NOTE TO USERS

Antitumor Properties of Kefir:

Possible Bioactive Component(s) and Mechanism(s)

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February, 2005

A thesis submitted to McGill University in partial fulfilment of the requirements of the degree of

DOCTOR OF PHILOSOPHY

Your file Votre référence
ISBN: 0-494-12819-4
Our file Notre référence
ISBN: 0-494-12819-4

To the memory of my mom, Mrs. Cuilian Zhang.

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ABSTRACT

Research on the putative health benefits has indicated that kefir, a traditional fermented milk, might have antimutagenic and antitumor properties. The major objective of the present thesis was to isolate and identify antitumor compounds in cow's milk kefir and investigate the possible mechanisms involved. High speed centrifugation (HSC), molecular weight cut-off filtration (MWCO), size exclusion high performance liquid chromatography (SEC-HPLC) and reverse phase-HPLC (RP-HPLC) were utilized for fractionation of kefir and a cell culture model was developed to screen for the antiproliferative effects of the kefir fractions. The antiproliferative effects of bacteria-free extracts from different fermentation stages of kefir production, as well as bacteria-free extracts from milk and yogurt were compared. The results showed that extracts from an early stage of fermentation (i.e., kefir mother culture) and the final commercial kefir product both exerted dose-dependent inhibition effects on human mammary tumor MCF-7 cells, yogurt extracts showed less potent antiproliferative effects, while pasteurized milk extracts showed no antiproliferative effects. No antiproliferative effects of the kefir extracts were observed on human mammary epithelial cells (HMEC) whereas the yogurt extracts showed antiproliferative action in HMEC cells at a high dose. A fraction of the kefir mother culture isolated by HSC, MWCO and RP-HPLC contained components that inhibited MCF-7 cell growth and had no effect on HMEC cells. Characterization of the bioactive fraction using mass spectrometry (MS) indicated that the main components in the fraction are likely fragments of kefiran and/or ceramide containing compounds such as gangliosides. The growth inhibitory effect may be mainly caused by the induction of TNF-alpha in MCF-7 cells. Whole extracts of kefir depleted glutathione (GSH) in MCF-7 cells, while the SEC-HPLC Fraction 7 and the RP-HPLC Fraction 30 induced GSH productions in MCF-7 cells. Fractions of kefir also enhanced ceramide toxicity on MCF-7 cells and increased the susceptibility of MCF-7 cells to tamoxifen. The potential of using the kefir extract as co-drug for chemotherapy should be explored. Comparison of RP-HPLC elution profiles and CE

electropherograms among kefir, milk, whey protein isolates, and their digests indicated that both microflora fermentation and in vitro enzymatic digestion have effects on protein and peptide profiles. These results provided scientific evidence for possible health benefits of kefir and provide valuable information regarding possible mechanisms involved in the antitumor effects of kefir.

RÉSUMÉ

La recherche sur les avantages de la santé ont démontré que le kéfir, un lait traditionel fermenté, aurait possiblement des propriétés antimutagènes et antitumeurs. L'objectif principal de cette thèse était d'isoler et d'identifier les composés antitumeurs du lait de vache kéfir et d'étudier les mécanismes impliqués. La centrifugation à grande vitesse, la filtration de coupure du poids moléculaire, la chromatographie liquide de rendement élevé de l'exclusion de la taille, et la phase renversée de la chromatographie liquide de rendement élevé ont été utilisées pour le fractionnement du kéfir et un modèle du culture de cellule a été développé pour identifier les effets antiproliphères des composés du kéfir. Les effets antiproliphères d'extraits exempts de bactéries, provenant de différentes étapes dans la fermentation de la fabrication du kéfir, ainsi que des extraits exempts de bactéries de lait et de yogourt ont été comparés. Les résultats de deux extraits pris au début de la fermentation (c.-à-d., la culture mère du kéfir) et lors du produit final du kéfir prêt à commercialiser, ont exercé des effets inhibiteurs dose dépendant sur les cellules humaines d'une tumeur mammaire MCF-7, les extraits de yogourt ont révélé une diminution des effets antiproliphères, tandis que les extraits du lait pasteurisé ont démontré aucun effet antiproliphères. Aucun effet antiprolifère dans les extraits de kéfir n'a été observé sur les cellules mammaires humaines épithéliales. Par contre, les extraits de yogourt ont démontré un mouvement antiprolifères dans ces mêmes cellules à forte dose. Une partie de la culture mère du kéfir a été isolée par centrifugation à grande vitesse, filtration de coupure du poids moléculaire et phase renversée de la chromatographie liquide de rendement élevé renfermait des composants qui ralentissaient la croissance de la cellule MCF-7 et n'avaient aucun effet sur les cellules mammaires humaines épithéliale. La caractérisation de la partie bioactive utilisant la masse spectrométrique a indiqué que les composants principaux de la partie sont surtout des fragments de kéfiran et/ou de céramide contenant des composés comme les gangliosides. L'effet ralentissant de la croissance est peut-être causé par l'induction de TNF-alpha dans les cellules MCF-7. Des extraits

entiers de kéfir ont réduit le glutathion, pendant que la chromatographie liquide de rendement élevé de l'exclusion de la taille partie 7 et la phase renversée de la chromatographie liquide de rendement élevé partie 30 ont déclenché la production de glutathion dans les cellules MCF-7. Des parties de kéfir ont accru aussi la toxicité de la céramide des cellules MCF-7 et ont augmenté la susceptibilité des cellules MFC-7 au tamoxiphène. La possibilité d'utiliser l'extrait de kéfir comme médicament parallèle pour la chimiothérapie devrait être explorée. La comparaison entre les profils de la phase renversée de la chromatographie liquide de rendement élevé et les éllectogrammes CE dans le kéfir, le lait, les protéines du petit-lait et leurs éléments digérés ont indiqué que la fermentation microflore et la digestion enzymatique in vitro ont des effets sur les profils protéiniques et peptide. Ces résultats ont fourni une preuve scientifique des bienfaits probables du kéfir et apportent des renseignements utiles sur des mécanismes possibles dans les effets antitumeurs du kéfir.

ACKNOWLEDGMENTS

I would like to thank both of my supervisors, Dr. Hing Man Chan and Dr. Stan Kubow, for their financial and scientific support throughout the Ph.D. program. Without their trust, fairness and help, I would not have been able to finish my graduate studies. I would also like to acknowledge Dr. Ted Farnwarth, for his advice and comments all along my graduate studies, Dr. Kobi Yeboah for mass spec analyses, and Dr. Xin Zhao and Dr. Gaetan Faubert for letting me use their facilities. Thanks to all the staff of CINE and School of Dietetics and Human Nutrition, my fellow students and friends for their kind help.

I would also like to thank Mrs. Donna Leggee and Mrs. Jenny Eny for their technical support, Mrs. Guylaine Charbonneau for translation of the thesis abstract in French, Mr. Nil Basu for proof reading the thesis.

Thanks also go to Liberte Inc. for providing the kefir samples and funding, NSERC for research grant.

Finally, I would like to thank my wife Cuixia, my sons Yuyuan and Yumeng, and my family in China for their understanding and support.

CONTRIBUTIONS OF AUTHORS

In accordance with McGill thesis submission guidelines the following paragraphs have been reproduced to inform external reviewers of faculty regulations:

This is a manuscript-based thesis and includes connecting texts between the manuscripts, one comprehensive reference list, a final conclusion and summary, a statement in regard to the contribution of authors and a statement of originality.

All of the manuscripts of this thesis are co-authored by Dr. Hing Man Chan and Dr. Stan Kubow who provided a supervisory role that included advice on the objectives, data analysis and presentation of results, editing and securement of funding for the laboratory procedures. All other aspects were the responsibility of the primary author, Chujian Chen.

CONTRIBUTIONS TO KNOWLEDGE/ STATEMENT OF ORIGINALITY

Breast cancer is the most commonly diagnosed cancer in women. Resistance to chemo- and radio-therapies is the major reason why most cancer treatments fail. Thus the development of novel effective therapeutic agents will improve patient outcome. One major accomplishment of the present study was the successful separation of a kefir fraction that has an antiproliferative effect on human breast cancer cells (MCF-7) while not on human normal mammary epithelial cells (HMEC). This fraction also increased the susceptibility of MCF-7 cells to tamoxifen, a commonly used anti-breast cancer drug. Extensive MS analysis suggested that the main components in this fraction were likely fragments of kefiran and/or ceramide containing compounds such as gangliosides. To my knowledge, this is the first study to document this.

Another achievement of this thesis was the exploration of possible cellular mechanisms involved in the antiproliferative properties of kefir. Due to the inherent complexity of the fractions of kefir, their effects on both MCF-7 and HMEC cells are different. Fractions of kefir extract enhanced cytotoxicity of ceramide on MCF-7 cells, and TNF-alpha had an important role in depression of cancer cell growth.

Furthermore, this thesis explored the effects of in vitro digestion and microflora fermentation on the profiles of protein and peptide in the kefir. This study provided useful information regarding the possible compositional change after gastrointestinal (GI) digestion for further research in animal and human clinical trials.

In summary, this thesis has contributed scientific evidence to demonstrate the health benefits of kefir and examine possible underlying mechanisms. The results suggested that bioactive compound in kefir may be used in functional foods for prevention of breast cancer, or a candidate agent for anticancer therapy.

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LIST OF ABBREVIATIONS

ANOVA: analysis of variance

AAS: flame atomic absorption spectrometry

CE: capillary electrophoresis

Da: dalton

DMEM: Dulbcco's modified eagle medium

FM: fermented milk

GSH: glutathione

HI-FBS: heat-inactivated fetal bovine serum

HMEC: human normal mammary epithelial cells

HPLC: high performance liquid chromatography

HSC: high speed centrifugation

MALDI-TOF: matrix assisted laser desorption/ionization- Time Of Flight mass spectrometry

MCF-7: human breast cancer cells

MEGM: mammary epithelial growth media

min: minute

MS: mass spectrometer

MWCO: molecular weight cut-off

n: number of samples or replicates

PBS: phosphate buffered saline

RP-HPLC: reverse phase high performance liquid chromatography

SAS: statistic analysis system

SEC-HPLC: size exclusion high performance liquid chromatography

SD: standard deviation

TNF-alpha: tumor necrosis factor-alpha

INTRODUCTION

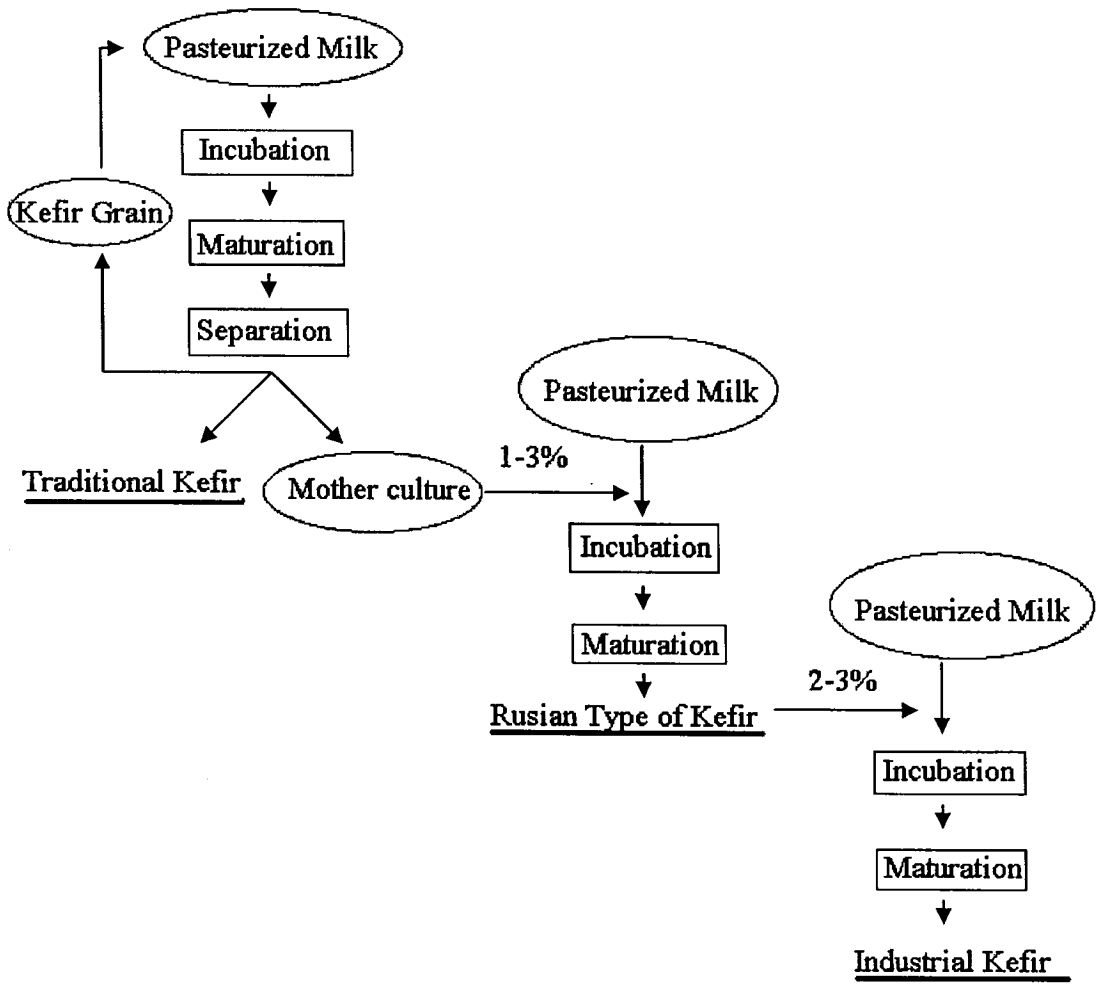
Kefir is a fermented milk (FM) that originated in and still widely consumed in the Caucasus region of the former Soviet Union. It is estimated that the kefir accounts for approximately 70% of the total amount of fermented milk consumed in the former Soviet Union (Kurmann *et al.*, 1992). Kefir is also well known in Hungary, Poland, Sweden, Norway, Finland, and Germany (Kroger, 1993), as well as in Japan, Greece, Austria, Brazil, Iran and Israel (Halle *et al.*, 1994). It is also commercially available in Canada.

Kefir distinguishes itself from the more known fermented milk product, yogurt, in that it is traditionally made only from kefir grains. Kefir is an acidic, mildly alcoholic, distinctly effervescent FM, and kefir grains are moist, gelatinous, whitish, or yellowish, irregular cauliflower-like granules. In general, kefir grains are stable conglomerates of lactic acid bacteria (primarily *lactobacilli*, *lactococci*, *leuconostocs*) and yeasts (predominantly *Torulopsis holmii* and *Saccharomyces delbruechii*) held together by a polysaccharide gum (kefiran) that it is produced by the predominant bacterial species, and thus the grains create a natural immobilized cell system. Recent studies have shown that grains from different geographical locations differ in composition, and a large variety and number of different species of yeasts and bacteria can be identified in kefir grains (Bottazzi, 1994, Halle *et al.*, 1994, Koroleva *et al.*, 1988).

Kefir can be made from the milk of goat, sheep, or cow with the addition of kefir grains. According to different manufacturing procedures, there are at least three possible kefir products: cottage style kefir, Russian process kefir and industrial kefir (Figure 1) (Farnworth, 1999).

The traditional method of producing kefir is achieved by directly adding kefir grains (2-10%) to milk that has been pasteurized and cooled to 20-25°C. After a period of fermentation lasting approximately 24 h, the grains are removed by filtration. The beverage, itself containing live microflora from the grains is then ready for consumption. The grains that grow in the process of kefir production are reused for subsequent fermentation (Halle *et al.*, 1994).

Figure 1 Diagram of different manufacturing procedures of kefir


Pasteurized Milk
Incubation
Maturation
Separation
Kefir Grain
Traditional Kefir
Mother culture
1-3%
Pasteurized Milk
Incubation
Maturation
Rusian Type of Kefir
2-3%
Pasteurized Milk
Incubation
Maturation
Industrial Kefir

A second method, known as the "Russian method", permits the production of kefir on a larger scale, and uses two fermentation steps. The first step is to prepare the cultures by incubating milk with grains (2-3%). The grains are then removed by filtration and the resulting mother culture is added to milk (1-3%), which is fermented for 12 to 18 h (Halle *et al.*, 1994).

Kefir-related products are produced in many countries (Kurmann *et al.*, 1992). Some examples include (a) freeze-dried kefir is made from concentrated milk (360 g/kg total solid) and fermented using traditional grains; (b) buttermilk kefir is a traditional product made from skimmed milk; (c) cultured milk kefir is produced from a special blend of baker's yeast and a cream or yogurt starter culture (none of which originated from kefir grains); (d) kefir-like products are manufactured using blends of micro-organisms which result in a varying range of sensory properties, but they lack the typical characteristics of traditional kefir. Products within this categories are known as Omaere (in South-West Africa), Rob or Roba (in some Arab countries), Kjalder Mjolk (in Norway), Kellermilch (in Germany) and Tarag (in Mongolia); and (e) a modified kefir produced in Russian from bovine milk low in fat, but with enriched protein, known as Osobyi.

Kefir enjoys a rich tradition of beneficial claims. Consumption of kefir has been used in the former Soviet Union for the treatment of a variety of conditions including metabolic disorders, atherosclerosis, cancer, and gastrointestinal disorders (Koroleva *et al.*, 1988). Although there is lack of clinical tirals or population based studies on kefir, in a case-control study done by Ronco *et al.* (2002) in Montevideo, Uruguay, consumption of ricotta cheese and skim yogurt were associated with a significant decreased risk of breast cancer. Low-fat and fermented products combined appeared to be the most protective dairy foods. Kefir contains a greater number of different bacteria than yogurt. Therefore consumption of kefir may have the similar effect. Encouraging results regarding the antitumor properties of kefir have been reported in animal studies (Cervikbas *et al.*, 1994; Furukawa *et al.*, 1990 and 2000; Shiomi *et al.*, 1982). Components in fermented milk such as conjugated linoleic acid (CLA) (Schonberg *et al.*, 1995), sphingolipids (Schmelz *et al.*, 1996; Dillehay *et al.*, 1994), polysaccharides

(Shiomi *et al.*, 1982), organic acids (Garrote *et al.*, 2000), and some proteins and peptides (Svensson *et al.*, 1999) have been shown to possess antimutagenic and antitumor effects.

Due to the complexity of bacteria and yeasts in kefir grains, kefir from different regions may vary in composition. Russian-type of kefir enjoys the longest history and rich health benefits. However, there is still a need for comprehensive scientific evidence to support the above mentioned health benefits.

Objectives

The general objective of this thesis is to identify the bioactive ingredient(s) that are responsible for the antitumor properties of kefir and to characterize their mechanism(s) of action(s). Specific objectives include:

(1) To fractionate extracts of kefir, mother culture used in the production of kefir, yogurt and milk; screen for antiproliferative effects of these fractions on human breast cancer cells (MCF-7) and human mammary epithelial cells (HEMC).

(2) To characterize the compositions of kefir mother culture and final kefir in terms of nutrients such as protein, fat, carbohydrates, minerals and lactic acid; the most active fraction(s) of kefir fermentation showing antiproliferative effects on MCF-7 cells with respect to major constituents (proteins and peptides) and potential physiologically active components (organic acids, sphingolipids, proteins, and peptides).

(3) To relate the presence of an ex novo soluble compound such as a functional peptide, sphingolipids produced by kefir during fermentation with the antiproliferative activity of kefir on MCF-7 cells.

(4) To explore the specific mechanisms resulting in the antitumor and antioxidative activity of the isolated kefir fractions in normal mammary cells and mammary tumor cells. Specifically, to investigate whether the antiproliferative effects of kefir on tumorigenic but not normal mammary

cells are due to differential effects of kefir on cellular concentrations of gluthathione (GSH) or tumor necrosis factor-alpha (TNF-alpha).

(5) To study the effect of in vitro digestion on protein and peptide profile of kefir. Kefir is usually consumed orally. As there is likely further generation of bioactive peptides following gastrointestinal (GI) digestion, it would be of interest to explore the further compositional changes in kefir after in vitro digestion.

Rationale

Breast cancer is the most commonly diagnosed cancer in women, and the American Cancer Society estimated there were approximately 213,000 new cases diagnosed in 2003. Resistance to therapy is the major reason for the failure of most cancer treatments. Chemo- and radio-therapies are thought to primarily exert antitumor effects through the activation of programmed cell death pathways (Mesner *et al.*, 1997), and resistance to these therapies is often the result of defects in the apoptotic cascade. Developments of new agents that can induce programmed cell death or overcome resistance mechanisms are predicated to improve patient outcomes, prevent relapse, and prolong patient survival.

Research on the putative health benefits of fermented milks (FM) has indicated that by-products of bacterial fermentation of proteins, lipids and carbohydrates present in FM exert health benefits beyond basic nutrition including antitumor action, immune system enhancement and antioxidant effects. The active ingredients in the FM products have not been fully characterized but several studies suggested that the antimutagenic effect of these cultured milk was due to the presence of the lactic acid bacteria themselves (Abdelali *et al.*, 1995; Hosono *et al.*, 1990; Renner *et al.*, 1991; Pol-Zobel *et al.*, 1993), casein and calcium (Abdelali *et al.*, 1995), organic acids (Garrote *et al.*, 2000), polysaccharides (Shiomi *et al.* 1982), certain proteins and peptides (Bourtourault *et al.*, 1991; McIntosh *et al.*, 1995; Svensson *et al.* 1999) and sphingolipids (Lemonnier *et al.*, 2003).

As suggested by the previous studies, dairy products such as whey protein isolates and fermented whey protein have been shown to diminish oxidative stress in normal tissues after their consumption (Nicodemo *et al.*, 1999; Zommara *et al.*, 1998). Similarly, kefir intake has been demonstrated to induce antioxidative effects in humans (Kubow and Fotouhinia, 2001). An induction of tissue GSH has been postulated to be the possible mechanism of action from the intake of native and fermented whey proteins for decreasing oxidative stress in normal cells (Nicodemo *et al.*, 1999; Zommara *et al.*, 1998; Kubow and Fotouhinia, 2001), as fermentation of milk proteins generates more peptides and enhances their GSH-inducing properties. This evidence suggests that functional peptides derived from the fermentation process could play a role in the stimulation of GSH synthesis. In this regard, protein fermentation products found in kefir could be exerting similar antioxidative effects since milk, as the substrate for kefir production, does not exert antioxidative action (Kubow and Fotouhinia, 2001). Hence, the decrease in oxidative stress in normal cells could be attributable to a possible induction of GSH synthesis. In addition, exposure to whey proteins causes depletion of glutathione in cultured human mammary carcinoma cells whereas glutathione concentrations in normal cells are induced by whey proteins (Baruchel and Viau, 1996). Thus selective alterations in GSH synthesis and transport in normal cells versus tumor cells in response to stimuli such as dietary whey proteins, pro-drugs (i.e. L-2-oxothiazolidine-4-carboxylate, OZT), may explain the different observed antiproliferative effects in normal and cancer cells (Bounous, 2000).

