.5% and a noticeable increase in S/M from 15.5 to 22.4% were detected (Figure 12A & B).

The same pattern of cell cycle shift was seen upon treatment of HT-29 cells with 10% kefir. The sub-G0/G1 population increased from 13.9 to 52.7%, accompanied by a significant decrease in S/M phase population from 24.8 to 1.5% (Figure 12D&F). In contrast, the milk-treated HT-29 cells showed a slight decrease in sub-G0/G1 cells from 13.9 to 12.6% and an obvious raise from 24.8 to 43.1% in the S/M phase population (Figure 12 D&F). The differences between kefir and milk treatment were significant, as determined by statistical analysis.

It is thus implied that kefir causes a cell cycle arrest at the G1 transition checkpoint. These results explain the anti-proliferative effect of kefir that was detected through the WST-1 proliferation assay.

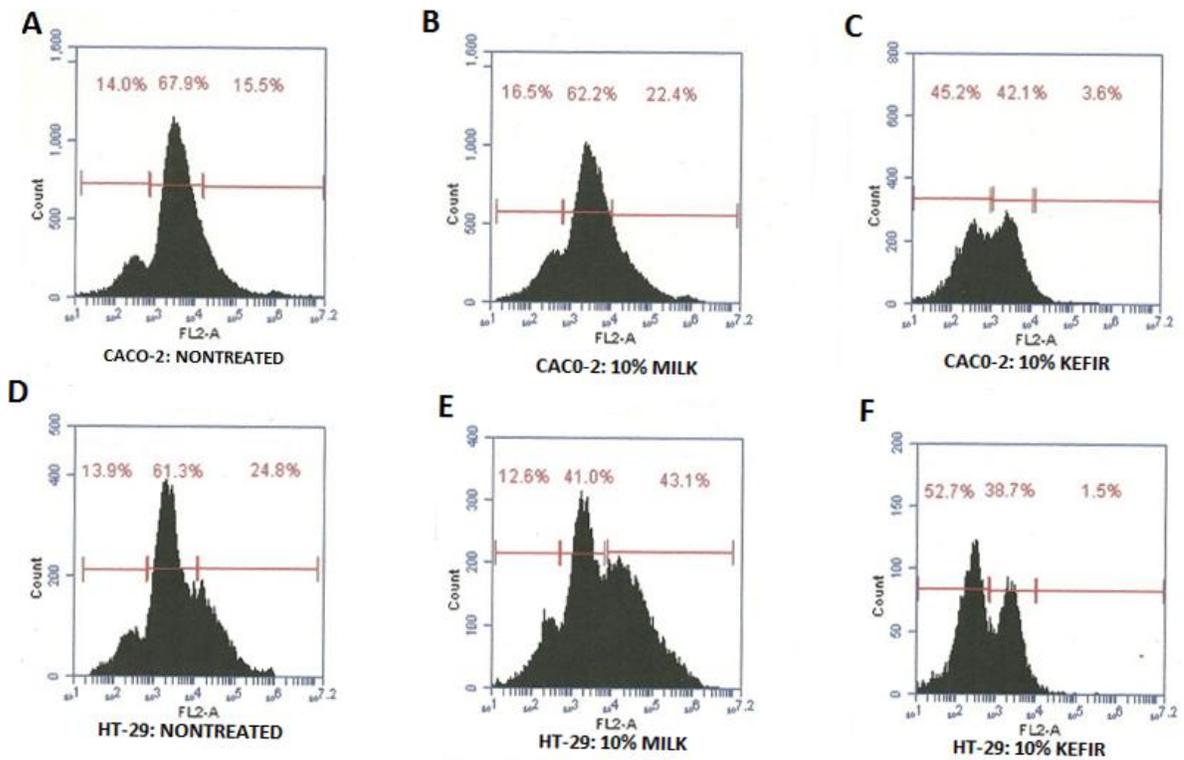


Figure 12: Cell cycle analysis by flow cytometry. Caco-2 (upper panels) and HT-29 (lower panels) cells were treated for 24 hours with 10% milk and kefir. Control cells were left untreated. After fixation overnight, cells were incubated with Propidium Iodide. Cells were analyzed using a C6 flow cytometer, which indicated the distribution of the cells into their respective cell cycle phases based on their DNA content. Sub-G0/G1 cells were less than 2n, G0/G1 cells were 2n, S/M phase cells were >2n.