Certain milk peptides (Yoo *et al.*, 1997), as well as whey proteins found in milk (Svensson *et al.*, 1999) and fermented milk products (Biffi *et al.*, 1997), have been demonstrated to exert antitumor activities in cell cultures without suppressing the growth of normal cells. Sulfur-containing antioxidants such as N-acetylcysteine and dimercaptopropanol have been shown to induce apoptosis in several transformed cell lines and transformed primary cultures but not in normal cells (Liu *et al.*, 1998). Baruchel and Viau (1996) demonstrated an apparent paradoxical action whereby whey proteins decreased GSH in tumor cells although GSH levels were raised in normal cells. As depression of cellular GSH has been

known to induce apoptosis (Hall *et al.*, 1999), it is conceivable that apoptosis may also play a role in the suppression of tumor cell proliferation observed after kefir treatment of tumor cells (Hursting *et al.*, 1999).

Previous work has indicated that redox potential may be influenced by GSH-modulating dietary peptides. This offers the prospect of altering the sensitivity of cancer cells to cytotoxic drugs, dietary components and immune system attack. Whey proteins could exert antitumor effects by modulation cellular GSH content in tumor cells, and depletion of GSH following kefir exposure could be a mechanism underlying the antiproliferative effects of kefir on these cells. In addition, some components of milk and fermented milk exhibited effects on TNF-alpha which can also lead to apoptosis. Those may also explain in part the observed antiproliferative effects on tumor cells.

TNF-alpha is a naturally occurring cytokine secreted by cells of the immune systems. Although TNF-alpha is cytotoxic to some tumor cells, it is rarely cytotoxic to normal cells. TNF-alpha induces intracellular signals that mediate cell death. One major signal induced by TNF-alpha is activation of neutral and acidic sphingomyelinases which catalyze the degradation of sphingomyelin to ceramide (Hannun, 1996).

Ceramide, or N-acyl-sphingosine, has been implicated in the acquired drug resistance that often characterizes breast cancer cells (Liu *et al.*, 1999). Two main routes have been defined for the generation of ceramide: (1) hydrolysis of sphingomyelin (SM), the most abundant lipid in the plasma membrane of mammalian cells, by the action of neutral or acidic sphingomyelinases (SMase); and (2) by *de novo* biosynthesis catalyzed by ceramide synthase. Ceramide functions as a second messenger to signaling cascades that promote differentiation, senescence, proliferation, and apoptosis. SMase activation by gamma radiation, ionizing radiation, TNF, Fas ligand, and daunorubicin leads to apoptosis. The de novo pathway for ceramide is also activated in response to TNF-alpha and paclitaxel agents, leading to apoptosis (Pettus *et al.*, 2002). Ceramide is also an inhibitor of cell proliferation in a variety of tumor cell lines and some

chemotherapy drugs such as doxorubicin mediate apoptosis by generation of ceramide (Hannun, 1996).

Dairy products are a rich source of sphingolipids. The sphingolipid contents of whole milk, butter, and cheese range from 0.5 to 1 μmol/g (Zeisel *et al.*, 1986). Their hydrolysis in the digestive tract, releases long chain bases that may protect the intestines from cancer formation (Borek *et al.*, 1991). Sphingosine and sphinganine inhibited protein kinase C and the transformation of mouse C3H 10T1/2 cells exposed to x-rays and phorbol 12-myristate-13-acetate (Borek *et al.*, 1991 and 1993). Sphingolipids reduce the metastatic potential and growth of several human cancer cell lines (Merrill *et al.*, 1995). Oral intake of sphingomyelin purified from powdered milk has been shown to reduce chemical induced colon tumors and suppress the conversion of adenomas to adenocarcinomas in mice (Dillehay *et al.*, 1994; Schmelz *et al.*, 1996). Kefir has been shown to have antitumor activity in cell culture and animal studies. Thus studying the interactions of kefir extract, anti-breast cancer drugs and ceramides may help explain the underlying mechanism involved in anticancer property.

Kefir is usually consumed orally, and is digested and absorbed in gastrointestinal tract (GI). Both microflora fermentation and in vivo enzymatic digestion will have impacts on the protein and peptide concentration of kefir. Kefir is made of milk fermented with kefir grains. Milk protein is the main source of protein in kefir. Most of the peptides showing bioactive properties from milk protein are in an inactive state in their peptide precursors. It has been shown that these bioactive peptides may be released during proteolysis induced by digestive enzymes and microflora fermentation (Matar *et al.*, 1996). Although some bioactive components in kefir have been studied in cell culture and animal study (Shiomi *et al.*, 1982; Rokka *et al.*, 1997; Cevikbas, 1994), the physical composition after gastrointestinal digestion are still unknown. Thus, an *in vitro* enzymatic hydrolysis model mimicking GI digestion will help us better understand the properties of kefir after GI passage.

In summary, kefir, or fractions from kefir have been shown to have antimutagenic and antitumor activities in cell culture and animal studies. However,

further research is necessary to characterize the bioactive component(s) in kefir that are responsible for these effects, and whether specific fractions of kefir generated during different stages of kefir production can exhibit more potent differential antitumor effects. Furthermore, possible mechanisms of action of kefir with respect to GSH, and TNF-alpha content in normal and tumor cells, the interactions of kefir extract and its subfractions, anti-breast cancer drug and ceramides need to be investigated.

CHAPTER 1

LITERATURE REVIEW

Kefir, as a traditional foodstuff in a nutrient diet, is widely consumed by Caucasus people. High consumption of kefir has been associated with longevity in the Caucasus regions (Koroleva *et al.* 1988). Van't Veer *et al.* (1989) observed a significantly lower consumption of fermented milk products (predominantly yogurt and buttermilk) among 133 breast cancer cases compared to 289 population control. In a case-control study by Ronco *et al.* (2002) in Montevideo, Uruguay, consumption of ricotta cheese and skim yogurt were associated with a significant decreased risk of breast cancer. Kefir, a fermented milk product (FM), is usually made of Cow's milk fermented with kefir grains. In the former Soviet Union, kefir has been used in hospitals and sanatoriums for a variety of conditions including metabolic disorders, atherosclerosis, cancer, and gastrointestinal disorders (Koroleva *et al.* 1988; Olesmall *et al.*, 1999). Research on the putative health benefits of FMs including kefir have showed that beyond basic nutrition, their health benefits may include blood cholesterol-lowering, immune system enhancement, antioxidant, antibacterial, antifungal, antimutagenic and antitumor activities (Cevikbas *et al.*, 1994; Nadathure *et al.*, 1994; Zacconi *et al.*, 1995; Biffi *et al.*, 1997; Matar *et al.*, 1997; Furukawa *et al.*, 1990 and 2000; Sanders, 2000). Some components derived from kefir are manufactured as health foods for prevention and control of obesity (Tokumaru *et al.*,1999), dietary supplements for the prevention of osteoporosis (Weissmahr *et al.*, 2000), and therapeutic use as a substitute of pancreatic stone protein (Obata *et al.*, 2000).

1 Health benefits of kefir

Fermented milks (FM) are animal milks incubated with either lactic acid bacteria or yeast or a combination of bacteria and yeast. Yogurt and kefir are two main FM. The production of yogurt is well defined., *Lactobacillus bulgaricus* (*Lb. bulguaricus*) and *Streptoccus thermophilus* (*S. thermohphilus*) are used for yogurt fermentation. These two strains are required by US FDA Standards in order for a product to be called Yogurt in the United States. Other cultures may be added but

are not required. In contrast to yogurt, kefir production may differ among manufacturers from different regions. Some companies use kefir grains, some use mixtures of pure cultures not found in kefir grains, and some use lyophilized cultures from grains. Further more, kefir grains from different region may contain different bacteria flora and yeasts. Thus, even those products are all called kefir, but each product may have different properties. This makes interpretation of results and comparison of studies difficult. The following literature review will focus on health benefits of kefir. Results from studies of other FM such as yogurt were also summarized as supportive evidence.

1.1 Improved lactose intolerance

Numerous studies appear to indicate that fermented milk products containing live bacteria are well tolerated by individuals who have lactose intolerance. Such individuals experience gastrointestinal symptoms due to a reduced ability to digest lactose, the sugar (a disaccharide) in milk, into its component sugars-glucose and galactose which can then be absorbed readily in the small intestine (Judith, 1997). Undigested lactose cannot be absorbed and travels to large bowel (colon) where it is digested by the resident microorganisms, causing excess gas production, intestinal discomfort and diarrhea. Lactose can be breakdown to its components or utilized by the culture bacteria and yeast during kefir fermentation, leads to decreased lactose content in the milk by 20-30% (Kuo and Lin, 1999). Kwak *et al.* (1996) reported that during kefir manufacture with grains, the lactic acid fermentation depleted lactose and lowered the pH value. In addition, during alcohol fermentation by yeast, lactose and other sugars were utilized to produce ethanol. Due to decreased lactose content in kefir, those with lactose intolerance can consume kefir. In a randomized block design study, 15 healthy adults (8 males and 7 females) were given 5 treatments consisting of 2% reduced-fat cow's milk, plain or flavored yogurt and kefir. Both yogurts and kefir reduced the perceived severity of flatulence by 71% relative to milk. Abdominal pain and diarrhea symptoms were negligible among 5 treatments (Hertzler and Clancy, 2003).

1.2 Antibacterial and antifungal activity

Consumption of kefir leads to the intake of large amounts of live bacteria. Alm (1983) and Korneva (1979) assessed the antibacterial activity of kefir against *Salmonella*, *Shigella* and *Staphylococcus* species. Batinkov (1971) reported that kefir was effective in the treatment of duodenal and peptic ulcers. The direct antibacterial and antifungal effect of kefir have also been investigated. Cevikbas *et al.* (1994) found that kefir and kefir grains showed strong antibacterial activities against gram-positive coccus, staphylococcus and gram-positive bacillus. The greatest activity of kefir was exhibited against the gram-positive bacillus. In every case, kefir grains showed higher antibacterial activity than kefir. Kefir exhibited antifungal activity against all types of fungi used in their study except *Candida parapsilosis* and *Cryptococcus neoformans*. Morgan *et al.* (2000) identified that kefir fermentates were capable of inhibiting the growth of *Listeria innocua* DPC1770 and *Escherichia coli* O157:H45.

1.3 Blood cholesterol lowering and antioxidative effects

Having a high blood cholesterol concentration is considered to be one of the four major risk factors for coronary heart disease. If blood plasma total and LDL cholesterol is lowered by 1%, the risk of cardiovascular disease is estimated to be decreased by 2-3%. Diet has been identified as a means of controlling serum cholesterol concentrations. Interest is growing in the possibility that some fermented milk products may facilitate a reduction in blood cholesterol level, specifically the low density lipoprotein (LDL). This interest was initially derived from the observation that Masai tribesmen in East Africa consume large quantities of yogurt-like foods and have lowered blood cholesterol in spite of a diet largely based on animal products (Mann, 1974). In an animal study, Beena and Prasad (1997) compared the effects of standard yogurt with a bifidus-containing yogurt on serum cholesterol concentrations in rats. All yogurts significantly decreased total cholesterol in rats fed a cholesterol-enriched diet supplemented with each of the yogurts ad libitum for 30 days. Agebaek and colleagues (1995) have reported a 10% reduction in LDL-cholesterol after regular consumption of a fermented product carrying a specific bacterial culture for 6 weeks. Renner (1991) and

Schaafsma (1996) have also demonstrated reductions in LDL-cholesterol of a similar magnitude (4.2% and 5-6% respectively). In a long-term (6 months) study with fermented milk, Richelsen *et al.* (1996) found that LDL-cholesterol concentrations in women were significantly reduced by 8% after 1 month of fermented milk consumption, and remained stable for the rest of the experiment. In men, a linear 10% reduction in LDL cholesterol was seen for the first 3 months of fermented milk consumption. Recently, Nagaoka (2001) identified a novel hypocholesterolemic peptide derived from bovine milk beta-lactoglobulin which can powerfully influence serum cholesterol levels and exhibit a greater hypocholesterolemic activity comparing to beta-sitosterol in animal studies. Kefir contains a greater number of different bacteria than yogurt, therefore, it is assumed that kefir may have hypcholesterolemic effect in humans. In a randomized crossover trial among 13 health mildly hypercholesterolemic males, St-Onge *et al.* (2002) reported that kefir had no effect on total cholesterol, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol or triglyceride concentrations nor on cholesterol fractional sunthesis rates after 4 wk of supplementation.

Kubow *et al.* (Patent pending with Kubow and Fotouhinia, 2001) investigated the possible antioxidative and anti-inflammatory effects of kefir in hypercholesterolemic subjects with total serum cholesterol levels between 6 and 10 mmol/L with no history of heart disease and diabetes. The experimental group received 500 mL of flavored kefir daily supplementing their habitual diet for 4 weeks and the control group received a supplement of 500 mL of 2% unfermented flavored milk. After the first 4 weeks of treatment the subjects were changed to the alternative treatment for another 4 weeks. The two phases were separated by a 4-week wash out period during which subjects did not consume any supplementation. They found that kefir intake was associated with a drop in concentrations of thiobarbituric acid reactive substances (TBARS), an index of lipid peroxidation and TNF-alpha, which they postulate are the result of induction of tissue GSH. A correlation analysis of plasma TBARS and TNF-alpha demonstrated a strongly association ($r=0.8305$; $P < 0.05$), which supports the

concept that the decrease in TNF-alpha concentrations was linked to a decrease in oxidative stress induced by kefir. It has been previously shown that oxidative processes are involved in the induction of TNF-alpha (Kubow, 1999). The above findings are supported by previous animal research indicating that fermentation of whey proteins promotes antioxidative effects (Zommara *et al.*, 1996).

1.4 Immune-enhancing benefits

Kefir has been shown to have immuno-modulatory properties. One such effect has been on delayed type hypersensitivity as examined by Murofushi *et al.* (1983). They found an enhancing effect on antibody response to thymus-independent antigens as well as a positive effect on delayed type hypersensitivity. In addition, immune system stimulation with kefir and with sphingomyelin isolated from the lipids of kefir (Osada *et al.* 1994) has been demonstrated in vitro. Kefir and sphingomyelin isolated from the lipids in kefir have been reported to stimulate the immune system, in vitro and in vivo (Furukawa *et al.*, 1991, Osada *et al.*, 1994). Karine (2001) examined the adjuvant effects of kefir fermented milk on the mucosal and systemic immune systems in young and old rats immunized intraduodenally with cholera toxin (CT). The nonspecific serum immunoglobulin (IgA) titers in kefir-fed and Control rats did not differ in either age group. The serum anti-CT IgA antibody concentrations were significantly higher in the kefir-fed young rats compared with their age-matched control. This difference was associated with enhanced in vitro antibody secretion by cultured lymphocytes isolated from Peyer's patches and the intestinal lamina propria. These enhanced responses were found only in young rats. However, the nonspecific serum IgG titer was higher and the anti-CT IgG titer was lower in both young and old kefir-fed rats compared with their respective controls. Their results indicated that orally administered kefir enhances the specific intestinal mucosal immune response against intraduodenal cholera toxin in young but not in old rats.

Similar effects were also observed in other fermented milk products. De Simone *et al.* (1992) noted an increase in the B-cell concentration of peripheral blood and a decrease in colonic inflammatory infiltration in a group of elderly subjects (>70 y) after ingestion of *Bifidobacterium* and *L. acidophilus*.

Donnet-Hughes *et al.* (1999) showed that fermented milk with 10^7 cfu/ml *L. johnsonii* La1 significantly enhanced the function of respiratory burst and phagocytic activity of peripheral blood leukocytes in healthy adult volunteers.

1.5 Antimutagenic and antitumor activity

The antitumor activity of kefir was first investigated in the Caucasus (Kubo *et al.*, 1992). As summarized in Table 1.1, several studies have demonstrated a significant *in vivo* reduction of tumor growth with kefir intake (Cervikbas *et al.* 1994; Furukawa *et al.* 1990, 1991 and 2000).

Furukawa *et al.* (1990) fed freeze dried kefir (2 g/kg body weight) for 9 days to mice with previously injected with Lewis lung carcinoma cells. The mice that received the kefir had 62% less carcinoma growth but no increase in spleen weight or number of leucocytes compared to controls. This same group also reported that mice receiving kefir (2 g/kg BW/day) had 10% increase in survival time when challenged with Meth-A, in spite of carrying a heavier tumor load (Furukawa *et al.*, 1991). Recently this group investigated the anti-metastatic effect of water soluble polysaccharide fraction (KGP) and water insoluble fraction (KGM) from kefir grains on Lewis lung carcinoma (3LL) and highly metastatic B 16 melanoma (B 16). In this study, cytotoxicity of splenocytes (natural killer cell activity) was detected by assessing lactate dehydrogenase (LDH) release from YAC-1 lymphoma cells. KGP had a protective effect against the pulmonary metastasis of 3 LL when mice (female BDF 1, 5 wk old) were treated twice with KGP either before or after injection of 3 LL (6.8-7.0 $\times$ 10^5 cells/mouse) in the left axilla. The low and high doses of KGP given to the aged mice (female BDF 1, 30 wk old) inhibited significantly not only the pulmonary metastasis but also the tumor growth. Six dosages of the small amount of KGP showed an effective inhibition of tumor growth at the injection site. In the treated mice with KGM for 9 consecutive days before the highly metastatic B 16 injection (2.5 $\times$ 10^5 cells/mouse), an average inhibition rate of metastasis was 39.4 $\pm$ 8.2% compared to control mice. In contrast to the mice treated with KGM, the inhibition of metastasis was not observed in the mice treated with KGP. Cytotoxicity of natural killer (NK) cells was significantly stimulated by pre-incubation in vitro with KGM at concentrations above 0.1 μg/mL,

but KGP at concentration less than 100 μg/mL did not show the activation of NK cells. The results supported the conclusion that the anti-metastatic effect of KGM on the experimental metastatic system resulted from NK cell activation and the inhibitory action of KGP on the metastasis resulted from the suppression of tumor growth at the injection site by the activated macrophage. (Furukawa *et al.*, 2000)

Kubo *et al.* (1992) observed inhibited proliferation of tumors cells in mice that had solid tumors of Ehrlich ascites carcinoma transplanted subcutaneously and been orally dosed with 100-500 mg/kg body weight kefir. Cevikbas *et al.* (1994) reported that the daily intraperitoneal administration of 0.5 ml kefir for 20 days to mice containing transplanted fusiform cell carcinomas resulted in a significant decrease in tumor size (2/11 complete disappearance of tumors, 5/11 tumor size reduction and 4/11 no change). Kefir-induced disappearance of tumoral necrosis was also evident. Specific bacterial cultures isolated from kefir were also shown to bind to mutagenic substances such as indole and imidazole (Hosono *et al.* 1990; Miyamoto *et al* 1991).

Other FMs such as yogurt also showed antituomor activities. In a case-control study by Ronco *et al.* (2002) in Montevideo, Uruguay, ricotta cheese and skim yogurt were associated with significant decreased risk of breast cancer. Low-fat and fermented products combined appear to be the most protective dairy foods. Martar *et al.* (1997) investigated the antimutagenic effects of whey, acetone extracts, and protein fractions isolated from milk fermented by *lactobacillus helveticus* L89 using mutagen 4-nitroquinoline-N'-oxide in the Ames test (*Salmonella typhimurium* TA 100). They found that whey fractions and acetone extracts of fermented milk significantly inhibited mutagenesis induced by 4-nitroquinoline-N'-oxide.

Table 1.1 Studies investigating the antitumor and immuno-enhancing properties of kefir

Effects	Experimental design	Results	Ref
Antitumor	*In vivo.* Oral doses of 100 or 500 mg/kg of kefir to mice with solid tumor of E-ascites carcinoma transplanted. Duration: 10 d. Control: Mitomycin C	Significant reduction in tumor size. Activated immunosuppressive activity of spleen	Kubo *et al.* 1992
	In vivo. Daily i.p. administration of 0.5 ml of kefir for 20 d to mice with fusiform cell sarcomas. Control: saline.	Noticeable disappearance of tumor size	Cevikbas *et al.* 1994
	In vivo: Water soluble polysaccharide isolated from kefir grain were given ad lib in water to mice with inoculated EC or sarcoma 180	The growth of EC was inhibited by 49-59 % The growth of S-180 was inhibited by 21-81%	Shiomi *et al.* 1982
	In vivo. Gastric intubation of mice with Lewis lung carcinoma cells with a pasteurized solution of yogurt and kefir, every day from d 1 to 9 after tumor cell inoculation	62% growth inhibition of tumor cells compared to non-treated mice. 4-fold increase in the weight of the spleen and the number of leukocytes in tumor-bearing mice on d 14 compared to normal mice	Furukawa *et al.* 1990
	In vitro: water soluble polysaccharide fraction (KGP) and water insoluble fraction (KGM) from kefir grain on Lewis lung carcinoma (3LL) and highly metastatic B 16 melanoma (B 16)	The anti-metastatic effect of KGM on the experimental. metastatic system resulted from NK cell activation and the inhibitory action of KGP on the metastasis resulted from the suppression of tumor growth at the injection site by the activated macrophage.	Furukawa *et al.* 2000
Immuno-enhancing	*In vivo.* Oral administration of kefir to BALB/c mice bearing Meth-A fibrinoma for 1-6 d after tumor inoculation	Induction of the delayed-type hypersensitivity response to sheep red blood cells. Increase of the weight of lungs and spleen of tumor mice compared to normal mice. Average survival period of tumor mice unchanged with or without treatment	Furukawa *et al.* 1991
	In vivo: Water soluble polysaccharides (KFG-C) isolated from kefir grain administered orally to mice	An enhancing effect on antibody response to thymus-independent antigens was observed. A positive effect on delayed type hypersensitivity Reponse: Overall KFG-C enhanced T-cell mediated immune response.	Murofushi *et al.* 1986
	In vitro. Measurement of IFN-beta secretion by sphingomyelin	14-fold enhancement of the IFN- beta secretion of osteosarcoma cells by sphingomyelin from kefir lipids	Osada *et al.* 1994
	In vivo: the adjuvant effect of kefir fermented milk on the mucosal and systemic immune systems in young and old rats immunized intraduodenally with cholera toxin (CT).	Orally administered kefir enhances the specific intestinal mucosal immune response against intraduodenal cholera toxin in young but not in old rats.	Karine *et al.*, 2001

2 Possible Bioactive Compounds in Kefir

Kefir is one kind of fermented milk product. A wide variety of bioactive compound(s) can be generated during milk fermentation, which may play a significant role in the possible antitumor properties of kefir and other FM products. Changes in milk composition after lactic acid bacteria fermentation are shown in Table 1.2. After fermentation, about 20-30% of lactose is consumed by bacteria. There is significant increase of lactic acid, acetic acid, peptides and free amino acids.