3.4 Kefir has a proapoptotic effect on Caco-2 and HT-29 cells

Kefir was shown to induce death through apoptosis in both colorectal adenocarcinoma cell lines. The results obtained from Cell Death Detection ELISA^{PLUS} confirmed that the decreased viability upon kefir treatment, detected using Trypan Blue Exclusion method, involves the induction of apoptosis. DNA fragmentation is the hallmark of apoptosis. When the cell lysates are incubated in the presence of anti-histone-biotin and anti-DNA-peroxidase, the broken nucleosomes will be caught by the antibodies, while also getting bound streptavidin-coated microplate. In Cell Death Detection ELISA, the absorbance of ABTS at 405 nm, reflects the quantity of Anti-DNA peroxidase, which in turn reflects that of the nucleosomes generated by apoptosis. The enrichment factor, ratio of the absorbance measured for each concentration to that of the untreated controls, was calculated. Both colorectal adenocarcinoma cell lines showed an increase in the enrichment factors calculated upon kefir treatment. The increase was dose-dependent. In Caco-2 kefir-treated cells, the enrichment factor increased around 2.3, 2.6, and 6 fold, 24 hours after treatment with 5%, 10%, and 15% kefir respectively (Figure 13A). Upon 48 hours of treatment, the calculated enrichment factors showed a 3.5, 4, and 5.6 fold increase with 5%, 10%, and 15% kefir respectively (Figure 13B). HT-29 cells also showed a significant increase in apoptosis induction upon kefir treatment, evident from the increase in the calculated enrichment factor. In the 24-hour kefir-treated HT-29 cells, the enrichment factors were approximately 1.9, 2.4, and 6.3 upon treatment with 5%, 10%, and 15% kefir respectively (Figure 14A). Similarly, after 48 hours of kefir treatment, the fold increase in enrichment factor was significant in this cell line as well. Treatment with 5%, 10%, and 15% kefir, yielded the following enrichment factors 1.3, 2.2, and 5.5 respectively (Figure 14B). As in the cytotoxicity and proliferation assays, cells were also treated with the different milk concentrations, same as those of kefir, to assess the effect on apoptosis levels. In both cell lines, milk treatment induced a decrease in the apoptotic levels as compared to the untreated controls. The enrichment factors calculated for various milk concentrations were all less than 1. The lowest value of enrichment factor was 0.5 for HT-29 cells, and 0.7 for Caco-2 cells obtained with 10%, 15% milk at 24 hours and 15% milk at 48 hours respectively (Figures 13 A,B and 14 A,B). The differences between kefir and milk treatment were significant, as determined by statistical analysis.

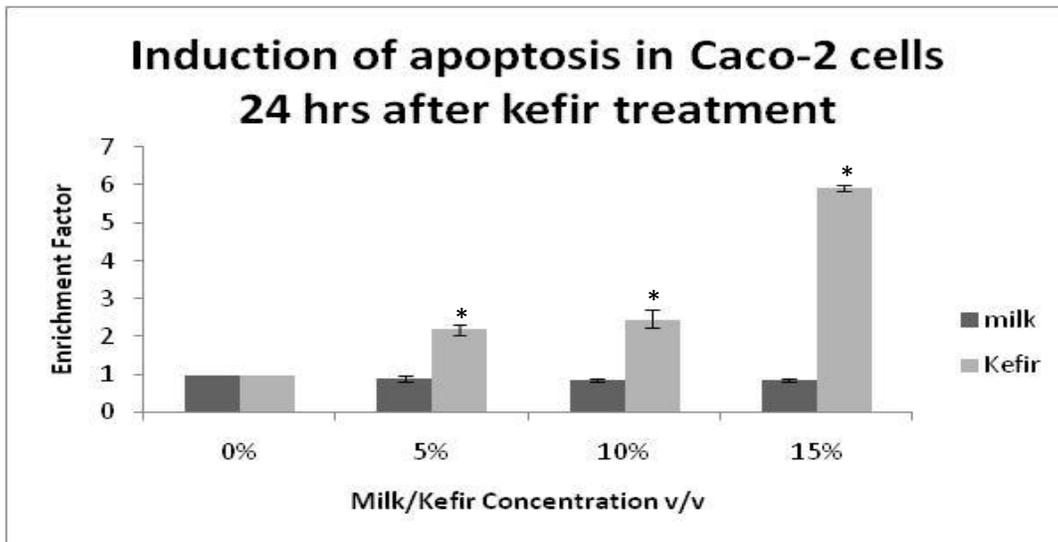


Figure 13A: Induction of apoptosis in Caco-2 cells 24 hr after kefir treatment. Cells were treated with noncytotoxic kefir concentrations and the corresponding milk concentrations. Absorbance was read at 405 nm after the addition of ABTS substrate. Results are shown as the enrichment factor (absorbance of sample divided by the absorbance of untreated control). The asterix signifies $p < 0.05$.

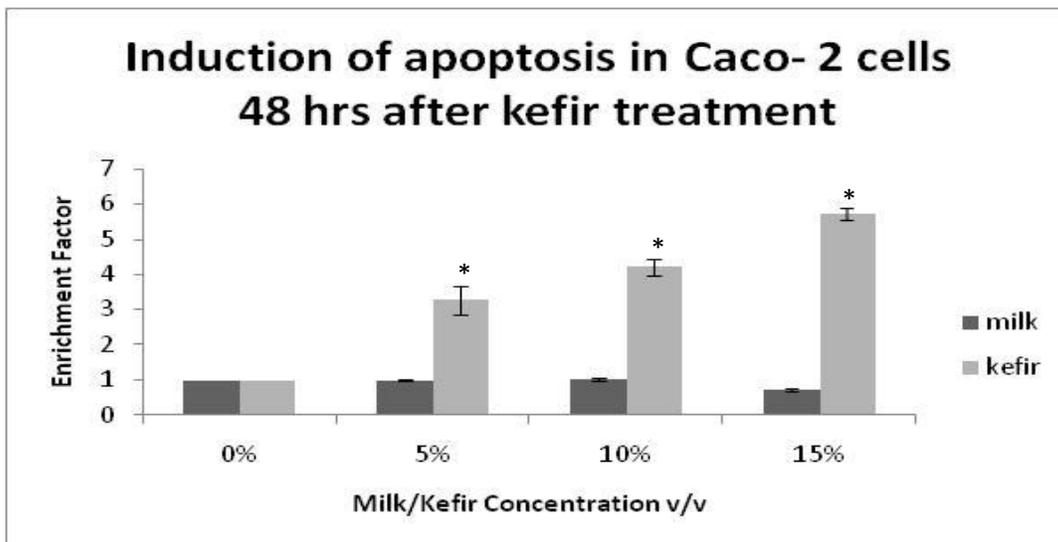


Figure 13B: Induction of apoptosis in Caco-2 cells 48 hr after kefir treatment. Cells were treated with noncytotoxic kefir concentrations and the corresponding milk concentrations. Absorbance was read at 405 nm after the addition of ABTS substrate. Results are shown as the enrichment factor (absorbance of sample divided by the absorbance of untreated control). The asterix signifies $p < 0.05$.