The composition of kefir can vary significantly, depending on a variety of factors including the source milk (cow, ewe, goat, mare) (Kneifel, 1991), fat content (regular fat, low fat, nonfat) of the milk, composition of the grains or starters, and the technological conditions of production (Zourari *et al.*, 1988; Wszolek *at al.*, 2001). Table 1.3 lists some of the known components of kefir. The major products formed during fermentation are lactic acid, CO_2, and alcohol. Many aromatic compounds, including diacetyl and acetaldehyde are also present in kefir (Zourari *et al.*, 1988). Diacetyl is produced by *Streptococcus lactis subsp. diacetylactis* and *Leuconostoc sp.* (Libudzisz *et al.*, 1990). The pH of kefir is from 4.2 to 4.6 (Odet, 1995). As in yogurt, the lactose content is reduced in kefir (Zourari, 1988), and the galactosidase level is increased as a result of fermentation. Information on vitamin and mineral contents is limited and sometimes contradictory, but overall, there do not seem to be significant variations from that of the milk source (Halle, 1994). There is also an increase in proteolysis, leading to an increase in free amino acids and peptides (Zourari, 1988). But comparison of amino acid profiles of milk, yogurt and Turkish kefir indicated that only slight differences in amino acid profiles exist among the 3 different products. Kefir had higher amounts of threonine ($P < 0.05$), serine ($P > 0.05$), alanine ($P > 0.05$), lysine ($P > 0.05$), and ammonia ($P > 0.05$) than milk or yogurt. Methionine and cysteine amino acids were not significantly different among the three samples (Guzel-Seydim *et al.*, 2003).

Table 1.2 Changes in milk composition after lactic acid bacteria fermentation (Graciela *et al.*, 1999; Judith, 1997; Bourlioux and Pochart, 1988)

Before fermentation	After fermentation	
	Decrease	**Increase**
Lactose	Lactose	Galactose, glucose, polysaccharides
Protein	Protein	Peptides, free amino acids
Fat	Fat	Volatile and long chain fatty acids
Urea	Urea	Ammonia
Vitamins	Vitamins B_{12}, C, choline	Folic acids
	Organic acids: Pyruvic acid, orotic acid	Organic acids (succinic, fumeric, benzoic, acetic acid, citric acid, lactic acids,)

Table 1.3 Kefir composition
(Wszolek, 2001; Halle *et al.*, 1994; Bottazi *et al.*, 1994; Duitschaever *et al.*, 1988)

Component	Concentration/comments
Fat	Depends on the source (cow, ewe, goat, mare) and on fat content (whole fat, low fat, nonfat) of milk used.
Protein	Content: 3 to 3.4 g / 100 g
Lactose	Partial consumption of lactose (about 25%) by lactic acid bacteria and yeast. Content: 2 to 3.5 g / 100 g
Lactic acid	Formed by lactic acid bacteria, primarily L (+) form. Content: 0.6 to 1 g / 100g
Other organic acids	Acetic, formic, propionic, succinic, pyruvic, isobutyric, caproic, capric, caprylic, lauric acids. Consisting less than 1% of FM
Ethanol	Produced by yeast. Content: 0.01 to 0.1 g /100 g using starters, 0.03 to 1.8 g/100 g using grains.
CO_2	Produced by yeast and heterofermentative lactic acid bacteria. Gives the typical carbonation associated with kefir.
Aromatic compounds	Acetaldehyde, diacetyl, acetoin which contribute to kefir's aroma
Vitamins	Increase of vitamin concentration of 20% observed for thiamin (only in ewe milk kefir), pyridoxine (kefir from ewe, goat, mare milk sources), folic acid (kefir from all milk sources)

2.1 Milk lipids

Dairy products are a rich source of sphingolipids. The sphingolipid content of whole milk, butter, and cheese range from 0.5 to 1 μmol/g (Zeisel *et al.*, 1986). A wide variety of sphingolipids found in milk fat that differ in their polar headgroups (i.e., sphingomyelin, glucosylceramide, lactosylceramide, and ganglioside GD3) have antitumorigenic properties (Lemonnier *et al.*, 2003). Both complex sphingolipids and their digestion products (ceramides and sphingoid bases such as sphingosines) have important roles in signal transduction pathways in differentiation, proliferation and apoptosis (Hannun, 1994) and in inhibition of the cancer cells growth and induction of differentiation and apoptosis in vitro (Merrill and Sandhoff, 2002).

Oral intake of sphingomyelin purified from powdered milk has been shown to reduce chemical-induced colon tumors and suppress the conversion of adenomas to adenocarcinomas in mice (Dillehay *et al.*, 1994; Schmelz *et al.*, 1996). In vitro studies showed that the milk phospholipid, sphingomyelin, through its biologically active metabolites ceramide and sphingosine, participates in three major antiproliferative pathways influencing oncogenesis, namely, inhibition of cell growth, and induction of differentiation and apoptosis (Hannun, 1994; Merrill, 1991; Zhang and Kolesnick, 1995). In vitro cell culture of mammary tumor cells, Wieder *et al.* (1998) demonstrated that induction of ceramide by hexadecylphosphocholine (HePC) leads to cell apoptosis and membrane-permeable ceramides additively increased the apoptotic effect of HePC. Specially, 25 μmol/L HePC, a phospholipid analog, induced apoptosis in HaCaT cells, and increased the amount of ceramide by 53% in HePC-treated cells compared with controls. HePC-induced apoptosis was blocked by fumonisin B1, an inhibitor of ceramide synthesis. Membrane-permeable ceramides additively increased the apoptotic effect of HePC. Osada *et al.* (1993) demonstrated that an active substance in kefir enhances interferon-beta (IFN) secretion of a human osteosarcoma line MG-63 treated with a chemical inducer, poly I: poly C. The active substance in kefir was indentified to be sphingomyelin (SpM) by a

combined use of a fast atom bombardment mass spectrometry (FAB-MS) and a fast atom bombardment tandem mass spectrometry (FAB-MS/MS). SpM from fermented milk (F-SpM) was a mixture of four molecular species of SpMs having C21-, C22-, C23- and C24-fatty acids. F-SpM enhanced the IFN secretion 14 times, SpMs from other sources also enhanced moderately (2-3 times). Recent evidence (Struckhoff *et al.*, 2004) suggested a role for aberrant ceramide levels in the pathogenesis of cancer and chemo-resistance, and indicated that manipulation of tumor ceramide levels may be a useful strategy in the fight against breast cancer. Ceramide analogs also showed potential antiproliferative effects in breast cancer cells. It is suggested that ceramide can act as an intracellular second messenger for tumor necrosis factor-alpha, IL-1beta, and other cytokines. Sphingosine, sphingosine 1-phosphate and other sphingolipid metabolites can modulate cellular calcium homeostasis and cell proliferation (Merrill *et al.*, 1997).

One of the components of milk fat with potential functional properties is the conjugated linoleic acid (CLA) which is a collective term to describe one or more positional and geometric isomers of the essential fatty acid, linoleic acid. In vitro (Schoberg and Kordan 1995) and experimental animal studies (Ip *et al.* 1994; Parodi 1994) indicate that CLA inhibits the development of a variety of tumors, particularly mammary tumors (Ip *et al.* 1994). Diet supplementation with CLA at 0.1 to 1.0% decreases the carcinogen-induced formation of some cancers in experimental animals (Ip *et al.*, 1994). However, the concentrations used to inhibit tumor development are much higher than those observed in milk products. At present, there is no direct evidence that CLA protects against cancer in humans, including colon or mammary cancer.

The possible antimutagenicity of milk fat extracts has been investigated and has suggested that liberated fatty acids during milk fermentation contribute to the increased antimutagenicity of fermented milk. Nadathure *et al.* (1997) found that the level of fatty acids in yogurt is 2 to 20-fold greater than that in milk, and lowest concentration of palmitic and stearic acid mixtures exhibited the greatest anti-mutagenic effect using Ames test. In another study, palmitic acid was indicated to be the major fatty acid responsible for significant anti MNNG

(N-methyl-N'-nitro-N-nitrosoguanidine) activity in yogurt (Nadathure *et al.*, 1996).

Butyrate is a four-carbon short chain fatty acid that is found uniquely in milk fat, at an average of 3-4% by weight. Butyrate is a potent inhibitor of proliferation and an inducer of differentiation and apoptosis in a number of cancer cell lines (Hague and Paraskeva, 1995; Lupton, 1995). Both these events are associated with down-regulation or inactivation of the oncogene expression. Butyrate has been demonstrated to inhibit tumor invasiveness and metastasis by inhibiting urokinase (Young and Gibson, 1994) in experimental animal studies and has been suggested to exert protective effects against colon cancer. Belobrajdic and McIntosh (2000) examined butyrate supplied as tributyrin (Tbn) on the development of nitrosomethylurea-induced mammary tumors in female Sprague-Dawley rats. They found that the addition of 1% and 3% Tbn reduced the tumor incidence by 20% and 52%, respectively, compare to the control group.

2.2 Milk protein

Milk casein and whey proteins have antitumor properties. For example, McIntosh *et al.* (1995) showed that whey protein or casein-based diets cause a decrease in tumorigenesis in laboratory animals when compared to soybean protein diets.

Whey protein fraction contains a number of high-affinity binding proteins that bind iron, folic acid, vitamin B_{12}, riboflavin, retinol and vitamin D. The binding of lactoferrin may make potential pro-carcinogen unavailable and prevent intestinal damage (Yoo *et al.* 1997), whereas the vitamin B binding proteins make their vitamins more bioavailable to the host and protect them from utilization by intestinal micro-organism (Parodi 1998; Fox and Flynn, 1992).

Whey also contains growth factors (transforming growth factor-β and basic fibroblast growth factor) and an inhibitor (mammary-derived growth inhibitor) with anti-cancer action at extremely low concentrations (Rogers *et al.*, 1996). These compounds may survive digestion in sufficient quantities to generate a physiological response.

Evidence from animal and cell culture studies suggests that milk protein, especially those associated with whey, have anti-carcinogenic properties. These properties appear to be related to components rich in the sulfur amino acids cysteine and methionine, and containing the gamma-glutamyl-cysteine residue which makes cysteine bioavailable for glutathione synthesis. Cell culture studies showed that whey protein or whey protein components selectively inhibit cancer cell growth. Whey protein cultured with the estrogen-responsive human breast cancer cell line (MCF-7) and a prostate cancer cell line significantly reduced cell growth (Bourtourault *et al,* 1991). In another study with breast cancer cells, Laursen *et al.* (1990) found that bovine serum albumin inhibited growth of the MCF-7 cell line. Bovine serum albumin, but not total whey protein, β-lactoglobulin or soybean protein exhibited a strong antimutagenic effect against the mutagen 4-nitroquinoline-N-oxide in a Chinese hamster epithelial cell line (Bosselaers *et al.* 1994). In animal studies, whey protein fractions also exerted inhibitory effects on the growth of several tumor types (Bounous *et al.* 1991). Hakkak *et al.* (2000) has demonstrated that AIN-93G diets, which are rich in whey protein, reduced the incidence of chemically induced mammary tumors by 38-46% compared with casein in female Sprague Dawley rats. They also found that diets formulated with whey as the only protein source significantly reduced the incidence of chemically induced colon cancer (azoxymethane) in rats compared with that of casein (Hakkak *et al.*, 2001).

Lactoferrin, a component of whey, is an iron binding glycoprotein, which is related to the transferrin family and is present in bovine milk at about 0.1 mg/ml (Lyer and Lonnerdal, 1993). Lactoferrin may have other protective functions, by activating NK cells and stimulating lymphokine-activated killer (LAK) cells (Shau *et al.*, 1992; Sekine *et al.*, 1997). Bezault *et al.* (1994) has shown that intraperitoneal injection of human lactoferrin inhibits the growth of two transplantable solid tumor lines in mice. The iron binding property of lactoferrin is considered a potential contributing factor in the anti-cancer activity of whey protein as iron is a pro-oxidant that can generate free radicals, which may lead to neoplastic cell growth. The protective activity of lactoferrin against chemically

induced rat tumours is consistent with results obtained using preparations of proteins from whey and other dairy products (Abdelali *et al.*, 1995). Yoo *et al.* (1997b) reported that the subcutaneous administration of bovine apo-lactoferrin (1mg/mouse) 1 day after tumor inoculation significantly inhibited liver and lung metastasis of L5178Y-ML25 cells, and lung metastasis of B16-BL6 cells. Sekine *et al.* (1997a) have shown that dietary supplementation with bovine lactoferrin, derived from bovine milk, can inhibit the development of azoxymethane (AOM)-induced aberrant crypt foci (ACF) as precursor lesions of tumor development, as well as carcinomas in the rat colon (Sekine *et al.*, 1997b), without any toxic effects on major organs. Tsuda *et al.* (1999, 2000) demonstrated that bovine lactoferrin (bLF) inhibited colon carcinogenesis in the post-initiation stage in male F344 rats treated with azoxymethane (AOM) without any overt toxicity. The incidence of adenocarcinomas in the groups receiving 2% and 0.2% bLF were 15% and 25%, respectively, in contrast to the 57.5% control value. Bovine lactoferrin also significantly inhibited spontaneous lung metastasis in BALB/c mice bearing subcutaneous implants of colon carcinoma 26 (approximately 43% of the control). The number of cytotoxic asialoGM1+ and CD8+ cells in white blood cells increased (171% and 122% of control, respectively) after treatment. These results indicate that bovine lactoferrin prevents colon carcinogenesis and lung metastasis of colon carcinoma cells, possibly due to increasing cytotoxic cells in peripheral blood. Bovine lactoferrin also exerts chemopreventive effects in the esophagus and lung cancers (Ushida *et al.*, 1999). Orally administered lactoferrin exerted an antimetastatic effect on the lung colonization by colon 26 carcinoma and enhanced production of interleukin-18 in the intestinal epithelium (Tetsuya *et al.*, 2000).

Several recent studies have attempted to identify the specific component(s) of milk, which may be responsible for the observed antitumor properties of milk. There are a number of studies having successfully isolated and tested the antiproliferative components of human milk but not in bovine milk. Svensson *et al.* (1999) characterized a protein complex in human milk that induces apoptosis in tumor cells, but spares healthy cells. The fraction was purified from

casein and showed a N-terminal amino acid sequence identical to human milk alpha-lactalbumin. In this study, it was suggested that there are differences in the biological properties between different folding variants of alpha-lactalbumin. In another study, the interaction of multimeric alpha-lactalbumin from human milk (MAL) with different cellular components was examined. MAL was found to cross the plasma membrane, enter the cell nuclus, and induce DNA fragmentation through a direct effect at the nuclear level (Svensson *et al.* 1999). Another possible mechanism for MAL-induced apoptosis might be by direct interaction of MAL with the mitochondria leading to the release of cytochrome C. This release may be important in the initiation and/or amplification of the caspase cascade in these cells (Kohler *et al.* 1999). Although the exact mechanism of apoptotic action of alpha-lactalbumin on tumor cells has not been elucidated, these studies confirm the presence of a bioactive component in human milk protein with antitumor properties.

2.3 Fermented milk protein

The antitumor and antioxidative activities of fermented milk protein and peptides have also been examined. It was reported that cell proliferation is significantly reduced when mammalian intestinal cells are treated with 100-500 Da fractions of yogurt (Ganjam *et al.*, 1997). There is also indication that by-products of casein fermentation could conceivably play an anti-cancer role as casein hydrolysates inhibit faecal glucuronidase activity in infants (Gourley *et al.*, 1997). The enzyme β-glucuronidase which is produced by intestinal bacteria deconjugates the pro-carcinogenic glucuronides to carcinogens. Matar *et al.* (1997) found that protein fractions from milk fermented by *lactobacillus helveticus* L89 significantly inhibited mutagenesis induced by 4-nitroquinoline-N'-oxide in the Ames test. But the milk fermented by a nonproteolytic variant of the same strain showed no inhibitory effects.

Although the antiumor properties of fermented milk have been documented, there is not enough evidence to identify the bioactive component(s) involved. Some studies have suggested the functional peptides may be responsible for the

beneficial effects of fermented milk proteins. However, further studies are needed to identify functional peptides associated with the antitumor effects of fermented milk proteins.

2.4 Functional Peptides

Although the overall biological value of cow's milk protein in fermented milk is virtually unchanged by fermentation (Alm, 1981), part of the milk protein is broken down into peptides and amino acids during this process. There is evidence that peptides generated from milk fermentation may exert potent physiological effects. There has been recent interest in the possibility that specific peptides generated from milk fermentation can act as bioactive agents once absorbed into the circulation. In a fashion similar to what occurs during digestion in the GI tract, peptides are produced from the proteins of milk by the enzymes of lactic bacteria during fermentation. Consequently, after consumption of fermented dairy products, some of the peptides formed during the fermentation process can be directly absorbed and transported in the blood.

Functional peptides are sequences of amino acids which are inactive within the original protein, but once they have been released by enzymatic activity they have physiological properties. Functional peptides are generally small, as they usually contain between 3 and 10 amino acids. The small size, combined with the fact that they are hydrophobic, may explain why they are more easily absorbed. One exception is caseinomacropeptide (CMP) which consists of 64 amino acids. The physiological role of functional peptides may take place elsewhere in the body, in which case they have to be absorbed from the intestine and carried in the blood stream, or it may occur locally within the digestive tract. In the later case, the peptides do not have to be absorbed to be active, although they must resist digestive enzymes for a sufficient length of time. For these small molecules, the sequence of the amino acids is a crucial factor in their biological activity.

There are four main fields in which the observed effect of consuming dairy products can be attributed to functional peptides: the digestive system, immune function, the cardiovascular system and the nervous system. Some of the studies

dealing with immuno-modulatory, anti-hypertensive and antitumor effects of functional peptides are briefly summarized in Table 1.4. Under certain conditions, the same effects could be produced by peptides from other sources, and a given peptide may have several effects.

2.4.1 Immunomodulatory and antihypertensive activity of functional peptides

Many peptides, which have demonstrated immunomodulatory activities in vitro, have been isolated from both bovine and human casein, or from lactalbumin. With regard to in vivo effects, however, it is found that it is often difficult to isolate the immunomodulatory agent.

In other studies, a blood pressure lowering effect has been observed, without investigating the mechanism. For example, peptides derived from milk fermented by *L. helveticus*, ingested by hypertensive rats, have lowered the animal's blood pressure (Masuda *et al.*, 1996; Yamamoto *et al.*, 1994). Similarly, a single dose of milk fermented by *L. helveticus* and *S. cerevisiae*, ingested by hypertensive rats at a dose of 5 ml/kg, led to a reduction in the systolic blood pressure, whereas it had no effect on normal rats (Nakamura *et al.*, 1995). This same fermented milk was administered to hypertensive human subjects at a dose of 95 ml/d for 8 weeks, and gave the similar results with a significant reduction of the systolic pressure, compared to the control group, which received acidified milk as a placebo (Hata *et al.*, 1996). In this study, both groups of subjects also received medication. Furthermore, the work of Hata *et al.* (1996) indicated clearly that experimentally measurable effects on blood pressure can be obtained with the level of functional peptides that are present in the blood after small amounts of fermented milk are ingested over prolonged periods.

Table 1.4 Physiological effects of functional peptides derived from milk

Effect	Peptide	Study Result	Reference
Immuno-modulator	Fragments of bovine α-lactalbumin and bovine κ-casein	Proliferation of human (PBL) lymphocytes activated by Con A	Kayer *et al.* 1996
	Casokinin 10 and synthetic β-casomorpine 7	Proliferation or suppression of PBL depending on concentration	Kayer *et al.* 1996
	Bovine β-casein 191-193 and 63-68	Stimulation of mouse peritoneal macrophages	Daniel *et al.* 1993
	Bovine κ-casein Casein macrophage (106-169)	Inhibition of proliferation of B-lymphocyte from Peyer patches in mice and rabbits	Otani *et al.* 1995
Anti-hypertensive	Enzymatic hydrolysates of β-lactoglobulin and α-lactalbumin	In vivo inhibition of ACE	Mullally *et al.* 1997
	Peptides from milk fermented by *L.helveticus* and *S. cerevisiae*	Hypertensive rats: ingestion of 10 ml fermented milk/kg body weight, peptides found in the aorta with ACE inhibition	Masuda *et al.* 1996
	Peptides from milk fermented by *L.helveticus*	Hypertensive rats: after ingestion, blood pressure lowered	Yamamoto *et al.* 1994
	Peptides from milk fermented by *L.helveticus* and *S. cerevisiae* Val-Pro-Pro-/Il-Pro-Pro	Hypertensive rats: after ingestion blood pressure lowered Normal rats: No effect	Nakamura *at al.* 1995
	Peptides from whey protein digests	In vitro inhibition of ACE	Pihlanto-Leppala *et al.*, 2000
Anti-mutagenicity Antitumor	Lactoferricin	Induce apoptosis in THP-1 human monocytic tumor cells	Yoo *et al.*, 1997ab
		Inhibite liver and lung metastasis of L5178Y-ML25 cells, and lung metastasis of B16-BL6 cells	
	10,000- and 500-Da dialysis fraction of yogurt	Decrease intestinal epithelial cell IEC-6 and Caco-2 cell proliferation	Ganjam *et al.*, 1997

2.4.2 Anti-mutagenic and antitumor activity of functional peptides

Only a few studies have attempted to identify the active peptides in FM which has anti-mutagenic and antitumor effects. Biffi *et al.* (1997) assessed and reported a significant growth inhibition of human mammary tumor cells treated with FM. The presence of bacteria, as well as the isolated milk proteins (beta-lactoglobulin or alpha-lactalbumin) and the whole milk used as the control were found to exert no antiproliferative activity in the MCF-7 cell culture. Yoo *et al.* (1997a) found that lactoferricin (a pepsin generated peptide of lactoferrin) and iron free lactoferrin, but not iron-bound lactoferrin, is able to induce apoptosis in THP-1 human monocytic tumor cells, and that its apoptosis-inducing activity is related to the pathway mediated by production of the intracellular reactive oxygen species (ROS). They also reported that the subcutaneous administration of bovine derived lactoferricin (0.5 mg/mouse) 1 day after tumor inoculation significantly inhibited liver and lung metastasis of L5178Y-ML25 cells, and lung metastasis of B16-BL6 cells (Yoo *et al.*, 1997b). Matar *et al.* (1997) showed that protein fractions of milk fermented by *lactobacillus helveticus* L89 significantly inhibited mutagenesis induced by 4-nitroquinoline-N'-oxide in the Ames test. One of the effective protein fractions was further purified and sequenced. They found four peptides were derived primarily from beta-casein.

Ganjam *et al.* (1997) reported that isolated yogurt fraction using 10,000- and 500-Da membrane dialysis decreased intestinal epithelial cell IEC-6 and Caco-2 cell proliferation, while a similarly produced milk fraction or lactic acid solution did not. The determination of cell kinetics by flow cytometry revealed a decrease in the number of cells in the initial growth phase in response to the yogurt fraction for the IEC-6 cells, but not the Caco-2 cells. Alpha-lactalbumin inhibited cell division of both cell lines, but beta-casein did not.

Above all, milk fermentation leads to the change of protein and formation of bioactive peptides in the fermented milk. Those studies have generally suggested that the active components are a combination of whey proteins fractions or peptides derived from fermentation of milk proteins. The proteins and peptides

with anti-mutagenic and antitumor effects still need further studies to completely characterize their composition.

2.5 Polysaccharides

Polysaccharides are the main ingredient in kefir grains. As early as 1982, Shiomi *et al.* published results of an experiment in which they treated mice with a fraction of kefir grains. The mice were inoculated with either Ehrlich carcinoma or Sarcoma 180 cells. Using hot water extraction and ethanol precipitation, they isolated a polysaccharide with a molecular weight of approximately 1,000,000. The mice were given the kefir grain fraction either in their drinking water (up to 0.3%) or injected intraperitoneally (on day 0 of the 7-day experiment). Both routes of administration were effective, as the growth of the Ehrlich carcinoma and the Sarcoma 180 was reduced by 40-59% and 21-81%, respectively. However, the polysaccharide was not cytotoxic to the two lines of cancer cells when they were incubated together in vitro.

Work on kefir grains polysaccharide was extended by Murofushi *et al.* in 1983, again using mice inoculated with Ehrlich carcinoma. The orally administered polysaccharide appeared to stimulate the cell-mediated immune response particularly in the mice that had been injected with the tumor cells. Murofushi *et al.* stated that based on their results, it appeared that the total dose of kefir grains polysaccharide was critical and not the duration or frequency of treatment. Their mice responded whether the polysaccharide was given before or after the cancer cell inoculation. This same group extended their work to show that at least 0.034% of orally administered kefir grain polysaccharide or its products reached the spleen or thymus of mice, where it could act on the immune system (Murofushi *et al.*, 1986). Immune enhancement appeared to be mediated through involvement of the T cell population, but not the B cell population.

In addition, polysaccharudes isolated from other fermented milk also showed antimutagenicity property. Sreelumar and Hosono (1998) studied the antimutagenicity and fermentation pattern of three *Bifidobacterium longum* strains (*B. Longum, B. longum PS+, and B. longum PS-*) in skim milk by using an Ames-

like test with streptomycin-dependent strain SD510 of *Salmonella typhimurium* TA98. They found that the isolated crude polysaccharide from *B. longum* PS+ showed a dose-dependent inhibition of the mutagenicity of 3-amino-1,4-dimethyl-5H-pyrido-[4,3-b]indole (Trp-P-1). Although bifidobacteria were not identified in kefir, the polysaccharides from different fermented milks showing antimutagenicity may have certain similarities.

2.6 Organic acids and bacteriocins

It is well known that many metabolic products from lactic acid bacteria have strong inhibitory power over the growth of saprophytic and pathogenic bacteria. This antagonistic activity may in part due to the production of inhibitory metabolites (hydrogen peroxide, organic acids, diacetyl, and bacteriocins) (Schillinger, 1990). Brialy and coworkers (Brialy *et al.*, 1995) demonstrated the intrinsic inhibitory power of fresh kefir toward *Staphylococcus aureus*, *Klebsiella pneumoniae*, and *Escherichia coli*, but not to *Saccharomyces cerevisiae* and *Candida albicans*. *Lactococcus lactis* DPC3147, a strain isolated from Irish kefir grains, produces a bacteriocin with a broad spectrum of inhibition. Ryan *et al.* (1996) showed that most gram-positive bacteria tested were sensitive to this bacteriocin but *Salmonella typhi*, *E. coli*, and *Pseudomonas aeruginosa* were not inhibited. Garrot *et al.* (2000) examined inhibitory activity of milk and MRS broth fermented with kefir grains toward gram-negative and gram-positive strains. They found that fermented milk obtained with 10 g per 100 ml of inoculum (final pH 3.32 to 4.25) and MRS broth fermented with 1 and 10 g per 100 ml of inocula (final pH 4.18 to 5.25) had inhibitory power demonstrated by spot tests and the agar well diffusion assay. This inhibitory effect could be assigned to the undissociated forms of lactic and acetic acid produced during the fermentation process. Kefir supernatants inhibited the growth of *Escherichia coli* 3 in a nutrient broth at 37 °C for 24 h. However, supernatants of yogurt or milk artificially acidified with lactic and acetic acids allowed the growth of *E. coli* in the same conditions. Bacteriostatic effect of milk fermented with kefir grains over *E. coli* was also demonstrated.

2.7 Calcium

Calcium has long attracted attention as an abundant component in milk, and both clinical and experimental studies have pointed to an inverse link between intake and colon cancer development. Calcium supplements have been reported to decrease ornithine decarboxylase activity and the colonic epithelial hyperproliferation induced by bile and fatty acids, as well as nutritional stress and enteric resection (Pence, 1993). Drinling milk fermented with *Lactobacillus helveticus* reduced serum parathyroid hormone and increased serum calcium compared to the control milk in postmenopause women (Narva *et al.*, 2004).

Calcium was emphasized to be a protective factor in the colon in a review by McIntosh (1993). In a study of volunteers, transfer from a dairy-product-rich to a dairy-product-free diet was associated with significant decrease in calcium intake and an increase in an accepted cytotoxicity-associated risk factor for colon cancer (Glinghammar *et al.*, 1997). Although a randomized controlled trial in out-patients demonstrated a reduction in colonic epithelial cell proliferative activity, a shift towards acidic mucins, as well as nuclear size towards normal levels with low-fat dairy products was observed (Holt *et al.*, 1998). Nonfat dried milk, as a source of calcium, was found in one rat model to be associated with a decrease in lesion development when given together with a high–fat diet (Pence *et al.*, 1996). The data appear to be relatively consistent for calcium's protective role in the colon, although an opposite effect was found in at least in one other organ. Recently, it has been proposed that 1,25-dihydroxyvitamin D3 (1,25-$(OH)_2$-D_3) plays a protective function in prostate cancer, and high serum levels of calcium and phosphorus, found after consumption of dairy products, can cause a reduction in circulating 1,25-$(OH)_2$-D_3 (Giovannucci, 1998). Convincing epidemiological evidence has been obtained to support the hypothesis that this may explain the observed positive association between milk products and prostate cancer (Chan *et al.*, 1998).

3 Possible Mechanisms of Tumor Inhibition by FM

Although the exact mechanism for the antiproliferative activity of kefir has not been examined, there are several possible pathways that can be suggested based on available evidence (Figure 1.1).

3.1 Live bacteria

It is widely known that kefir grains, the starter of making kefir, have a complex microflora consisting of bacteria and yeasts (Marshall *et al.*, 1984; Rosi, 1978, 1978a, 1978b; Takizawa *et al.*, 1998). Oral intake of kefir is accompanied by the ingestion of large numbers of live bacteria which have been suggested to exert health benefits beyond basic nutrition, such as anticancer and prevention of cancer recurrence effects (Reddy and Rivenson, 1993; Lee and Salminen, 1995). Among the live bacteria, lactic acid bacteria (LAB) are believed to be most important. Protection effects of fermented milk products against breast cancer may due to LAB's preservation of normal balance of the intestinal microflora, interfering with the enterohepatic circulation (Gorbach *et al.*, 1984, Murray *et al.*, 1980; Marquina *et al.*, 2003). Some experimental studies showed that LAB may reduce the risk of developing colon tumors in humans. LAB likely play an important role in retarding colon carcinogenesis by influencing metabolic, immunologic, and protective functions in the colon (Roberfroid *et al.*, 1995). Kohwi *et al.* (1978) found that injection of live bifidobacterium cells into sarcomas prevented lethal effects and caused tumor regression in mice. Goldin *et al.* (1980) reported that colon cancers induced by dimethylhydrazine were decreased in rats fed viable acidophilus cells and speculated that a similar effect could occur in humans. Studies in vitro have shown that LAB have a capacity to absorb mutagens from cooked foods (Zhang & Ohta, 1993). This finding is consistent with an experimental study in humans in which it was observed that ingestion of *L. acidophilus* significantly reduced the excretion of mutagens following consumption of meat heavily browned or burnt by cooking at high-temperature (Lidbeck *et al.*, 1992). Postulated protective mechanisms of

probiotics in the development of colon tumors were summarized by Wollowski (2001) as shown in Table 1.5.

3.2 The Glutathione Paradox

Glutathione (GSH) accounts for more than 90% of total intracellular non-protein sulfhydryl, and is critical in a variety of cellular defense functions including protection from toxic oxygen species and detoxification of various xenobiotics. Numerous studies have demonstrated that GSH can be differently manipulated in normal versus tumor cells line (Russo *et al.*, 1986; Roberts *et al.*, 1991; Baruchel and Viau, 1996). Depending upon the method of GSH manipulation, protection could be demonstrated in normal but not in tumor cell line. The induction of GSH in normal cells has been shown to exert beneficial antioxidant effects, while the presence of high level of GSH in tumor cells can render them resistant to the oxidative damage of anti-cancer drugs. Therefore, the selective modulation of GSH in normal versus tumor cells has received considerable attention. Rotesein and Slaga (1988) demonstrated the effect of exogenous glutathione on tumor progression in the mouse skin model. Reduced glutathione (GSH) applied topically to skin papillomas inhibited tumor progression to a significant degree. Trickler *et al.* (1993) reported that adult Syrian hamsters receiving reduced glutathione orally had fewer and smaller tumors than control.

Kuchan and Milner (1992) found that depression of intracellular GSH in mammary tumor cells in vitro resulted in an increase in the anticancer activity of selenium. GSH plays an integral role in the detoxification of selenium and various drugs. Diane *et al.* (1993) demonstrated that oral intake of GSH significantly inhibited 7,12-dimethylbenz(a)anthracene (DMBA) induced buccal pouch carcinogenesis in hamsters. Baruchel and Viau (1996) showed that when whey protein concentrate was cultured with normal lymphocytes there was an increase in cell proliferation which was accompanied by an increase in cellular glutathione levels. However, when cultured with rat mammary tumor or Jurkat T tumor cells, cellular proliferation was inhibited.

Figure 1.1 Possible pathways for in vivo antiproliferative actions of kefir after oral consumption.

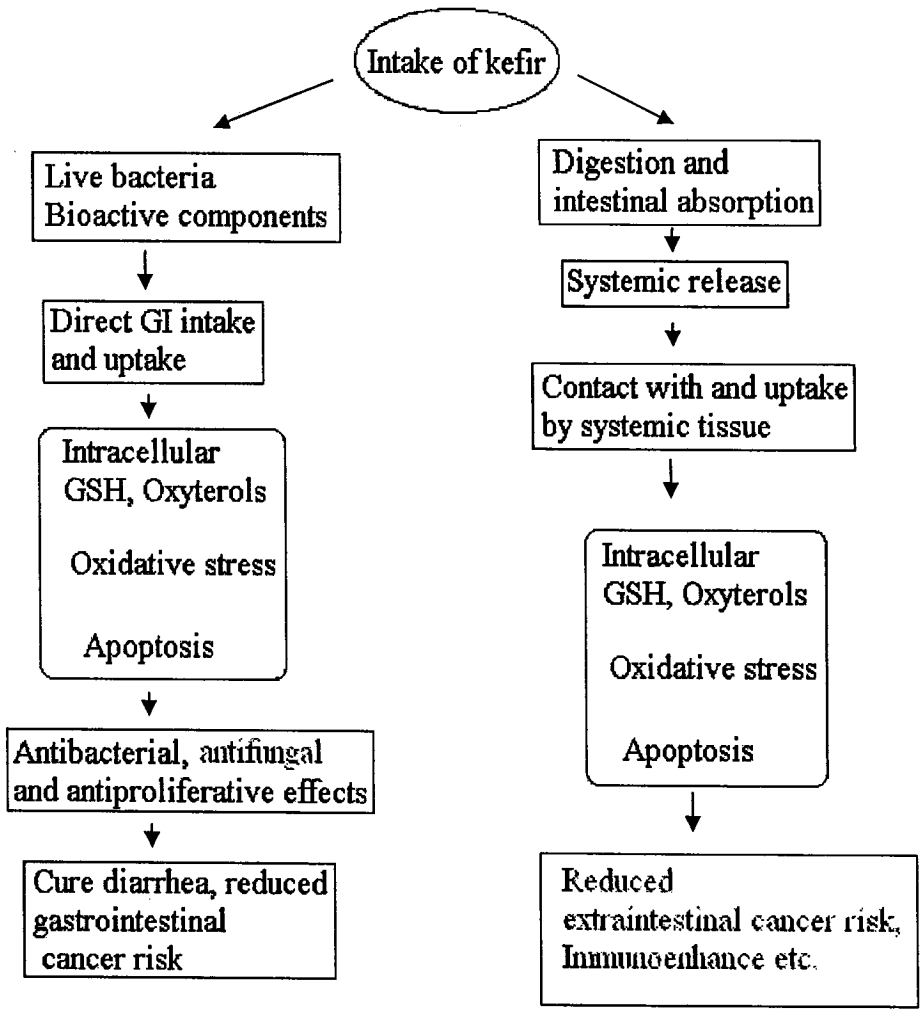

Intake of kefir
Live bacteria
Bioactive components
Direct GI intake
and uptake
Intracellular
GSH, Oxyterols
Oxidative stress
Apoptosis
Antibacterial, antifungal
and antiproliferative effects
Cure diarrhea, reduced
gastrointestinal
cancer risk
Digestion and
intestinal absorption
Systemic release
Contact with and uptake
by systemic tissue
Intracellular
GSH, Oxyterols
Oxidative stress
Apoptosis
Reduced
extraintestinal cancer risk,
Immunoenhance etc.

Table 1.5 Postulated protective mechanisms of probiotics in the development of colon tumors

References	Ingestion or investigation of	Protective mechanisms
Pool-Zobel *et al.* (1993), Bodana and Rao (1990)	*Lactobacillus casei*, omniflora, or yogurt	Mutations in the Ames test decreased
Pool-Zobel *et al.*(1993, 1996), Wollowski(1998), and Ji(1997)	Various strains of *Lactobacillus* and *Bifidobacterium*, cellular components and metabolites of LAB	DNA damage in colon cells decreased (antigenotoxicity)
Goldin and Gorbach, (1984) Goldin *et al.* (1992), Benno and Mitsuoka (1992), Bouhnik *et al.* (1996)	*Bifidobacterium* fermented milk; fermented milk with *Lactobacilus acidophilus, Bifidobacterium bifidum, Sreptococcus lactis, and Streptococcus cremoris*; lactulose	Procarcinogenic enzyme activity decreased: beta-glucuronidase. Nitroreductase, azoreductase, and detoxifying enzyme activity increased; GST
Morotomi and Mutai (1986), Zhang and Ohta (1991), Orrhage *et al.* (1994)	*L. acidophilus, S. cremoris*, cell wall of LAB	Binding of mutagens
Lidbeck *et al.* (1992)	Milk fermented with *L. acidophilus*	Excretion of mutagens decreased
Link-Amster *et al.* (1994)	Milk fermented with *L. acidophilus* La1 and bifidobacteria	Immune stimulation increased

Oral supplementation of sulfur amino acids has been used to replete normal tissue GSH, but cysteine and methionine are toxic at high dose levels. In addition, cysteine is readily catabolized. The limitation of sulfur amino acids administration can be overcome by cysteine prodrugs that are converted intracellularly to cysteine. L-2-oxothiazolidine-4-carboxylate (OTZ) is a 5-oxo-L-proline (5-OPase) analog that is metabolized by 5-OPase and has been employed to provide cysteine intracellularly. Baruchel *at al.* (1995) demonstrated that OTZ could increase GSH in normal tissue while depleting GSH in tumor tissue. Chen and Batist (1998) observed the lowering cellular GSH in vitro with human mammary MCF-7 tumor cells whereby the toxicity of melphalan was increased in the presence of OTZ as compared to the cells treated with melphalan alone. They concluded that OTZ increases melphalan toxicity by limiting glutamate production from 5-OPase for GSH synthesis thereby sensitizing the tumors to the alkylating agent, melphalan. There is evidence that glutamate is a rate-limiting substrate in tumor cells due to a selective increased requirement for glutamate in the energy metabolism of transformed compared with normal cells (Regan *et al.* 1973; Matsuno, 1991). Rose *et al.* (Rose *et al.*, 1995) examined the effects of oral OTZ on tumor and host tissue with reduced GSH levels in fasting and radiated models of GSH depletion. They found that oral OTZ selectively increases reduced GSH levels in normal tissues compared to tumor tissues following fasting and whole abdominal radiation in tumor-bearing rats. They suggested that OTZ may have a role in protection against toxicity associated with oxidative injury by selective repletion of normal host tissue GSH levels. Buthionine sulfoximine (BSO), a glutathione-S-transferase inhibitor, can inhibit intracellular GSH synthesis and affect cell growth. Donenko *et al.* (1995) reported that preincubation of cells of BDF1 hybrid mice with P388 leukemia with doxorubicin and BSO leads to the manifestion of a therapeutic effect of the antibiotic. Injection of BSO and ethacrinic acid (a glutathione inhibitor) into mice with leukemiadoes did not alter the therapeutic effect of the antibiotic. They indicated that some tumor cells with multiple drug resistance phenotypes and

resistant to alkylating agents may be mainly due to high activity of glutathione-S-transferase which can increase GSH synthesis.

There is evidence that exposure to whey proteins also can cause an apparent different effect on glutathione synthesis in normal versus tumor cells. Whey protein, a constituent of milk, is made of strum albumin, lactoferrin, beta-lactoglobulin, alpha-lactalbumin, and immunoglobulin. The glutathione modulating activity of dietary whey protein is dependent on cysteine which can act as a substrate for glutathione synthesis present in bovine serum albumin, alpha-lactalbumin, lactoferrin and the glutamyl-cysteine groups and hemoglutathione. Administration of whey protein obviates the toxic effect of other known agents for increasing the intracellular level of GSH such as N-acetyl-cysteine (Bounous *et al.*, 1988). The GSH-enhancing and protective effect of milk protein concentrates on normal cells has been previously reported (Nicodemo *et al.*, 1999; Bella *et al.*, 1996). Recent work by Zommara *et al.* (1998) has demonstrated that fermentation of whey proteins induce higher tissue glutathione concentrations than native whey protein concentrates when fed to rats. Presumably the greater induction of tissue glutathione could be the result of a greater availability of the peptide, gamma-glutamyl-L-cysteine, that could conceivably be generated during whey fermentation. On the other hand, exposure to whey proteins causes depletion of glutathione in cultured human mammary carcinoma cells whereas glutathione concentrations in normal cells are induced by whey proteins (Baruchel and Viau, 1996). Bounous *et al.* (1991) have suggested that the gamma-glutamyl-L-cysteine peptide present in whey proteins was the factor that mediated the protective action of whey proteins against colon cancer development in mice. In addition, treatment of cancer patients with whey protein has been shown to cause a depletion of high circulating tissue glutathione levels which was correlated with tumor regression (Kennedy *et al.*, 1995). This latter finding is of relevance as cellular GSH levels have been found to be several times higher in human cancer cells than in adjacent normal cells (Russo *et al.*, 1986). The presence of high glutathione cellular concentration in tumor cells may aid such cells to resist immune system attack due to the potent antioxidant potential

of glutathione, and thereby allow a greater proliferation of tumor cells to occur. Therefore, it is conceivable that fermented whey proteins could increase glutathione induction in normal cells and deplete glutathione in tumor cells.

Recent efforts elucidating the molecular mechanism(s) of selective alterations in GSH synthesis and transport in normal cells versus tumor cells in response to stimuli such as dietary whey proteins, pro-drugs (OZT), have focused on apoptosis as the mechanism of action (Bounous, 2000).

3.3 Apotosis

Apoptosis is a programmed form of cell death. It receives its primary boost with the identification of internucleosomal DNA breakdown during apoptosis (Wyllie, 1992). This form of DNA breakdown suggested the action of an endonuclease. This singular finding may have convinced many investigators that apoptosis is the manifestation or outcome of a biochemical process.

Apoptosis is now recognized as a mechanistically driven form of cell death that is either developmentally regulated (Wyllie, 1992), launched in response to specific stimuli (such as the cytokines tumor necrosis factor-alpha) (Smith *et al.*, 1994), or activated in response to various forms of cell injury or stress (Gerschenson and Rotello, 1992). In developmental biology, programmed cell death is responsible for eliminating superfluous or redundant precursor or mature cells. Apoptosis also appears to play an important role in tissue remodeling and reaction to the environment whereby unnecessary cells may undergo cell death to allow the growth and differentiation of cells that are better geared to deal with the changing environmental demands (Wyllie, 1992). In cancer biology, it is becoming increasingly apparent that many cancer cells circumvent the normal apoptotic mechanisms to prevent their self-destruction, which would have normally occured because of the many mutations they may have harbor (Kerr *et al.*, 1994; Williams, 1991).