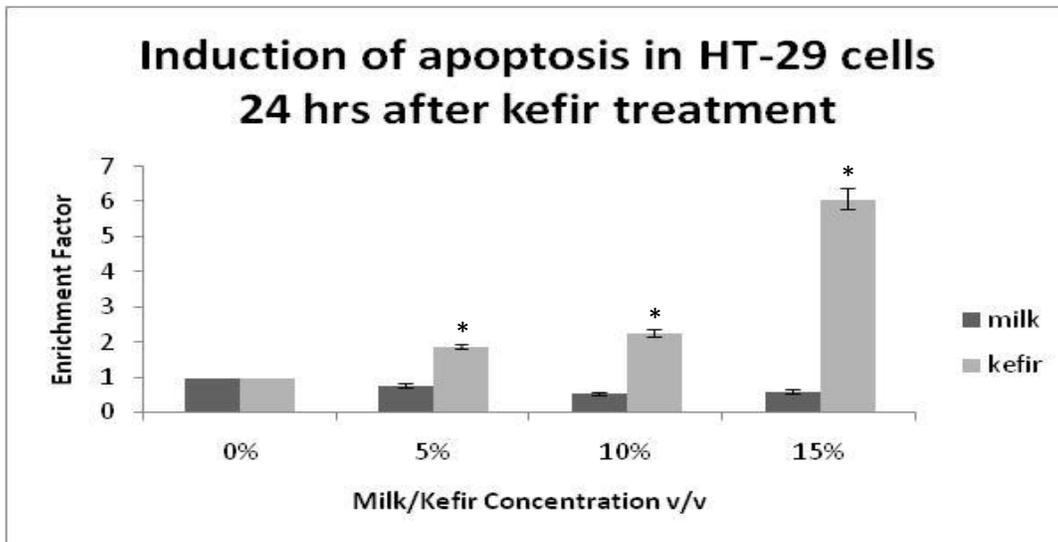


Figure 14A: Induction of apoptosis in HT-29 cells 24 hr after kefir treatment. Cells were treated with noncytotoxic kefir concentrations and the corresponding milk concentrations. Absorbance was read at 405 nm after the addition of ABTS substrate. Results are shown as the enrichment factor (absorbance of sample divided by the absorbance of untreated control). The asterisk signifies $p < 0.05$.

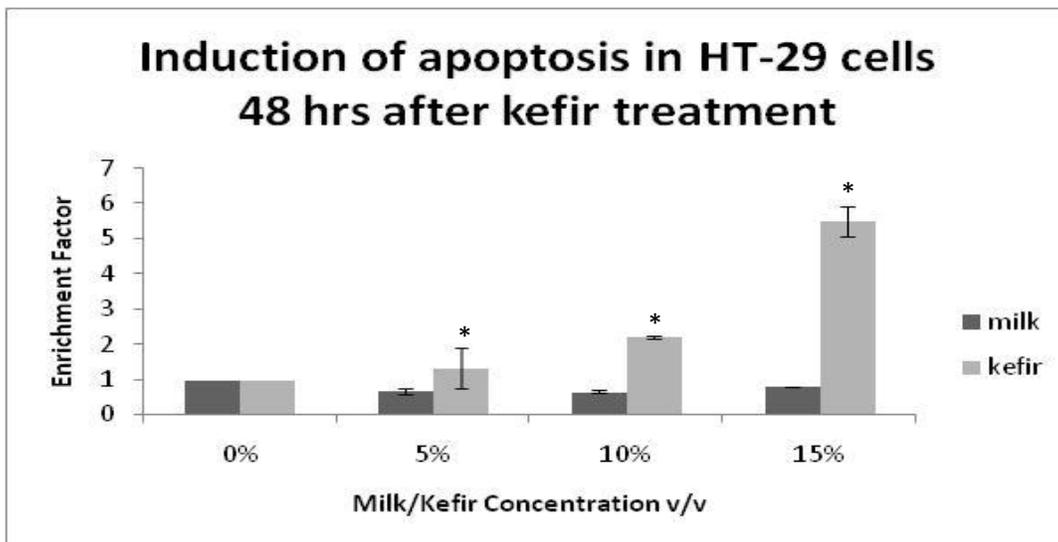


Figure 14B: Induction of apoptosis in Caco-2 cells 48 hr after kefir treatment. Cells were treated with noncytotoxic kefir concentrations and the corresponding milk concentrations. Absorbance was read at 405 nm after the addition of ABTS substrate. Results are shown as the enrichment factor (absorbance of sample divided by the absorbance of untreated control). The asterisk signifies $p < 0.05$.