Apoptosis has recently emerged as a critical target for diet- and drug-mediated chemoprevention. For example, the synthetic retinoid, fenretinide, which has shown promising chemopreventive activity against several cancers, appears to

exert its antitumor effects primarily by inducing apoptosis in damaged cells (Lotan, 1995). Similarly, calorie restriction has also been shown to increase apoptotic cell death in heavily damaged cells, thereby accelerating the elimination of cells with irreparable DNA damage (James and Muskhelishvili, 1994). Sovensson *et al.* (1999) isolated a fraction from human milk contained alpha-lactalbumin complexes of increasing molecular size that induced apoptosis in L1210 leukemia cells. The localization analysis of the fraction in the L1210 cells by using biotinylated material in confocal microscopy demonstrated that the fraction bind to the cell surface, enter the cytoplasm and finally to accumulate in the nuclei, and thus induced DNA fragmentation. Inhibition of nuclear uptake with wheat germ agglutinin rescued cells from DNA fragmentation. This suggested that the active fraction can transported into cells and the interaction of the active fraction with the nucleus is critical for the induction of DNA fragmentation. Kohler *et al.* (1999) also reported that MAL (a folding variant of human alpha-lactalbumin) isolated from human milk initiated caspase cascade leading to cell apoptosis in Jurkat and A549 cells. Although MAL did not directly activate caspases in the cytosol, it colocalized with mitochondria and induced the release of cytochrome *c* which is an important step in the initiation and/or amplification of the caspase cascade in these cells.

Altered cellular levels of GSH have been indicated to change the susceptibility to apoptosis through a number of different putative mechanisms. Changes in the redox status of the cell are regulated by cellular concentrations of GSH and reactive oxygen species which can alter the function of the mitochondrial membrane which plays a pivotal role in the initiation of cell death and apoptosis (Hall, 1999). Apoptosis may occur under anaerobic conditions, when the production of reactive oxygen species (ROS) is halted because of the lack of aerobic respiration (Shimizu *et al.*, 1995; Jacobson *et al.*, 1993). GSH depletion within the mitochondria has also been suggested to be an important trigger of the apoptotic pathway. Hayter *et al.* (2000) found that in L929 cells TNF-alpha induced a depletion of GSH, and exogenous addition of GSH blocked TNF-induced sphingomyelin hydrolysis as well as TNF-induced cell death.

Certain milk peptides (Yoo *et al.*, 1997) as well as whey proteins found in milk (Svensson *et al.*, 1999) and fermented milk products (Biffi *et al.*, 1997) exert antitumor activities in cell cultures without suppressing the growth of normal cells. Studies have suggested that the stimulation of cellular thiol levels could be an important mediator of the differential effects of whey proteins on tumor versus normal cells (Baruchel and Viau, 1996). In support of this concept, sulfur-containing antioxidants such as N-acetylcysteine and dimercaptopropanol have recently been shown to induce apoptosis in several transformed cell lines and transformed primary cultures but not in normal cells. These apoptosis-inducing antioxidants elevated the total cellular thiol levels and it was shown that apoptosis required the p53 tumor suppressor gene (Liu *et al.*, 1998).

Tumor necrosis factor-alpha (TNF-alpha) induces intracellular signals that mediate cell death. One major signal induced by TNF-alpha is activation of neutral and acidic sphingomyelinases which catalyze the degradation of sphingomyelin to ceramide (Hannun, 1996). Ceramide has been proposed to be an important intracellular second messenger for many of the early cellular responses elicited by TNF-alpha (Cai *et al.*, 1997; Schutze *et al.*, 1994; Verheij *et al.*, 1996; Westwick *et al.*, 1996). One of the most clearly illustrated targets of ceramide is cell death, and their mode of action varies among different ceramides (Obeid *et al.*, 1995; Okazaki *et al.* 1998; Selzner *et al.* 2001; Fillet *et al.* 2003). Modification of ceramide metabolism increases the sensitivity of cancer cells to cytotoxic compounds (Lucci *et al.*, 1999). Lucci *et al.* (1999) demonstrated that MCF-7 adriamycin resistant cells were insensitive to ceramide, wheras MCF-7 wild-type cells were sensitive. Blocking of ceramide glycosylation potentiated cellular sensitivity to ceramide and chemotherapeutic drugs.

Recently, several lines of evidence suggested that ceramide can inhibit a variety of tumor cell lines (Hannun 1996; Simstein *et al.*, 2003). Ceramides can act as a second messenger to regulate or mediate pro-apoptotic signaling involved in the cell death induced by a wide variety of stressors (Hannun and Luberto, 2000; Mathias *et al.*, 1998). It targets ceramide-activated protein kinase (CAPK), ceramide-activated protein phosphatases (CAPPs), and the mitochondria (Gudz *et*

al., 1997). CAPK is stimulated by ceramide through an unknown mechanism, but it has been linked to interleukin-1, TNF receptor binding, and to Raf activation, which stimulates extracellular signal-related kinases (ERK). CAPPs are the best characterized of the potential ceramide targets, with possible implications for apoptosis.

Ceramide can induce oxidative damage by increasing reactive oxygen species (ROS) generation (Kondo *et al.*, 2000; Schwandner et al., 1998; Huang *et al.*, 1997; Wang *et al.*, 1997) or by inhibiting ROS scavenger glutathione (GSH) (Davis *et al.*, 2000; Garcia-Ruiz *et al.*, 1997). Ceramide induction of oxidative stress could play a role in the induction of apoptosis. The addition of exogenous *N*-acetylsphingosine (C_2-ceramide) and the generation of intracellular ceramide via agents such as TNF-alpha and lipopolysaccharides were shown to increase oxidative damage in mitochondria to induce apoptosis (Kondo *et al.*, 2000; Garcia-Ruiz *et al.*, 1997). In this regard, it has been suggested that ceramide could induce apoptosis not only by enhancing the pro-apoptotic signal, caspase-3, but also by depleting the anti-apoptotic catalase via caspase-3 (Iwai *et al.*, 2003). Activation of caspase-3, a pro-apoptotic signal, inhibits catalase activity via proteolysis. Increased oxidative damage by catalase depletion is closely related to ceramide-induced apoptosis. For example, ceramide-induced inhibition of catalase results in increased apoptosis and TBARS production at least 12 and 24 h after ceramide treatment, respectively. These results suggested that ceramide-activated caspase-3 increased oxidative damage through the inhibition of catalase is at the level of protein (Luberto *et al.*, 2002). In further support of these concepts, antioxidant agents such as N-acetylcysteine, GSH, catalase, and glutathione peroxidase (GPx) inhibit ceramide generation caused by apoptosis induced via oxidative stressors, such as tumor necrosis factor-alpha, interleukin-1-beta, hypoxia, and daunorubicin (Liu and Hannun 1997; Liu *et al.*, 1998; Singh *et al.*, 1998; Lavrentiadou *et al.*, 2001).

Although kefir and some components or fractions from kefir have been demonstrated to have antimutagenic and antitumor properties, the underlying mechanisms are largely unknown. The current available literature suggests that

GSH, TNF-alpha and ceramides may play roles of therapeutic interest, and in observed antitumor effects. Kefir contains bioactive components, each component or fraction may act differently. Comparing the effects of different fractions of kefir on MCF-7 cells and quantifying biochemical response will help us understand the machamisms involved.

CHAPTER 2

INTRODUCTION TO CHAPTER 2

Epidemiological studies have indicated that consumption of fermented milk products (FM) reduced risk of breast cancer (Reddy *et al.*, 1983; Veer *et al.*, 1989). The FM product, kefir, enjoys a rich tradition of beneficial claims. Encouraging results regarding an antitumor activity of kefir in animal studies have been reported (Shiomi *et al.*, 1982; Cevikbas *et al.*, 1994; Furukawa *et al.*, 1990; Kubo *et al.*, 1992). Effects on human tumor cell cultures, however, have not been examined. The present study was designed to determine the effects of extracts from kefir, yogurt and milk on the growth of human breast cancer cells (MCF-7) and normal mammary epithelial cells (HMEC).

MANUSCRIPT A

Kefir Extracts Suppress in Vitro Proliferation of Estrogen-Dependent Human Breast Cancer Cells But not Normal Mammary Epithelial Cells

Chujian Chen, Stan Kubow and Hing Man Chan

ABSTRACT

Background: Epidemiological studies have indicated that consumption of fermented milk products (FM) reduced risk of breast cancer. The purpose of this study was to determine the effects of kefir, a traditional FM originated from Russia, on human cancer cells or normal mammary epithelial cells. **Materials and methods:** The extracts of kefir, yogurt and pasteurized milk made by centrifugation and membrane filtration were tested on human breast cancer cells (MCF-7) and normal human mammary epithelial cells (HMEC). **Results:** Both kefir and yogurt extracts suppressed the proliferation of MCF-7 cells in a dose-dependent manner but the inhibitory effects of the kefir extract were significantly greater ($P < 0.01$). After 6 days of culture, kefir extract of 0.6 % (v/v) and 2.5% (v/v) decreased the cell numbers by 29% and 56%, respectively while yogurt extract at 0.6% (v/v) showed no anti-proliferative effects and the 2.5 % (v/v) dose decreased the cell numbers by only 14%. Milk extracts exerted no effect. The antiproliferative effect of kefir extracts was not observed in HMEC cells whereas the yogurt extracts exerted anti-proliferative effects at the higher doses. Peptide analyses and capillary electrophoresis profiles showed that kefir-mediated milk fermentation led to an increase in peptide concentrations and a change in peptide profiles in comparison to milk or yogurt. **Conclusion:** These results suggest that kefir extracts contain constituents that specifically inhibit the growth of human breast cancer cells, and therefore might eventually be useful in the prevention or treatment of breast cancer.

***Key words*:** kefir, proliferation, breast cancer cells

INTRODUCTION

Research on the putative health benefits of fermented milks (FM) has grown dramatically in the past 20 years. In particular, by-products of bacterial fermentation of proteins, lipids and carbohydrates present in FM have been implicated to exert health benefits beyond basic nutrition including antitumor action, immune system enhancement and antioxidant effects. Epidemiological studies have indicated a reduced risk of breast cancer in women who consumed FM products (Reddy *et al.*, 1983; Veer *et al.*, 1989). Antimutagenesis of FM has also been widely demonstrated (Cassand *et al.*, 1994; Abdelali *et al.*, 1995). The active ingredients in the fermented milk products have not been fully characterized but several studies suggested that the antimutagenic effect of these cultured milk could be due to the presence of the lactic acid bacteria (LAB) (Abdelali *et al.*, 1995; Hosono *et al.*, 1990; Renner *et al.*, 1991; Pol-Zobel *et al.*, 1993) or by-products of LAB fermentation of cow's milk constituents. Proteins and peptides from FM have been shown to have antitumor activities in tumor cells (Bourtourault *et al.*, 1991; McIntosh *et al.*, 1995; Svensson *et al.* 1999). A wide variety of sphingolipids found in milk fat that differ in the polar headgroup (i.e., sphingomyelin, glucosylceramide, lactosylceramide, and ganglioside GD3) have shown antitumorigenic effects (Lemonnier *et al.*, 2003). Both complex sphingolipids and their digestion products (ceramides and sphingoid bases such as sphingosines) isolated from dairy products have been demonstrated to play important roles in signal transduction involved in various responses such as differentiation, proliferation and apoptosis (Hannun, 1994) and to inhibit the growth of cancer cells and induce differentiation and apoptosis in vitro (Merrill and Sandhoff, 2002). Sphingomyelin purified from powdered milk has been shown to reduce chemical-induced colon tumors and suppress the conversion of adenomas to adenocarcinomas in mice (Dillehay *et al.*, 1994; Schmelz *et al.*, 1996). In vitro cell culture of mammary tumor cells also demonstrated that induction of ceramide by hexadecylphosphocholine (HePC) leads to cell

apoptosis and membrane-permeable ceramides additively increased the apoptotic effect of HePC (Wieder *et al.*, 1998).

The FM product, kefir, enjoys a rich tradition of health claims, as consumption of kefir has been used in the former Soviet Union for the treatment of a variety of conditions including metabolic disorders, atherosclerosis, cancer, and gastrointestinal disorders (Shiomi et al., 1982; Koroleva, 1988; Olesmall et al., 1999). In the former Soviet Union, kefir accounts for 70% of the total amount of FM consumed (Komai et al., 1992). Kefir distinguishes itself from the more well known FM product, yogurt, in that it is traditionally made only from kefir grains. Kefir grains are clusters of microorganisms held together by a matrix of polysaccharides and protein. The grains include a stable and specific balance of primarily a variety of lactic acid bacteria (i.e., lactobacilli, lactococci, leuconostocs) and yeasts, which exist in a complex symbiotic relationship. The polysaccharides are an integral part of the grain, and without their presence, kefir grains cannot be propagated. The grains are formed in the process of making kefir and only from pre-existing grains. They resemble small cauliflower florets, and each grain is 3 to 20 mm in diameter (Bottazzi et al., 1994). Hence, in kefir production the milk undergoes a dual fermentation process under the action of both lactic acid bacteria and yeasts. While yogurt can readily be made from the lactic acid bacteria present in fresh yogurt, kefir can only be made from kefir grains and mother cultures prepared from grains.

Encouraging results regarding an antitumor activity of kefir in animal studies have been reported (Shiomi *et al.*, 1982; Cevikbas *et al.*, 1994; Furukawa *et al.*, 1990; Kubo *et al.*, 1992). For example, Shiomi *et al.* (1982) has shown that polysaccharides extracted from kefir grains had antitumor activity in mice. Also, oral doses of 100 or 500 mg/kg of kefir to mice with solid tumor of E-ascites carcinoma (EC) transplanted were shown to cause a significant reduction in transplanted tumor size and activate the immunosuppressive activity of the spleen (Kubo *et al.*, 1992). Effects on human tumor cell cultures, however, have not been examined. The present study was designed to determine the effects of extracts

from kefir, yogurt and milk on the growth of human breast cancer cells (MCF-7) and normal mammary epithelial cells (HMEC).

MATERIALS AND METHODS:

Cell culture

MCF7-E3 human breast cancer estrogen-sensitive cells were provided by Dr. D. Desaulniers of Health Canada, Ottawa (Wade *et al.*, 1997). Cells were routinely propagated as a monolayer culture in Dulbcco's Modified Eagle Medium (DMEM) (Gibco, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA), in a 75-cm^2 plastic dish at 37 °C in a humidified atmosphere with 5% CO_2, and passaged every 3-4 days. Normal human mammary epithelial cell line was kindly provided by Dr. M. R. Stampfer (Lawrence Berkeley National Laboratory, Berkeley, USA). Cells were routinely propagated as a monolayer culture in Mammary Epithelial Growth Media (MEGM, Clonetics, San Diego, USA) supplemented with 10% heat-inactivated FBS, in 75-cm^2 plastic dish at 37 °C in a humidified atmosphere with 5% CO_2, and passaged every week. For the experiments, both cells were harvest from the dish using 0.25% trypsin-EDTA solution (Sigma, St Louis, MO, USA).

Preparation of extracts

Four kefir products (K1-K4) of three different batches collected at various stages of kefir production at Liberty Food Inc. (Brossard, Canada) were used in this study. The large scale production of kefir involves a two-step fermentation process. The first step is to prepare the cultures by incubating milk (K1) with kefir grains (2-10%) and fermented for 24 h. The grains are then removed by filtration and the resulting mother culture (K2) is added to pasteurized milk (K3), which is further fermented for 24 h and this final product (K4) is packaged for the consumer market. A pasteurized milk sample (M1) and two yogurt products: mixture of yogurt bacteria, pasteurized milk and milk powder (Y1) and the final yogurt product after 12 h of fermentation (Y2) were included for comparison. The yeast and bacteria in the samples were removed by centrifugation and filtration.

About 35 mL each of the 7 samples was centrifuged (32000 x g, 60 min, 4 °C) and the supernatant was filtered through a 0.45 μm Millipore filter followed by a filtration using a 0.2 μm Millipore filter (Millipore Corporation, Bedford, USA). Extracts from three separate batches of kefir and yogurt were used. Protein concentrations were determined by using a micro protein determination kit (Cat. No. 690) obtained from Sigma (Sigma, St Louis, MO, USA). Peptide concentrations in solution were analyzed by the method of Church et al. (1983). Lactic acid concentration was determined by using a lactic acid assay kit (Cat. No. 735) from Sigma Diagnostics Inc. (Sigma, St Louis, MO, USA).

Capillary zone electrophoresis

Capillary zone electrophoresis (CZE) was performed using P/ACE TM 2200 HPAC instrument controlled by System Gold software (Beckman, Fullerton, CA, USA) coupled to IBM PC 486 computer (IBM Corp., Portsmouth, England) for data acquisition and analysis. A neutral uncoated fuse silica capillary column (57 cm x 50 μm; the length from inlet to detector is 50 cm, Polymicro Technologies, Phoenix, Arizona USA) was assembled in the P/ACE cartridge for capillary separations. Injection volume, buffer concentration and running voltage were optimized to achieve the best resolution with the shortest running time.

The gold-coated fused-silica tube was flushed with 1 M sodium hydroxide, followed by nanopure water, 0.1 M sodium hydroxide, nanopure water, 1 M hydrochloric acid, and again water, each for 5 min at a pressure of 40 psi. A solution of 5% polybrene and 2% ethylene glycol was then passed for 10 min at 40 psi. Excess coating was then removed by flushing with water for 2 min. Further capillary flushing was then performed for an additional 10 min using 200 mM formic acid buffer. The polybrene-modified fused-silica tube was then ready for a Beckman P/ACE 2200 (Beckman Instruments, Inc., Fullerton, California, USA). Before each sample application, the capillary was rinsed with 1 min water, 1 min 0.1 M sodium hydroxide, 1 min water and 3 min separation buffer. After the completion of each run, the capillary was rinsed with nanopure water for 1 min, 0.1 M sodium hydroxide for 1 min and nanopure water for 1 min. Peptide standard and sample injection were carried out at the anode end of the capillary

using N_2 pressure (0.5 psi) for 5 sec, and was separated at a constant temperature of 20°C. On-line detection was performed using an UV detector at 200 nm. Twenty kilovolts was applied across the run buffers for best separation. After every 5 runs, the capillary was re-coated.

Cell proliferation experiments

Cells previously harvested were seeded in 24-well plates; 10,000 cells for MCF-7 per well in DMEM supplemented with 10% FBS and 5,000 cells for HMEC per well in MEGM supplemented with 10% FBS. The cells were allowed to attach for 24 h. After that period, old media were removed and fresh media and extracts were added to each well. To study the dose response, a serial dilution of each the extract using the culture media was made to achieve final concentrations of extracts at 10%, 5%, 2.5%, 1.3%, 0.6%, and 0.3% (vol/vol) respectively. Because the kefir and yogurt extracts were acidic (pH 4.5). Dulbecco's Phosphate Buffered Saline (PBS) buffer (Gibco BRL, Grand Island, NY) was added to the culture media to adjust the pH between 7.0 and 7.4. Cells were incubated at 37°C in a humidified atmosphere with 5% CO_2 for 6 days. The culture media were collected and centrifuged at 1900 x g for 10 min at 4°C. The supernatant was isolated for lactic acid measurement. The cells were rinsed with 500 µl of PBS with the subsequent addition of 250 µl of hypotonic buffer (0.01M HEPES, 1.5 M $MgCl_2$, pH 7.5) to each well. After 2 min, cells showed a slight swelling. At this time, 250 µl of cell lysing solution (10% ethylhexadecyldimethylammonium bromide, 3% glacial acetic acid, in water) was added. The plate was shaken lightly every minute for 5 min. Cell lysis was confirmed microscopically as indicated by a suspension of clean nuclei. The suspension was mixed vigorously using a micropipette just prior to counting. Cell nuclei were counted using Coulter Counter (Z1, Coulter Counter Corporation, Miami, FL). Each sample was run in quadruplicate. Control cells were incubated with the culture medium with the dosing vehicle (PBS).

Statistics

The cell numbers were calculated as the percentage of control from different kefir/milk/yogurt treatments and doses, and expressed as means ± SD of three batches. Two-way ANOVA was used to analyze the effects of treatment and dose. The difference among doses and treatments were determined by Student-Newman-Keuls (SNK) multiple comparison test. The comparison of protein and peptide concentrations before and after fermentation was done using Student's t-Test. Statistical significance was considered at $P < 0.05$. All statistics test were performed using SAS 8.2 for PC (SAS, Cary, NC).

RESULTS

Table 2.1 showed effects of different kefir samples (K1 –K4) on proliferation of MCF-7 cells. The effects of both treatment and dose were significant ($P < 0.01$); however, the interaction between treatment and dose was also significant ($P < 0.01$). The fermented mother culture (K2) and the final kefir product (K4) showed a significantly dose-dependent inhibitory effects ($P < 0.01$ and $P < 0.01$). Dilution of the kefir mother culture with milk (K3) resulted in elimination of inhibitory effects ($P < 0.05$). The calculated IC_{50} of extracts of kefir mother culture and final kefir were 0.62 μg/mL and 0.77 μg/mL, respectively.

Extracts of yogurt (Y2) also showed inhibitory effects (Table 2.2) but to a lesser degree than those observed using extracts of either the kefir mother culture (K2) or the final kefir product (K4). Thus, yogurt extract at 0.6% (v/v) showed no anti-proliferative effect and the 2.5 % (v/v) dose of yogurt decreased the cell numbers by only 14%, while kefir (K4) extract of 0.6 % (v/v) and 2.5% (v/v) decreased the cell numbers by 29% and 56%, respectively. Moreover, extracts of mother culture (K2) and final kefir product (K4) showed significant depression of cell growth at the concentration of 0.3% (v/v), while extract of yogurt only began to show anti-proliferative effects at 2.5% (v/v). On the other hand, milk extract (M1) showed a significant stimulation of cell growth at a concentration of

0.63% (v/v). The mixture of milk and yogurt bacteria (Y1) showed a significant stimulation of growth at the dose of 2.5% (v/v).