3.5 Kefir induces apoptosis in a Bax/Bcl-2 dependent manner.

To elucidate the mechanism through which kefir was causing the apoptotic death of the treated cells, Western Blot analysis was performed for Bax and Bcl-2. The results show that kefir promotes the expression of Bax. With respect to the nontreated controls (Figure 15 upper panel lane 1), and the milk treated samples (Figure 15 upper panel lanes 2 and 3), the kefir treated samples (Figure 15 upper panel lanes 4 and 5) showed a significantly higher level of Bax. On the other hand, Bcl-2, the anti-apoptotic partner, was downregulated by kefir. As shown, the level of Bcl-2 detected in the kefir samples (Figure 15 middle panel lane 4 and 5) is much lower than in the non-treated control (Figure 15 middle panel lane 1) and the milk-treated samples (Figure 15 middle panel lanes 2 and 3). β -Actin was used as an internal control to verify equal loading among the different samples (Figure 15 lower panel).

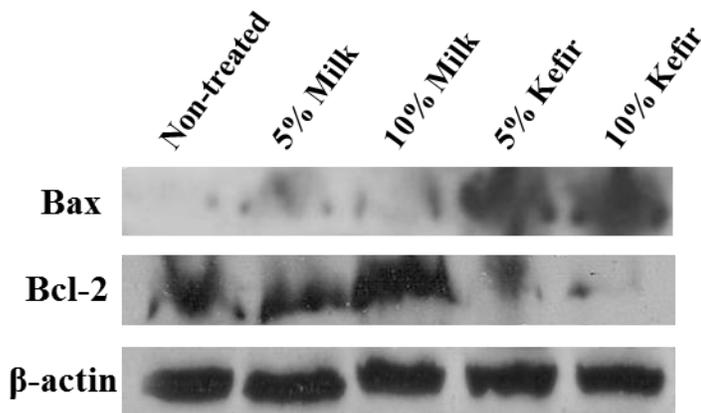


Figure 15: Western Blot analysis for Bax and Bcl-2. Caco-2 cells were treated with 5% and 10% milk and kefir for 48 hours. Control cells were left untreated. At the end of the treatment period, cells were lysed and blotted for Bax (upper panel), Bcl-2 (middle panel), and β -Actin (lower panel) as internal loading control.

Chapter IV

DISCUSSION

Since its early origins, kefir has been shown to be beneficial to human health in many aspects (Adriana & Socaciu, 2008; Angulo, et al., 1993; Farnworth, 2005). It has been proven that kefir has potential as an antimicrobial, immunostimulatory, antimutagenic, and anti-inflammatory agent (Matsuu, et al., 2003; Nagira, et al., 2002; Rodrigues, et al., 2005; Silva, et al., 2009; Thoreux & Schmucker, 2001; G. Vinderola, Perdigon, Duarte, Farnworth, et al., 2006). Most importantly, many studies have highlighted an anti-cancerous role of kefir. The mechanisms behind this role are complex and still not fully known. Several *in vivo* studies on Sarcoma 180, Ehrlich carcinoma, Lewis Lung Carcinoma, and B16 melanoma, all showed that the administration of kefir retarded tumor growth in murine or rat cancer models (C. Chen, et al., 2007; de Moreno de Leblanc, et al., 2007; Furuwaka, et al., 2000; Kubo, et al., 1992; Liu, et al., 2005; Maalouf, et al., 2011; Murofushi, et al., 1983; Rizk, et al., 2009; Shiomi, et al., 1982). In the present study, we aimed to investigate whether kefir's anti-cancerous effect, previously proven on several types of cancers, both *in vivo* and *in vitro*, also applies to colorectal cancer cell lines, *in-vitro*. The results obtained from this study are in accordance with previous *in-vitro* studies, on breast and leukemic cancer cell lines, which showed that kefir treatment possesses an antiproliferative and proapoptotic effect on these cancer cell lines (Chen, et al., 2007; Maalouf, et al., 2011; Rizk, et al., 2009).