In contrast to their anti-proliferative effects in mammary tumor cells, kefir extracts (both K2 and K4) did not have any inhibitory effect on proliferation of the HMEC cells (Table 2.3). On the other hand, extracts of yogurt (Y2) showed a slight inhibitory effect (Table 2.4) ($P < 0.01$) at the 5% (v/v) and 10% (v/v) concentrations. Likewise, the non-fermented milk products (K1, K3, M1 and Y1) all showed a slight stimulation of cell growth (Table 2.3 and 2.4).

Protein and peptide concentrations were analyzed in the extracts of milk, kefir mother culture, final kefir and yogurt products (Table 2.5). After kefir fermentation, protein concentration decreased significantly from 7.55 to 2.45 mg/mL ($P < 0.05$) and peptide content increased significantly by 172%, from 2.42 to 6.58 μM/L ($P < 0.05$). After yogurt fermentation, protein concentration decreases from 7.5 to 2.04 mg/mL ($P < 0.05$), while peptides content increased by 119%, from 2.41 to 5.27 μM/L ($P < 0.05$). Capillary electrographs showed that more peaks appeared after kefir fermentation comparing to yogurt fermentation (Figures 2.1 and 2.2). Lactic acid concentrations varied between 0.2 to 0.4 mg/mL in the media. There was, however, no significant difference in concentration of lactic acid in the culture media dosed with different milk or fermented milk extracts (data not shown).

Table 2.1 Effects on cellular proliferation of different kefir fractions at varying doses on MCF-7 cells

Treatment	Dose (v/v)						
	Control	0.31%	0.63%	1.25%	2.5%	5.0%	10.0%
[1]K1	[2]100.0±9.2 [a]	105.6±2.4 [a#]	115.9±10.9 [b#]	112.9±11.3 [b#]	120.2±8.0 [b#]	122.6±10.3 [b#]	112.6±4.9 [b#]
K2	100.0±8.7[a]	91.8±5.3 [a*§]	76.7±15.1 [ab*]	62.1±12.2 [b*]	38.9±8.9 [c*]	24.0±5.4 [c*]	12.0±6.9 [c*]
K3	100.0±6.1	100.9±7.1 [#§]	96.6±5.3 [#]	106.2±7.9 [#]	103.4±7.2 [#]	106.5±11.4 [#]	102.9±5.8 [#]
K4	100.0±10.3[a]	88.7±6.6 [a*§]	71.0±7.3 [b*]	57.6±9.8 [bc*]	43.7±5.4 [c*]	37.6±10.5 [c§]	17.8±6.8 [d*]

[1]K1- K4 refer to pasteurized milk, mother culture, mixture of kefir mother culture and pasteurized milk (time=0), final kefir product, respectively

[2] The cell numbers were calculated as the percentage of the Control, and expressed as Mean±SD, n=3.

Means with the same letter are not significantly different for comparison among doses at the same treatment.

Means with the same symbols are not significantly different for comparison among treatments at the same dose.

Table 2.2 Effects on cellular proliferation of different yogurt fractions at varying doses on MCF-7 cells

Treatment	Dose (v/v)						
	Control	0.31%	0.63%	1.25%	2.5%	5.0%	10.0%
[1]M1	[2]100.0±5.7 [a]	105.6±2.4 [a]	115.9±10.9 [b]	112.9±11.3 [b]	120.2±8.0 [b#]	122.6±10.3 [b#]	112.6±4.9 [b#]
Y1	100.0±6.5 [a]	106.6±7.0 [a]	100.6±10.0 [a]	104.6±5.1 [a]	111.8±14.5 [b#]	104.5±12.9 [a#]	108.8±11.4 [a#]
Y2	100.0±4.8 [a]	98.6±2.3 [a]	101.6±6.9 [a]	94.8±4.2 [a]	86.6±3.4 [a*]	80.7±3.4 [b*]	55.4±13.3 [c*]

[1]M1, Y1, Y2 refer to pasteurized milk, mixture of yogurt starter and pasteurized milk (time=0), and final yogurt product, respectively.
[2] The cell numbers were calculated as the percentage of the Control, and expressed as Mean±SD, n=3.
Means with the same letter are not significantly different for comparison among doses at the same treatment.
Means with the same symbols are not significantly different for comparison among treatments at the same dose.

Table 2.3 Effects on cellular proliferation of different kefir fractions at varying doses on HMEC cells

Treatment	Dose (v/v)						
	Control	0.31%	0.63%	1.25%	2.5%	5.0%	10.0%
[1]K1	[2]100.0±6.9[a]	110.7±5.6 [b#]	122.6±6.6 [b#]	141.9±9.9 [c#]	113.5±6.0 [b#]	105.7±2.5 [b#]	104.0±3.5 [b#]
K2	100.0±8.1	98.9±14.2 [*]	99.1±6.8 [*]	100.4±9.2 [*]	101.6±7.1 [#]	104.7±7.1 [#]	103.4±7.1 [#]
K3	100.0±9.7[a]	124.6±7.0 [b#]	112.1±10.9 [b#]	115.4±6.9[b§]	125.4±5.8 [b*]	125.2±2.9 [b*]	121.2±2.4 [b*]
K4	100.0±8.6	108.7±8.9 [#]	105.5±2.7 [#]	116.8±9.4 [*]	108.7±4.3 [#]	100.7±8.8 [#]	98.3±9.6 [#]

[1]K1- K4 refer to pasteurized milk, mother culture, mixture of kefir mother culture and pasteurized milk (time=0), final kefir product, respectively

[2] The cell numbers were calculated as the percentage of the Control, and expressed as Mean±SD, n=3.

Means with the same letter are not significantly different for comparison among doses at the same treatment.

Means with the same symbols are not significantly different for comparison among treatments at the same dose.

Table 2.4 Effects on cellular proliferation of different yogurt fractions at varying doses on HMEC cells

Treatment	Dose (v/v)						
	Control	0.31%	0.63%	1.25%	2.5%	5.0%	10.0%
[1]M1	[2]100.0±6.9 [a]	110.7±5.6 [b#]	122.6±6.6 [b#]	141.9±9.9 [c#]	113.5±6.0 [b#]	105.7±2.5 [a#]	104.0±3.5 [a#.]
Y1	100.0±6.1 [a]	98.1±6.5 [a*]	94.8±11.7 [a*]	98.7±7.[2 a*]	112.4±4.6 [b#]	115.8±5.2 [b#]	116.5±3.2 [b*]
Y2	100.0±8.0 [a]	110.0±12.2 [a#]	105.8±5.5[a*]	104.5±7.7 [a*]	97.7±5.7 [a*]	85.5±4.4 [b*]	85.7±5.1 [b§]

[1]M1, Y1, Y2 refer to pasteurized milk, mixture of yogurt starter and pasteurized milk (time=0), and final yogurt product, respectively.
[2] The cell numbers were calculated as the percentage of the Control, and expressed as Mean±SD, n=3.
Means with the same letter are not significantly different for comparison among doses at the same treatment.
Means with the same symbols are not significantly different for comparison among treatments at the same dose.

Table 2.5 Protein concentration in extracts of milk, kefir, and yogurt

	Protein (mg/mL)	Peptide (μM/L)
[1]K1	[2]7.78±0.16	2.36±0.19
K2	2.03±0.26	5.53±0.26
K3	7.55±0.23	2.42±0.16
K4	2.45±0.05	6.58±0.15
Y1	7.50±0.26	2.41±0.15
Y2	2.04±0.22	5.27±0.18

[1]K1 - K4 refer to pasteurized milk, mother culture, mixture of kefir mother culture and pasteurized milk (time=0), final kefir product, respectively. Y1, Y2 refer to mixture of yogurt starter and pasteurized milk (time=0) and final yogurt product
[2]Mean±SD, n=3.

Figure 2.1 Capillary electropherograms of extract of milk (A), kefir mother culture (B) and final kefir (C). The capillary column (57 cm x 50 μm; the length from inlet to detector is 50 cm) was coated with a solution of 5% polybrene and 2% ethylene glycol. Separation was done on Beckman P/ACE 2200 (Beckman Instruments, Inc., Fullerton, California, USA) with 200mM formic acid as running buffer. On-line detection was performed using an UV detector at 200 nm. Twenty kilovolts was applied across the run buffers for best separation.

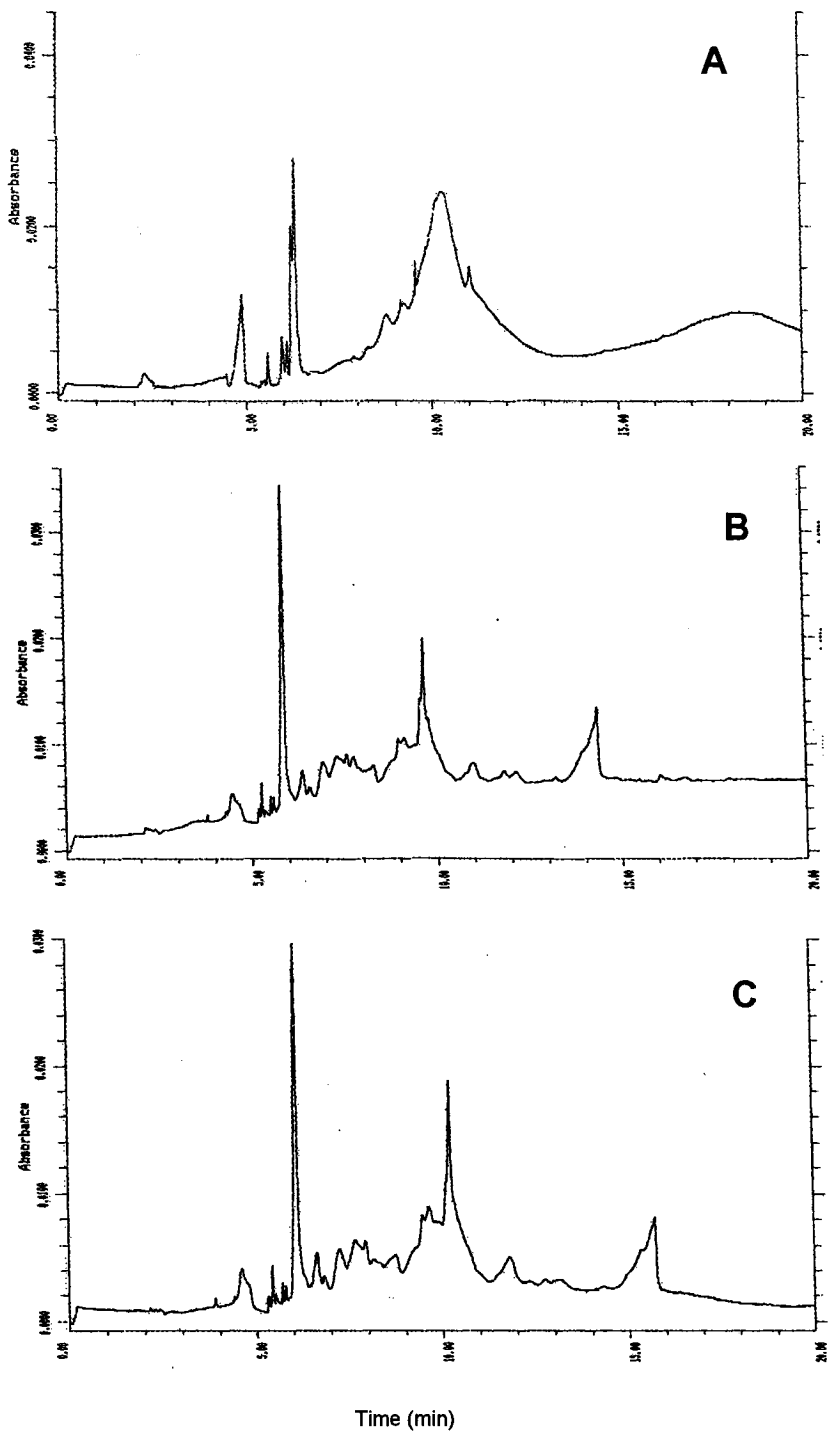
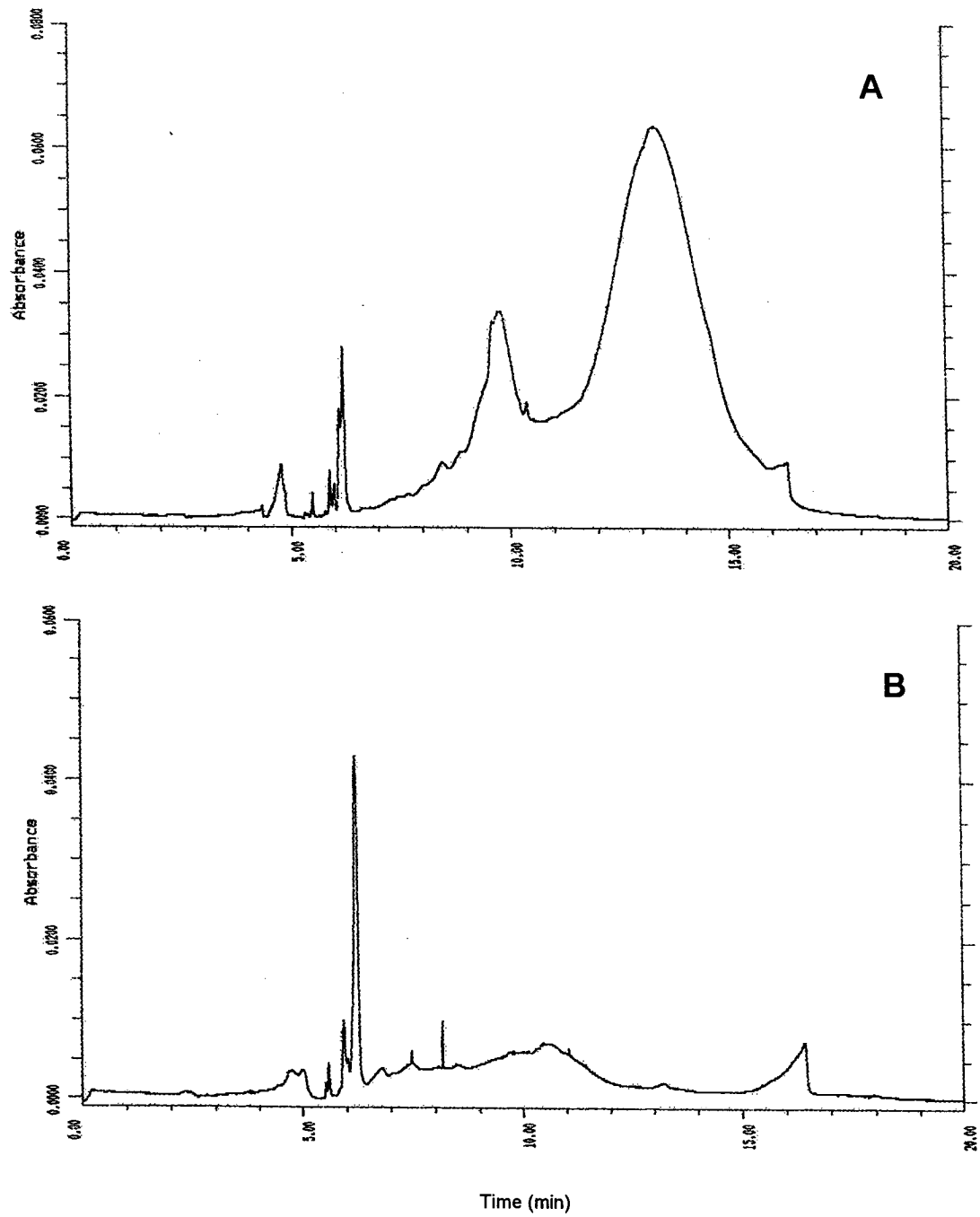

A
B
C
Absorbance
Absorbance
Absorbance
Time (min)

Figure 2.2 Capillary electropherograms of extract of milk (A) and yogurt (B). The capillary column (57 cm x 50 μm; the length from inlet to detector is 50 cm) was coated with a solution of 5% polybrene and 2% ethylene glycol. Separation was done on Beckman P/ACE 2200 (Beckman Instruments, Inc., Fullerton, California, USA) with 200mM formic acid as running buffer. On-line detection was performed using an UV detector at 200 nm. Twenty kilovolts was applied across the run buffers for best separation.


A
B
Absorbance
Absorbance
Time (min)

DISCUSSION

Previous results have shown that extracts of yogurt exert antiproliferative properties in MCF-7 cells (Biffi *et al.*, 1997). The present findings demonstrate that although both yogurt and kefir extracts depressed MCF-7 cellular growth, the antiproliferative potency of kefir was markedly more potent than that of yogurt. Moreover, the anti-proliferative effects of yogurt extracts were also observed in normal mammary cells, whereas kefir extracts exerted anti-proliferative effects on mammary tumor cells only. A similar selective cytotoxicity against MCF-7 cells has been observed previously when MCF-7 cells and HMEC cells were treated with a red grape wine flavonoid fraction (Hakimuddin *et al.*, 2004). Likewise, Svensson *et al.* (1999) recently characterized a protein complex in human milk that induces apoptosis in tumor cells but spares the healthy cells.

The antiproliferative activity was not caused by the presence of microbes in the kefir or yogurt extracts as the test samples underwent sterilization filtration and thus the bioactive ingredients must thus be products of the fermentation process. Similarly, Biffi *et al.* (1997) has demonstrated the antiproliferative effect of milk fermented by *Bifidobacterium infantis*, *Bifidobacterium bifidum*, *Bifidobacterium animalis*, *Lactobacillus acidophilus*, and *Lactobacillus paracasei* all showed a growth inhibition on MCF-7 cells. This observed antipfoliferative effect was not related to the presence of bacteria in fermented milk. It is noteworthy that the mother culture of kefir (K2) was found to be the most potent in decreasing tumor cell growth suggesting that the degree of fermentation could impact on the anti-proliferative effects of kefir.

The observation that the antiproliferative activity of kefir extracts was observed only in the MCF-7 cells but not the normal human mammary epithelial cells suggest that the bioactive component(s) bind to or trigger responses that are specifically found in tumor cells. The bioactive antiproliferative component in kefir could include a number of possible agents including polysaccharides, peptides and sphingolipids.

Kefiran, a soluble gel-polysaccharide discovered in kefir grains exhibited antitumor properties in mice (Shiomi *et al.*, 1982). Milk proteins and their peptide

fragments, especially those associated with whey, have anti-carcinogenic properties (McIntosh *et al.*, 1995). Whey proteins cultured with the estrogen-responsive MCF-7 human breast cancer cell line and a prostate cancer cell line reduced significantly cell growth (Bourtourault *et al.*, 1991). Similarly, Laursen *et al.* (1990) found that bovine serum albumin inhibited growth of the MCF-7 cell line. Yoo *et al.* (1997) found that lactoferricin (a pepsin-generated peptide of lactoferrin) and iron free lactoferrin were able to induce apoptosis in THP-1 human monocytic tumor cells. Our results showed that after kefir and yogurt fermentation, protein concentration significantly decreased while peptide concentration increased significantly suggesting that the products of fermentation rather than the milk proteins per se were responsible for the observed antiproliferative effects. Likewise, in previous studies by Biffi *et al.* (1997), the antiproliferative effect of fermented milk on MCF-7 cells in previous studies was not related to whole milk (crude or ultrahigh temperature sterilized) nor its main fractions (lactalbumin or beta-lactoglobulin fraction).

Although the same pasteurized milk was used for yogurt and kefir fermentations, major differences in the antiproliferative effects were observed between the two fermented milk products despite similar total peptide concentrations in kefir and yogurt. In this regard, as CE peptide profiles indicated more peaks in the kefir extracts relative to the extracts of milk and yogurt, it is conceivable that the presence of different peptide components in kefir could have played a role in the more potent antiproliferative effects of kefir. In that regard, Rokka *et al.*, (1997) described the generation of peptides via *Lactobacillus acidopholus* GG fermentation. The fermentation starter culture used for kefir fermentation (kefir grains) consists of variety of lactic acid bacteria species and yeasts as opposed to *Lactobacillus bulgaricus*, *Streptococcus thermophilus,* and *Lactobacillus acidopholus* commonly used in yogurt fermentation. It is thus conceivable that the different variety of bacterial and yeast microflora in kefir may generate different new peptides or other bioactive compounds, which could have anti-cancer activities.

Apart from peptides, sphingolipids are another class of compounds that could be produced through kefir fermentation that could exert antiproliferative effects since dairy products are a rich source of sphingolipids (Zeisel *et al.*, 1986). Sphingomyelin purified from powdered milk has been shown to inhibit 1,2-dimethylhydrazine (DMH) induced aberrant colonic crypt formation and suppress the conversion of adenomas to adenocarcinomas in female CF1 mice (Dillehay *et al.*, 1994; Schmelz *et al.*, 1996). In vitro studies have shown that the milk phospholipid, sphingomyelin, through its biologically active metabolites ceramide and sphingosine, participates in three major antiproliferative pathways influencing oncogenesis, namely, inhibition of cell growth, and induction of differentiation and apoptosis (Hannun, 1994; Merrill, 1991; Zhang and Kolesnick, 1995). There is evidence that the generation of ceramide by hydrolysis of sphingomyelin can induce anti-proliferative or lethal responses on tumor cells via its action as an intracellular second messenger for tumor necrosis factor-alpha, IL-1beta, IFN-gamma and other cytokines. Whether or not kefir fermentation induces higher concentrations or different molecular species of sphingolipids that play anti-proliferative effects in mammary tumor cells was not determined; however, previous work has indicated that kefir contains unique sphingomyelins that enhance markedly greater secretion of the anti-proliferative cytokine, IFN-beta, in human osteosarcoma cells than sphingomyelins from other sources (Osada *et al.*, 1993).

In summary, kefir mother culture and extracts of kefir have been demonstrated an antiproliferative effect on cultured breast cancer cells. Although the bioactive component(s) and the mechanism of the antitumor activity are not clear, the present study suggests the potential of kefir fermentation to produce compounds with antiproliferative activity useful in the prevention and therapy of solid tumors like breast cancer. Further analysis of the composition and the mechanisms of action of the direct responses of these extracts in cell culture should provide valuable information with respect to the pharmacological and therapeutic uses of these extracts.

ACKNOWLEDGMENT

This study was supported in part by grants from Liberty Food, Inc. and the Natural Science and Engineering Research of Canada grant to Drs. Stan Kubow and Hing Man Chan.