We first determined, through treating cells with increasing kefir concentrations, that the IC-50 of kefir was reached with 18%, 10%, and 11% v/v at 24, 48, and 72 hours respectively, for Caco-2 cells, and 12% and 10% v/v at 48 and 72 hours respectively, for HT-29 cells. Treatment with higher concentrations was cytotoxic to the cells. The two cell lines showed a similar pattern in the viability decrease in response to kefir treatment. However, with HT-29 cells, the IC-50 was not attained 24 hours after treatment, even with the highest dose used (20% v/v). The Trypan Blue Exclusion method does not differentiate, among dead cells,

between those that have died from apoptosis and those from necrosis. However, since 6 hours post-treatment, the viability of both cell types was not reduced, it was assumed that the cells were dying through apoptosis. The fact that the milk treated cells showed no decrease in viability, suggests that kefir's effect is due to products produced by the microorganisms during fermentation.

Chen et al (2007), Maalouf et al (2011), and Rizk et al (2009) documented an inhibition of proliferation upon in vitro treatment of kefir. We aimed to see, if kefir has this same effect on colorectal cancer cell lines as well. As with breast and leukemic cancer cell lines, a notable decrease in proliferation was detected in the cells using the WST-1 reagent. The figures show that the decrease in proliferation is consistent with the decrease in viability. Caco-2 cells exhibited a 91%, 74%, and 96% decrease in proliferative activity upon treatment with 18%, 12%, and 10% v/v at 24, 48, and 72 hours respectively (concentrations representing the IC-50). For HT-29 cells, treatment with the concentrations at which the IC-50 was attained (12% and 10% v/v at 48 and 72 hours respectively), caused 64% and 40% decrease. In addition, as with all other assays, cells were treated with various milk concentrations. WST-1 assay results show an increase in proliferation. Milk is a rich source of many amino acids, vitamins, and minerals, which explains its ability to enhance proliferation. Similar results were obtained by Chen et al (2007), where a reduction in proliferation was seen upon treatment of MCF-7 mammary cells with kefir, but proliferation increased upon their treatment with milk. Rizk et al (2009), and Maalouf et al (2011), as well, detected a significant reduction in proliferation levels, accompanied by an induction of apoptosis upon treatment of HTLV-1-negative malignant T-lymphocytes with kefir (Maalouf, et al., 2011; Rizk, et al., 2009).

We then aimed to examine if this antiproliferative effect was through interference in the cell cycle progression. Through flow cytometry, it was verified that kefir causes a shift in the cell cycle of the treated cells towards the sub-G0/G1 phase, by inducing cell cycle arrest at the G1 checkpoint. Kefir did not only increase the percentage of cells in the sub-G0/G1 phase, but caused a reduction in the S/M cell population as well. The G1 arrest implies that kefir induces death in colorectal cancer cell lines.

To verify that the cells were dying through the mechanism of apoptosis, as assumed, the Cell Death Elisa Kit assay was performed. The results of this assay confirmed that kefir was indeed inducing apoptosis in Caco-2 and HT-29 cells. When these cells were treated with higher kefir

concentrations, the level of apoptosis induction showed a significant increase. Hence, the reduction in viability, which was seen in the Trypan Blue Exclusion Method, was achieved through apoptosis, rather than necrosis. Unfermented milk, when applied to Caco-2 and HT-29 cells, didn't show any induction of apoptosis. This explains the lack of any reduced cell viability seen in the Trypan Blue Exclusion Method in the milk-treated cells. In addition, the results of the milk treatment again suggest a possible role for the fermentation products in the proapoptotic role.