CHAPTER 3

INTRODUCTION TO CHAPTER 3

In the first study, kefir and kefir mother culture were demonstrated to have dose-dependent antiproliferative effects on MCF-7 cells, and the mother culture of kefir was found to be the most potent in decreasing tumor cell growth. The CE electropherograms showed that the profiles of the extracts of kefir mother culture and kefir were similar suggesting similar profiles of bioactive components. We hypothesized that the active ingredients are products of the kefir fermentation process. The major objective described in the following manuscript was the fractionation and characterization of extracts derived from kefir mother culture in order to identify antiproliferative kefir components. High speed centrifugation, molecular weight cut-off filtration, SEC-HPLC and RP-HPLC were used for the separation of kefir fractions, which were screened for antiproliferative effects in the *in vitro* cell culture test system developed in the first manuscript. The fraction(s) that had antiproliferative effects were further characterized with MALDI and ESI MS-MS.

MANUSCRIPT B

Fractionation and Characterization of Bioactive Components in Kefir Mother Culture

Chujian Chen, Stan Kubow and Hing Man Chan

ABSTRACT

The mother culture of Russian-type of kefir has shown antiproliferative effects in MCF-7 cells but the bioactive component remains to be identified. In this study, components of kefir mother culture showing antiproliferative effects in cultured MCF-7 cells were separated and isolated using high speed centrifugation, molecular weight cut-off filtration, size exclusion high performance liquid chromatography (SEC-HPLC) and reverse phase liquid chromatography (RP-HPLC). Fraction of MWCO less than 3000 Dalton and 5 separate HPLC fractions all showed dose-dependent antiproliferative effects. One specific fraction (Fraction 30) significantly increased the susceptibility of MCF-7 cells to tamoxifen. RP-HPLC analysis of this fraction suggested that it likely contains a complex mixture of compounds. Although the exact molecular structures of these compounds were not identified, MS analysis suggested that the compounds may be sphingomyelin complexes. We suggest that these sphingomyelin complexes could play a role in the antiproliferative effects of kefir mother culture on MCF-7 cells.

Key words: kefir, fractionation, cell proliferation, MCF-7 cells

INTRODUCTION

Breast cancer is the most commonly diagnosed cancer in women. Resistance to therapy is the major reason for failure of cancer treatment (Mesner *et al.*, 1997). There is a critical need to identify new chemotherapeutic agents that can increase susceptibility to anti-breast cancer drugs or overcome resistance mechanisms, which would improve patient outcomes, prevent relapse, and prolong patient survival.

Kefir is an acidic and mildly alcoholic fermented milk which originated in the Caucasian mountains of former Soviet Union, and enjoys a rich tradition of health benefits. Consumption of kefir has been associated with lower incidence of breast cancer (Koroleva *et al.*, 1988, Ronco *et al.*, 2002.). Research on the putative health benefits of fermented milks suggested that by-products of bacterial fermentation of proteins, lipids and carbohydrates present in fermented milks exert health benefits beyond basic nutrition including antitumor action, immune system enhancement and antioxidant effects (Reddy *et al.*, 1983). Conjugated linoleic acid (Schonberg *et al.*, 1995), sphingolipids (Dillehay *et al.*, 1994; Schmelz *et al.*, 1996), polysaccharides (Shiomi *et al.*, 1982), organic acids (Garrote *et al.*, 2000), and some proteins and peptides (Svensson *et al.*, 1999) have been shown to have antimutagenic and antitumor effects. Promising results regarding antitumor activity of yogurt extracts in cell culture (Biffi *et al.*, 1997) and kefir extracts in animal feeding studies (Cervikbas *et al.*, 1994; Furukawa *et al.*, 1990 and 2000; Shiomi *et al.*, 1982) have been reported. In our recent studies, kefir extracts exerted potent anti-proliferative effects on cultured human mammary tumor cells as compared to extracts of yogurt or milk (Chen *et al.*, 2005. submitted). The aim of the present study was to fractionate and characterize bioactive components in kefir mother culture that exert antiproliferative effects in MCF-7 cells.

MATERIALS AND METHODS

Kefir samples were provided by Liberte Inc. (Brossard, Canada). The large scale production of kefir involves a two-step fermentation process. The first

fermentation is achieved by directly adding kefir grains (2-10%) to milk that has been pasteurized and cooled to 20-25°C. After a period of fermentation lasting around 24 h, the grains are removed by filtration. The filtrate (kefir mother culture) is added to milk (1-3%), which is further fermented for 24 h and packaged for the consumer market (final kefir commercial product). Samples from three different batches were used. Upon receipt of the samples, they were immediately well stirred, and centrifuged at 4°C, 32,000 x g, for 60 min (Sorvall RC 5C Centrifuge, rotor ss-34, Sorvall Instruments, Wilmington, USA). The supernatant was filtered with a 0.45 μm membrane filter followed by a 0.2 μm filter. The filtrates were stored at -80 °C for future use.

Macronutrients and minerals

A Flexi-Dry MP lyophilizer (FTS Systems Inc., Stone Ridge, USA) was used for triplicate determination of moisture. Ten grams of homogenized sample was transferred into pre-weighed aluminium weigh boat , frozen at -80°C for approximately 1 h and then freeze dried for 48 h. The boat was weighed again and the moisture was calculated. A LECO FP-428 Nitrogen Determination System (LECO Corporation, St. Joseph, USA) was used to determine nitrogen content in triplicate for freeze-dried samples. Crude protein content was calculated using a conversion factor of 6.25. Protein in solution was determined by using Bio-Rad protein assay kit arrording to the instruction with the kit (Bio-Rad Laboratories, Hercules, USA). Peptides concentrations were analyzed by the method of Church *et al.* (1983) (opthialdehyde; OPA). Crude fat was analyzed in triplicate with an automatic Soxtec extraction system (Soxtec HT6 Tecator AB, Hoganas, Sweden). Three grams of freeze-dried, well-mixed sample was loaded and analyzed. Proper amount of freeze-dried samples were digested in 70% (w/v) nitric acid (Fisher Scientific, trace metal grade) and minerals (i.e. Ca, Mg, Zn, Fe, Na and K) were determined by using Hitachi Z-8200 Zeeman polarized atomic absorption spectrophotometer (Nissei Sango Ltd., Missassauga, ON, Canada).

Organic acids

Lactic acid content was analyzed using a Sigma lactate kit assay (Sigma Diagnostics, Cat. No. 735-10, St. Louis, USA). Organic acids were determined by HPLC according to the method of Guzel-Seydim *et al.* (2000). Five mL of each sample was diluted with 25 mL 0.01 M H_2SO_4, vortexed for 1 min followed by centrifugation at 2000 x g for 10 min. Supernatants were collected and filtered through 0.2 µm filter. Volumes of 20 µl of samples and standards were injected into a Beckman Gold HPLC system (Beckman Coulter, Fullerton, USA) equipped with an Aminex HPX-87H (300 mm x 7.8 mm) organic acid column (Bio-Rad Laboratories, Hercules, USA) . Degassed 8 mM sulfuric acid (H_2SO_4) was used as the mobile phase. The organic acids oxalate, citrate, malate, succinate, formate and acetate were detected at 215 nm. Organic acids were quantified using external standards (organic acid analysis standard, Bio-Rad Laboratories, Hercules, USA).

Molecular weight cut-off fractionation (MWCO)

Centriplus centrifugal filter devices were used to get MWCO fractions at 3000 Da (Millipore, Bedford, USA). Ten milliliters whole extract were loaded to the sample reservoir and the assembled device was centrifuged at 4 °C, 3000 x g for 290 min. The filtrates were collected for further analysis.

Size exclusion HPLC (SEC-HPLC) separation

One hundred microliters of kefir mother culture extract was injected into a TSK G2000SWXL column (78 mm x 30 mm, SUPELCO, Bellefonte, USA) and separated with a Shimadzu LC-6AD Liquid Chromatograph system (Shimadzu Scientific Instruments, Inc. Columbia, USA) with UV detection at 210 nm. The separation buffer used was a mixture of 45% acetonitrile in 0.1% trifluorocetic acid (TFA) with a flow rate of 0.4 mL/min for 40 min. Nine fractions were collected for each HPLC run, and fractions from five to ten runs were pooled. The above nine fractions were evaporated with N_2 and then freeze dried, stored at -80 °C for cell culture incubations and for further analytical analyses.

Reverse Phase HPLC (RP-HPLC) fractionation

The fraction(s) collected with SEC HPLC that showed antiproliferative effects on MCF-7 cells were further analyzed with a Prosphere 300 C4 column (5μm, 250 mm x 4.6 mm) (Alltech Associate, Inc. Deerfield, USA) using a Beckman Gold HPLC System (Beckman Coulter, Fullerton, USA). After the column was equilibrated with buffer A (0.1% TFA in water) at a flow rate of 1 mL per min, the fractions were eluted with a linear gradient of buffer A (0.1% TFA in water) and buffer B (60% of acetonitrile in 0.1% TFA : 40% of 0.1% TFA in water) as follows: 0 to 60 min, 0 to 90% B; and 61 to 65 min, 90 to 0% B. Dual channel absorbance was monitored at 210 nm (channel A) and 280 nm (channel B).

Preparative HPLC fractionation

Three batches of extracts of kefir mother culture were pooled and fractionated using the Centriplus centrifugal filter devices to obtain fractions of compounds with molecular weights less than 3000 Da. The fractions of MWCO less than 3000 Da were freeze-dried using a FLEXI-DRY MP Freeze Dryer (FTS Systems, Inc. Stone Ridge, U SA). Five g of the lyophilized MWCO fractions were dissolved in 20 mL of water. Ten milliliters of reconstituted solution were loaded on a C4 preparative column (300 Å, 5 μm, 300mm × 50mm) (Vydac Company, Herperia, CA) and separated with a Water Delta Prep 4000 HPLC system (Waters Corporation, Milford, USA). After the column was equilibrated with buffer A (0.6% Acetic acid in water) at a flow rate of 13 mL/min, the fractions were eluted with a linear gradient of buffer A and buffer B (0.6% acetic acid in acetonitrile) as follows: 0 to 70 min, 0 to 60% B; 70 to 80 min, 60 to 70% B; 80 to 100 min, 70 to 80% B; 100 to 105 min, 80 to 0% B.

A total of 100 fractions were collected between 0 and 100 min, with samples taken at 1-min intervals. The fractions were then lyophilized and kept at −80 °C for further cell culture and analysis. The fractions were reconstituted by adding water and peptide and protein concentrations were determined before cell culture assays.

Capillary electrophoresis

Capillary zone electrophoresis (CE) was performed using a P/ACE ™ 2200 HPAC instrument controlled by System Gold software (Beckman, Fullerton, CA, USA) coupled to an IBM PC 486 computer (IBM Corp., Portmouth, England) for data acquisition and analysis. A neutral uncoated fused silica capillary column (57 cm × 50 μm, the length from intake to detector was 50 cm) was assembled in the P/ACE cartridge (Polymicro Technologies, Phoenix, Arizona USA) for capillary separations. Injection volume, buffer concentration and running voltage were optimized to achieve the best resolution with the shortest running time.

The capillary was flushed with 1 M sodium hydroxide, followed by nanopure water, 0.1 M sodium hydroxide, nanopure water, 1 M hydrochloric acid, and again water, each for 5 min at a pressure of 40 psi. A solution of 5% polybrene and 2% ethylene glycol was then passed for 10 min at 40 psi. Excessive coating was then removed by flushing with water for 2 min. Further capillary flushing was then performed for additional 10 min using 200 mM formic acid buffer. Before each sample application, the capillary was rinsed with 1 min water, 1 min 0.1 M sodium hydroxide, 1 min water and 3 min separation buffer (200 mM formic acid in water, pH 2.0). After the completion of each run, the capillary was rinsed with nanopure water for 1 min, 0.1 M sodium hydroxide for 1 min and nanopure water for 1 min. Peptide standard and sample injections were carried out at the anode end of the capillary using N_2 pressure (0.5 psi) for 5 seconds, and was separated at a constant temperature of 20 °C with a 200 mM formic acid (pH 2.0) as separation buffer. On-line detection was performed using an UV detector at 200 nm. Twenty kilovolts was applied across the run buffers for best separation. The capillary was re-coated after every 5 runs.

Matrix assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF) for estimation of molecular weight

Reconstituted SEC-HPLC and RP-HPLC fractions were analyzed using a MALDI-TOF mass spectrometer (Voyager DE-STR; Applied Biosystems, Palo-Alto, CA, USA) with a laser at 337 nm and an acceleration voltage of 20.000 V.

Mass spectrometry

Mass spectrometric analysis in the positive ion mode was performed on a triple quadupole mass spectrometer (SCIEX API III Biomolecular mass analyzer, Thornhill, Ontario, Canada). Lyophilized reversed phase HPLC fractions having antiproliferative effect on MCF-7 cells were reconstituted in 0.5 mM ammonium acetate in methanol, or in 10% acetic acid in 20% aqueous methanol. The resulting solution was then infused into the electrospray ion-source by a syringe pump (Harvard Apparatus Model 22, South Natick, MA) at a flow rate of 1.5μL/min. The ion-spray voltage was set at 5.5 kV, and the orifice potential was set at 50 V. Argon was used as the collision gas at a collision gas thickness (CGT) of 1.5×10^{14} for collision-induced fragmentation MS-MS analysis.

Cell Culture screening for antiproliferative effects

MCF-7 cells were purchased from ATCC (ATCC, Manassas, USA). Cells were routinely propagated as a monolayer culture in Dulbcco's Modified Eagle Medium (DMEM) (Gibco, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA), in a 75-cm^2 plastic dish at 37°C in a humidified atmosphere with 5% CO_2, and passaged every 3-4 days. Normal human mammary epithelial cell line (HMEC) was graciously provided by Dr. M.R. Stampfer (Lawrence Berkeley National Laboratory, Berkeley, USA). Cells were routinely propagated as a monolayer culture in Mammary Epithelial Growth Media (MEGM, Clonetics, San Diego, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS), in 75-cm^2 plastic dish at 37 °C in a humidified atmosphere with 5% CO_2, and passaged every week. For the experiments, both MCF-7 and HMEC cells were harvest from the dish using 0.25% trypsin-EDTA solution (Sigma, St Louis, MO, USA).

Cell proliferation experiments in 24-well plates

Cells previously harvested were seeded in 24-well plates, i.e., 10,000 cells for MCF-7 per well in DMEM supplemented with 10% FBS and 5,000 cells for HMEC per well in MEGM supplemented with 10% FBS. The cells were allowed

to attach for 24 h. After that period, old media were removed and fresh media and extracts were added to each well. To study the dose response, a serial dilution of each the extract using the culture media was made to achieve final concentrations of extracts at 5%, 2.5%, 1.3%, 0.6%, and 0.3% (vol./vol.), respectively. Because the kefir extracts were acidic (approximately pH 4.5), Dulbecco's Phosphate Buffered Saline (PBS) buffer (Gibco BRL, Grand Island, NY) was added to the culture media to adjust the pH between 7.0 and 7.4. Cells were incubated at 37°C in a humidified atmosphere with 5% CO_2 for 6 d. Cell nuclei were counted in order to eliminate the difficulty in counting whole cells due to clumping. Furthermore, a more uniform distribution of nuclei over the grids of a hemacytometer was seen compared with whole cells. Media was aspirated from wells and cells were rinsed with 500 µl of PBS. 250 µl of hypotonic buffer (0.01M HEPES, 1.5 M $MgCl_2$, pH 7.5) was added to each well. After 2 min, 250 µl of cell lysing solution (10% ethylhexadecyldimethylammonium bromide, 3% glacial acetic acid, in water) was added. The plate was shaken lightly every minute for 5 min. Cell lysis was confirmed microscopically as indicated by a suspension of clean nuclei. The suspension was mixed and the nuclei were counted using a Coulter Counter (Coulter Counter Corporation, Fullerton USA). Each sample was run in quadruplicate. Control cells were incubated with the culture medium with the dosing vehicle (PBS). To standardize results, each plate had its own control wells with cells treated with PBS and cell proliferation of different treatment was expressed as a percent of control.

Cell proliferation experiments in 96-well plates

Cells previously harvested were seeded in 96-well plates at 1,000 cells per well for MCF-7 in DMEM supplemented with 10% FBS and for HMEC in MEGM supplemented with 10% FBS. The cells were allowed to attach for 24 h. After that period, old media were removed and fresh media and extracts were added to each well. A serial dilution of each test fraction was made to study the dose-response. PBS buffer was added to the culture media to keep the final pH between 7.0 and 7.4. Cells were incubated at 37°C in a humidified atmosphere with 5% CO_2 for 6 d and the cell numbers in each well were determined by using

a CellTiter 96® Aqueous One Solution Cell Proliferation Assay kit (Promega, Madison, USA). Each sample was run in quadruplicate. Control cells were incubated with the culture medium with the dosing vehicle (PBS). To standardize results, each plate had its own control wells with cells treated with PBS and cell proliferation of different treatments was expressed as a percent of control.

Statistics

All statistics test were performed using SAS 8.2 for PC (SAS, Cary, NC USA). Means were compared with Student's t test. Two-way ANOVA was used to analyze the effects of treatments and doses for the cell culture experiments. The difference among doses and treatments were determined by the Student-Newman-Keuls (SNK) multiple comparison test. Statistical significance was considered at $P< 0.05$.

RESULTS AND DISCUSSION

Moisture, macronutrients and some minerals in kefir mother culture and final kefir described in Table 3.1. No significant differences among nutrients and organic acids were found, except that lactic acid in the kefir commercial product was higher than that of kefir mother culture (Table 3.2). Capillary electrophoresis analyses showed that the profiles of extracts of mother culture and kefir were similar (Figure 3.1). In our earlier study, the extract of kefir mother culture showed a tendency of having stronger antiproliferative effect on MCF-7 cells than other extracts (submitted manuscript, Chen *et al.*, 2005). Hence, mother culture was chosen for further fractionation and cell culture tests to identify the bioactive component(s).

Extracts of kefir mother culture that were fractionated via the 3000 Da MWCO were analyzed for protein and peptide content (Table 3.3). Both protein and peptide concentrations were significantly lower in the fraction of MWCO less than 3000 Da ($P < 0.05$) relative to the whole kefir mother culture extract. Both the whole extract and the fraction with the MWCO less than 3000 Da were

screened by MCF-7 cell culture using 24-well plates. As shown in Figure 3.2, the filtrate of MWCO less than 3000 Da had a dose dependent antiproliferative effect on MCF-7 cells comparable to that of the whole mother culture extract.

The kefir mother culture filtrate that contained the MWCO less than 3000 Da was further separated with SEC-HPLC. As shown in the elution profile in Figure 3.3, nine fractions were collected with each run, and 10 runs of SEC-HPLC fractions were pooled, freeze dried and reconstituted in 2 mL water. The estimated molecular weight, protein and peptide concentrations were shown in Table 4. All the nine fractions were tested with MCF-7 cells in 96-well plates for their antiproliferative effects. Fractions 7 and 8 were the only fractions to demonstrate a dose-dependent antiproliferative effects on MCF-7 cells with the most potent effect exhibited with fraction 7 (Figure 3.4). Fraction 7 was also tested with HMEC cells, and no antiproliferative effects were observed (Figure 3.5). Fraction 7 was further analyzed with RP-HPLC, which showed the presence of about 11 peaks in Fraction 7 (Figure 3.6). SEC-HPLC Fraction 7 was also analyzed via MALDI-TOF, which indicated that the peak masses ranged from 659 to 2074 Da (Figure 3.7).

Table 3.1 Nutrient composition from three different batches of kefir mother culture and final commercial kefir product (per 100 g wet weight)

Composition	Batch 1		Batch 2		Batch 3	
	Mother culture	Kefir	Mother culture	Kefir	Mother culture	Kefir
Moisture (g)	[1]89.78 ± 0.09	90.40 ± 0.06	91.22 ± 0.19	90.03 ± 0.02	89.56 ± 0.07	90.17 ± 0.08
Ash (g)	0.68±0.005	0.67±0.004	0.66±0.006	0.70±0.003	0.68±0.002	0.67±0.003
Protein (g)	3.05±0.16	2.81 ± 0.03	2.78±0.03	3.14 ± 0.04	3.28±0.01	2.77 ± 0.02
Fat (g)	2.32±0.06	1.77 ± 0.04	2.46±0.06	1.46 ± 0.02	2.36±0.04	1.48 ± 0.01
Calcium (mg)	78.20 ±0.66	77.70 ±0.71	78.93 ± 0.58	77.40 ± 0.64	81.53 ± 0.73	80.40 ± 0.56
Iron (mg)	0.02 ± 0.001	0.02 ± 0.001	0.02 ± 0.001	0.02 ± 0.001	0.02 ± 0.001	0.02 ± 0.001
Zinc (mg)	0.28 ± 0.02	0.29 ± 0.02	0.28 ± 0.02	0.30 ± 0.02	0.31 ± 0.03	0.29 ± 0.03
Magnesium (mg)	8.42 ± 0.55	8.65 ± 0.35	8.88 ± 0.56	8.40 ± 0.71	8.97 ± 0.48	8.70 ± 0.42
Sodium (mg)	28.51 ±1.86	29.0 ±0.71	28.60 ± 2.03	30.05 ± 2.05	30.99 ±1.08	31.05 ± 0.72
Potassium (mg)	60.28 ± 0.89	60.60 ± 0.42	59.36 ± 1.16	61.30 ± 1.34	60.40 ± 1.08	60.95 ± 0.78

[1]Mean±SD, n=3

Table 3.2 Organic acid concentrations (mmol/L) in mother culture and final kefir commercial product

	Mother culture	Kefir
Lactic acid[1]	[3]77.86±8.94	106.87±9.36*
Uric acid[2]	0.014±0.003	0.012±0.002
Pyruvic acid[2]	0.54±0.15	0.52±0.10
Oxalic acid[2]	[4]ND	ND
Citric acid[2]	3.27±0.17	3.22±0.28
Malic acid[2]	54.79±9.25	57.62±11.01
Succinic acid[2]	1.61±0.24	1.71±0.08
Formic acid[2]	Not detectable	Not detectable
Acetic acid[2]	3.81±0.74	3.46±0.47

[1]Determined by lactic acid kit assay method
[2]Determined by HPLC method of Guzel-Seydim *et al.* (2000).
[3]Mean±SD, n=3; *$P < 0.05$
[4]ND = Not detectable

Figure 3.1 Capillary electropherograms of extracts of kefir mother culture and final commercial kefir product. A: Extract of kefir mother culture. B: Extract of final commercial kefir product. The capillary column (57 cm x 50 μm; the length from inlet to detector is 50 cm) was coated with a solution of 5% polybrene and 2% ethylene glycol. Separation was done on Beckman P/ACE 2200 (Beckman Instruments, Inc., Fullerton, California, USA) with 200mM formic acid as running buffer. On-line detection was performed using an UV detector at 200 nm. Twenty kilovolts was applied across the run buffers for best separation.