Bcl-2 acts as a repressor of the apoptotic pathway, while Bax, counters this action, and promotes apoptosis. The ratio of Bax and Bcl-2 in a cell reflects the activation of the survival or apoptotic pathways (Oltvai, Milliman, & Korsmeyer, 1993). Because kefir was clearly inducing apoptosis in both colorectal cancer cell lines, we aimed to find out if the apoptotic pathway that was activated by kefir involved Bax and Bcl-2. Western Blot analysis was used, and the results showed that treating the cells with noncytotoxic kefir concentrations had triggered the expression of Bax, the proapoptotic protein, while simultaneously inducing a downregulation of Bcl-2, the anti-apoptotic protein. The milk-treated cells, on the other hand, showed the opposite response, an upregulation of Bcl-2 and a downregulation of Bax. These results suggest that kefir, which was shown to cause the death of the cancer cells (through the Trypan Blue Exclusion Method and the Cell Death Elisa Kit), was doing so through the activation of the Bax/Bcl-2 apoptotic pathway. The apoptotic pathway, which involves the couple Bax and Bcl-2 begins with the activation of AMPK. The latter is known to control apoptosis in several cancers, including colorectal cancer (Oda, et al., 2000). Downstream of AMPK is p53, whose up-regulation in turn causes the up-regulation of the pro-apoptotic Bax and the anti-apoptotic Bcl-2 (Oda, et al., 2000; Prives & Hall, 1999). The disturbance in the normal balance between Bax and Bcl-2, in favor of Bax, triggers the activation of Caspase-3. This in turn causes the proteolytic cleavage of PARP, thus triggering apoptosis (Green & Amarante-Mendes, 1998). Since kefir was found to modulate the expression of Bax and Bcl-2, it would be interesting to verify if its effect on these two proteins is direct or indirect, possibly through effectors in this pathway (Figure 16).

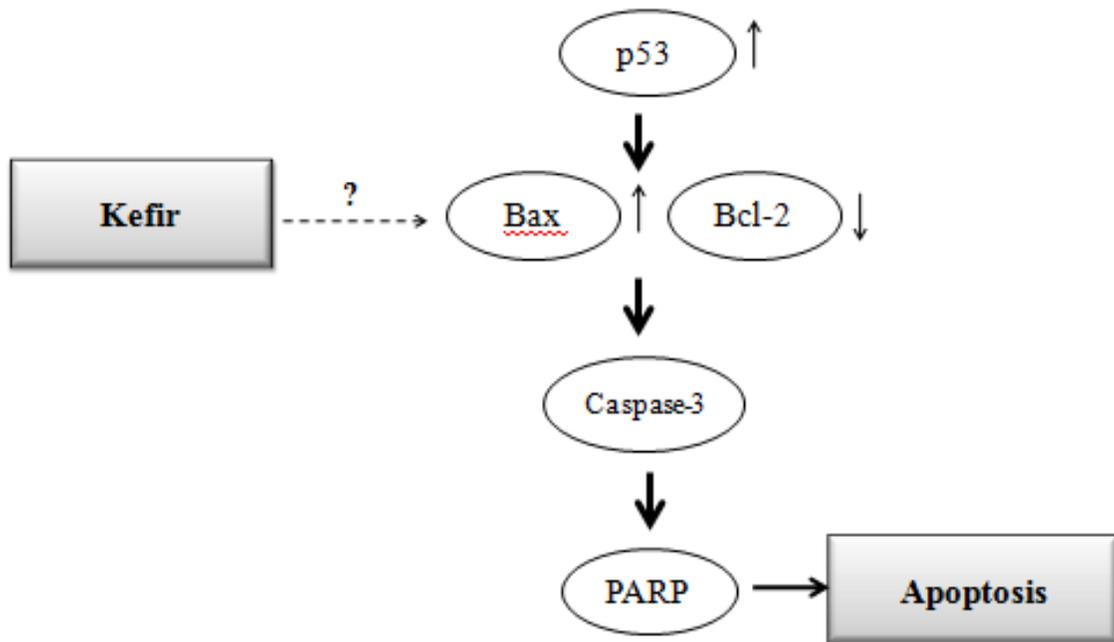


Figure 16: Possible pathway for kefir's proapoptotic effect

Rizk et al (2009) and Maalouf et al (2011) who showed an antiproliferative effect of kefir in HTLV-1-negative malignant T-lymphocytes, used RT-PCR to study the transcriptional level of TGF- α , TGF- β 1, and MMP-2. Their results showed that kefir inhibited TGF- α and upregulated TGF- β 1, thus explaining the decreased proliferation of the kefir-treated lymphocytes. Further investigation, should examine if kefir has similar mechanisms in colorectal cancer cell lines. Our results, along with those just mentioned, suggest that kefir may

have a pleiotropic effect, altering the expression of different proteins and genes, involved in various pathways, all leading to an antiproliferative and proapoptotic effect in cancer cells.

The active component behind kefir's anti-cancerous and other beneficial effects is still unidentified. The potential constituents include bacterial cell wall components, microbial DNA, peptides, and exopolysaccharides (Hong, et al., 2010; Thoreux & Schmucker, 2001; G. Vinderola, Perdigon, Duarte, Farnworth, et al., 2006). Chen et al (2007) who demonstrated the antiproliferative role of kefir on mammary cancer cells, detected a change in peptide content during kefir fermentation, to which the anti-cancer effects could be attributed (Chen, et al., 2007). Future work should aim to identify and isolate kefir's active ingredient.

In the present study, we were unable to determine the cytotoxicity of kefir in normal colorectal cells, due to the unavailability of such cell lines. However, kefir is a highly consumable product, without any known negative side effects on the gastrointestinal tract. Rather, kefir has been shown to be beneficial the digestive system, and hence it was assumed to be non-cytotoxic to normal colorectal cells. Further studies, should however, assert this assumption, through determining effect of the different kefir concentrations, on normal colon cell lines.

There have been several studies, similar to the current one, which aim to identify and test potential antiproliferative and proapoptotic agents on colorectal cancer cell lines in vitro. Such agents and compounds include osteopontin, fungal extracts, berry extracts, curcumin, and quercetin (Kim, et al., 2010; Lavi, Friesem, Geresh, Hadar, & Schwartz, 2006; Lavi, et al., 2010; Likui, Hong, Shuwen, Yuangang, & Yan, 2011; McDougall, Ross, Ikeji, & Stewart, 2008; Shehzad, Wahid, & Lee, 2010). Most of these studies were done on Caco-2 and/or HT-29 cells, and they all aimed to find if the compound or extract of interest could, someday, become a therapeutic target for CRC. One such compound, curcumin (diferuloylmethane), has entered several clinical trials, and has completed phase I in some trials for its potential employment in CRC treatment. One study showed that curcumin's IC₅₀ on HT-29 cells was 50 μ M (Singh, Shrivastav, & Sharma, 2009). Various studies showed that curcumin was responsible for the overexpression of Bax and p53, and the underexpression of Bcl-2 (Shehzad, et al., 2010; Su, et al., 2006). Kefir's anti-cancerous potential, demonstrated by this and many other studies, is comparable to that of curcumin, which suggests that clinical trials for kefir may be feasible.

In all the experiments that were done in this study, both cell lines, Caco-2 and HT-29 cells, showed a similar pattern in their responses to kefir and milk treatment. Although there were differences in the extent of the response in some cases, both cell lines exhibited the same results. This implies that kefir's mechanism is not cell-line specific, but rather, applies to colorectal cancer cells in general.

Chapter V

CONCLUSION

Kefir has become globally known as a complex probiotic, to which many health benefits have been attributed. These include anti-microbial, anti-inflammatory, immunomodulatory, and metabolic benefits. In this study, our main concern was kefir's anti-cancerous potential. Through several experiments, we have established that kefir exhibits proapoptotic and antiproliferative properties on colorectal adenocarcinoma cells, namely Caco-2 and HT-29, *in vitro*. It was also demonstrated that kefir causes cell cycle arrest at the G1 phase. Western Blot analysis demonstrated that kefir induces the overexpression of Bax, while repressing Bcl-2, thus depicting a possible pathway through which kefir intervenes to cause apoptosis of the malignant cells. The results of all experiments, which were correspondingly done milk treatment, affirm that kefir's beneficial effects are due to products produced by the microorganisms during fermentation. Further work should attempt to determine the exact pathway on which kefir works to cause apoptosis, cell cycle arrest, and reduced proliferation. Expression levels should be studied at the level of RNA and proteins. On the other hand, the active component of kefir should be identified.

The results obtained by this study are promising, and can pave way to *in-vivo* studies, and later on clinical trials.

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