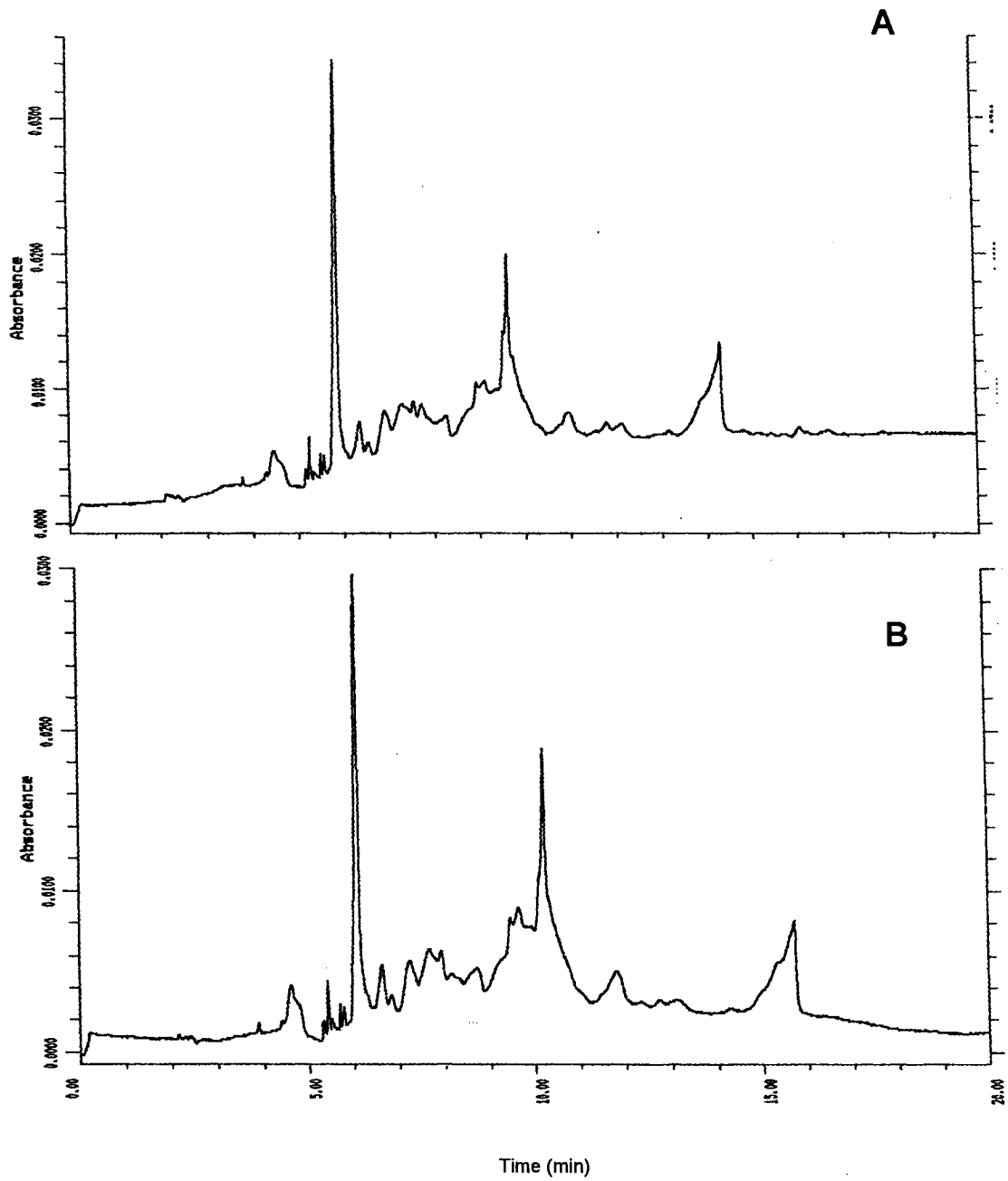

A
B
Absorbance
Absorbance
Time (min)

Table 3.3 Protein and peptide concentrations in different MWCO fractions of extracts of kefir mother culture

Samples	Moisture (g/100g)	Crude protein[1] (g/100g)	Protein[2] (μg/mL)	Peptides[3] (mmol/L)
Whole extract	[4]96.39±0.24	0.27±0.03	152.91±11.53	5.53±0.26
MWCO<3000	[5]ND	ND	5.10±0.07*	4.21±0.32*
MWCO>3000	ND	ND	195.94±4.11*	7.16±0.36*

Notes: [1]Determined by using Nitrogen Determinator
[2]Determined by Bradford method (1976)
[3]Determined by OPA method of Church *et al.* (1983)
[4]Mean±SD, n=3
[5]ND: No data is available
*Significant different at $P < 0.05$ when compared to protein or peptide concentration in whole extract.

Table 3.4 Estimated molecular weight, protein and peptide concentration of the nine SEC-HPLC fractions

Fraction	Retention time (min)	Molecular Weight (Dalton)	Protein (μg/mL)	Peptides (μM/L)
1	14.4-17	13116-29507	145.7±5.12	274.53±2.19
2	17.1-19	7618-12745	118.56±4.89	284.70±5.31
3	19.1-21	4672-7425	43.62±2.84	208.33±2.26
4	21.1-23.7	2588-4565	16.80±1.56	491.14±6.67
5	23.8-25.6	1776-2535	3.23±0.26	422.01±5.17
6	25.7-27.3	1297-1742	2.92±0.2	655.11±4.58
7	27.4-29.1	949-1274	1.38±0.12	1168.33±8.22
8	29.2-31.6	635-934	4.77±0.16	1053.71±5.17
9	31.7-33.7	470-625	3.84±0.22	224.59±1.93

Figure 3.2 Antiproliferative effects on MCF-7 cells induced by extracts of: (A) whole kefir mother culture; and (B) fraction of kefir mother culture obtained with MWCO less than 3000 Da. Cell proliferation was analyzed in 24-well plate. Values were graphed as Mean+SD, n=4.

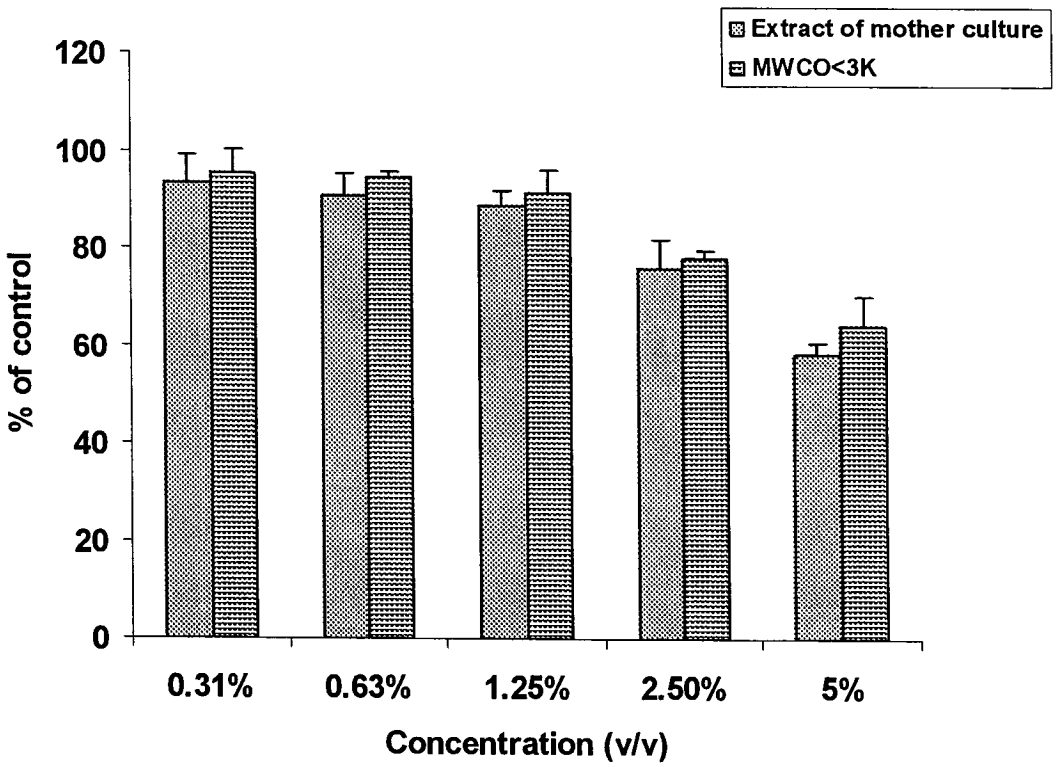

Extract of mother culture
MWCO<3K
120
100
80
60
40
20
0
% of control
0.31%
0.63%
1.25%
2.50%
5%
Concentration (v/v)

Figure 3.3 Elution profile of extract of kefir mother culture using SEC-HPLC. A TSK G2000SWXL 78 mm X 30 mm column was used. Separation buffer was 45% acetonitrile in 0.1% trifluorocetic acid (TFA) with flow rate of 0.4 mL/min for 40 min. Peaks were detected at 210 nm with UV detector. Nine fractions with retention times of 14.6 to 17.2, 17.2 to 19.2, 19.2 to 21.2, 21.2 to 23.8, 23.8 to 25.7, 25.7 to 27.4, 27.4 to 29.2, 29.2 to 31.7, 31.7 to 34 min respectively were collected as indicated in the graph.

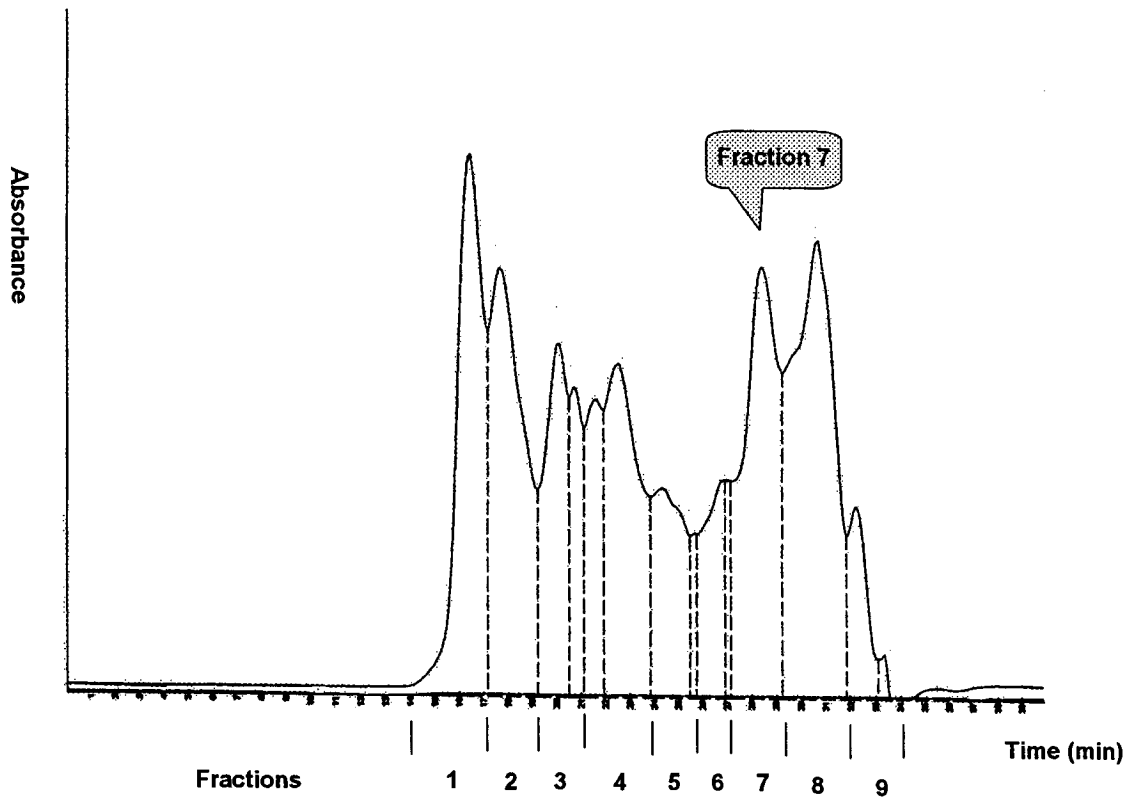

Absorbance
Fraction 7
Time (min)
Fractions
1
2
3
4
5
6
7
8
9

Figure 3.4 Antiproliferative effects of 9 SEC-HPLC fractions on MCF-7 cells. Cell proliferation were analyzed in 96-well plate using a CellTiter 96® Aqueous One Solution Cell Proliferation Assay kit. Values were graphed as Mean+SD, n=4.

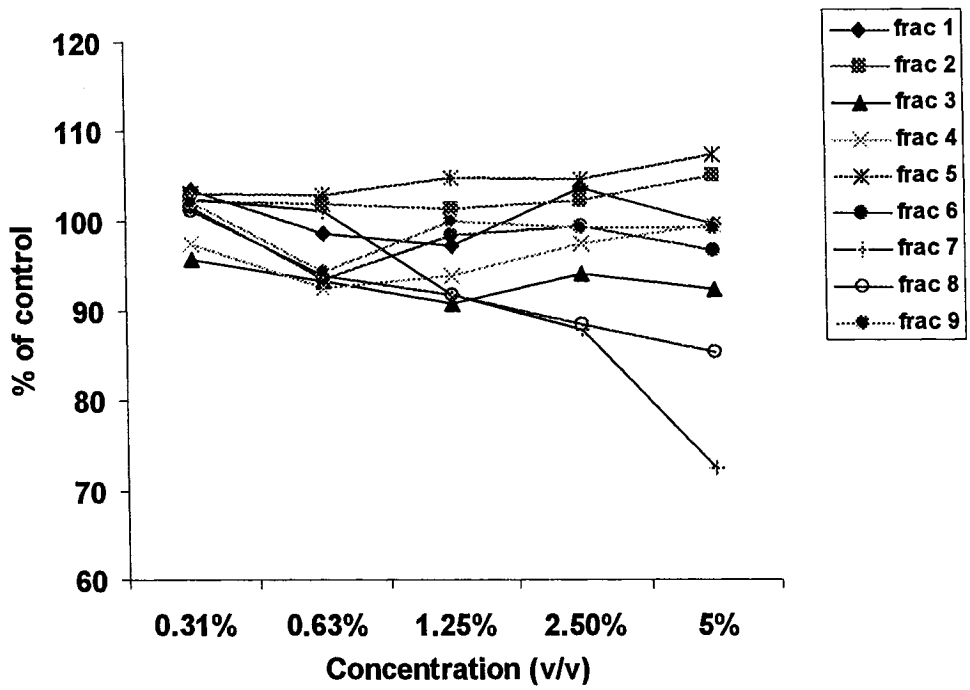

120
110
100
90
80
70
60
% of control
0.31%
0.63%
1.25%
2.50%
5%
Concentration (v/v)
frac 1
frac 2
frac 3
frac 4
frac 5
frac 6
frac 7
frac 8
frac 9

Figure 3.5 Antiproliferative effects of SEC-HPLC Fraction 7 on MCF-7 and HMEC cells. Cell proliferation was analyzed in 96-well plate using a CellTiter 96® Aqueous One Solution Cell Proliferation Assay kit. Values were graphed as Mean+SD, n=4.

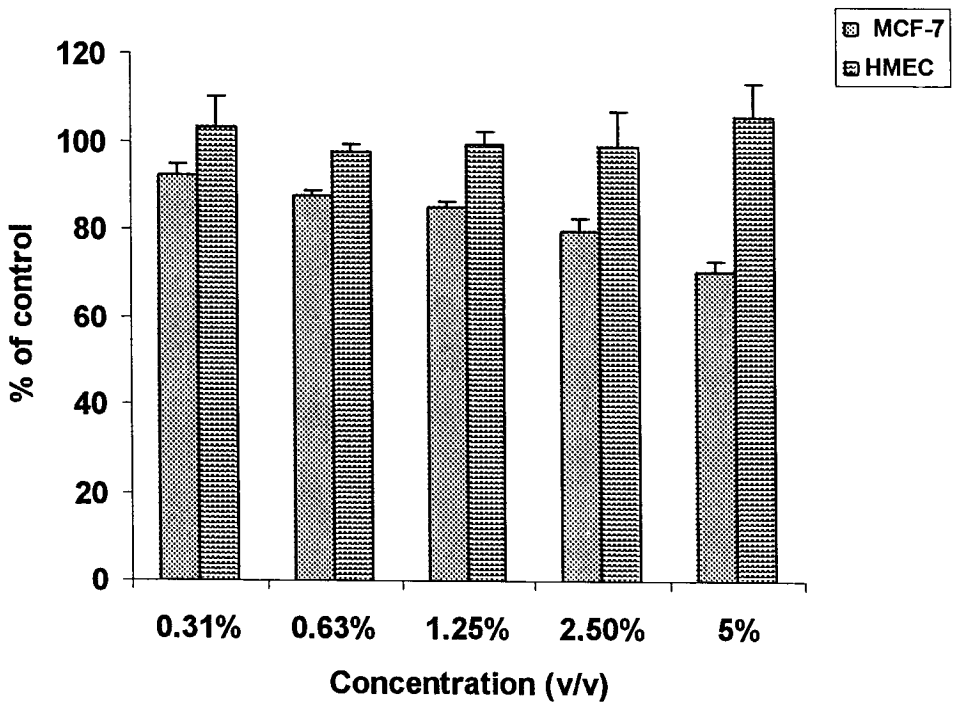

MCF-7
HMEC
120
100
80
60
40
20
0
% of control
0.31%
0.63%
1.25%
2.50%
5%
Concentration (v/v)

Figure 3.6 Elution profile of SEC-HPLC Fraction 7 of kefir mother culture using RP-HPLC. A Prosphere 300 C4, 5μm, 250 mm X 4.6 mm column was used. After the column was equilibrated with buffer A (0.1% TFA in water) at a flow rate of 1 mL per minute, the fractions were eluted with a linear gradient of Buffer A (0.1% TFA in water) and Buffer B (60% of Acetonitrile in 0.1% TFA: 40% of 0.1% TFA in water) as follows: 0 to 60 min, 0 to 90% B; and 61 to 65 min, 90 to 0% B. Absorbance was monitored at channel A 210 nm and channel B 280 nm. The retention times of the peaks 1 to 11 are 15.4, 18.6, 21.1, 23.3, 26.1, 39.1, 43.7, 44.5, 45.2, 46.3, and 50.1 min respectively.

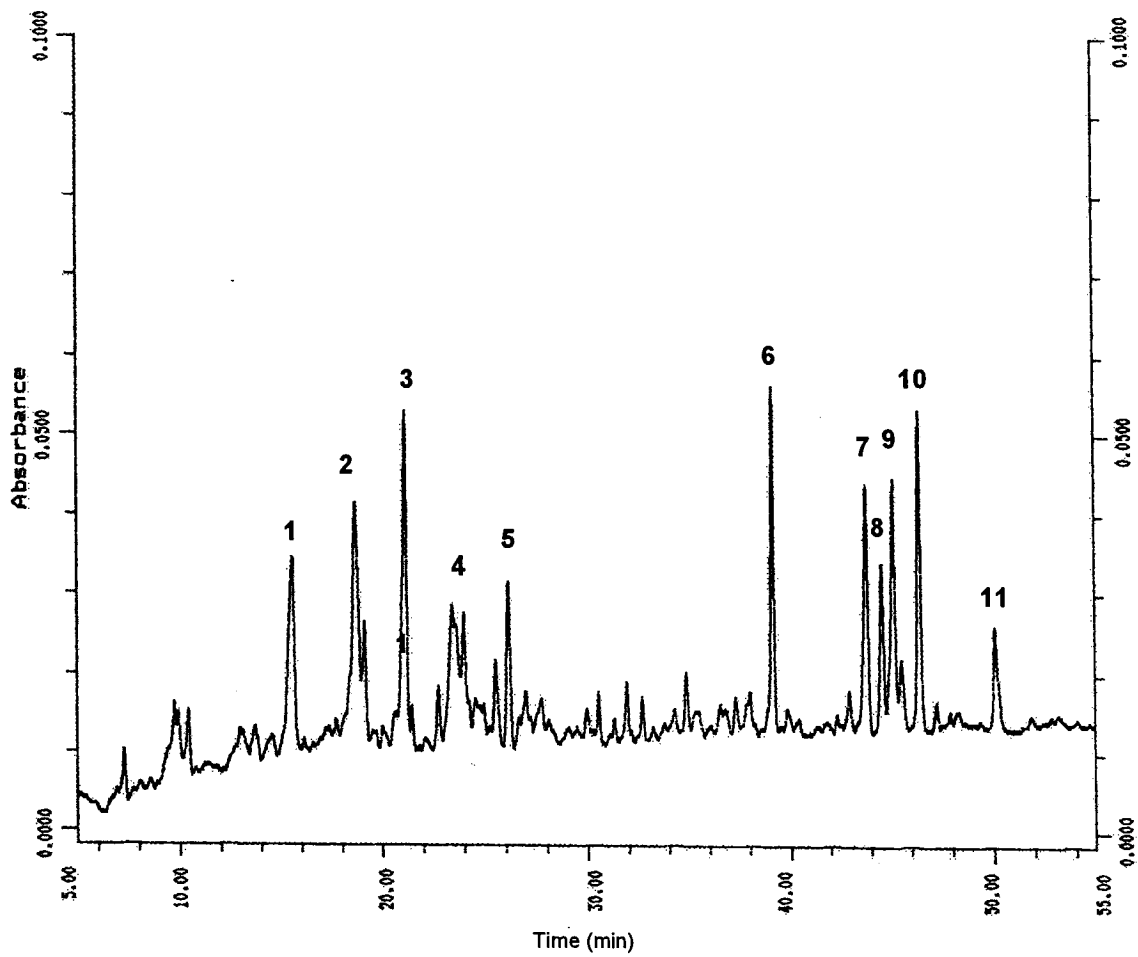
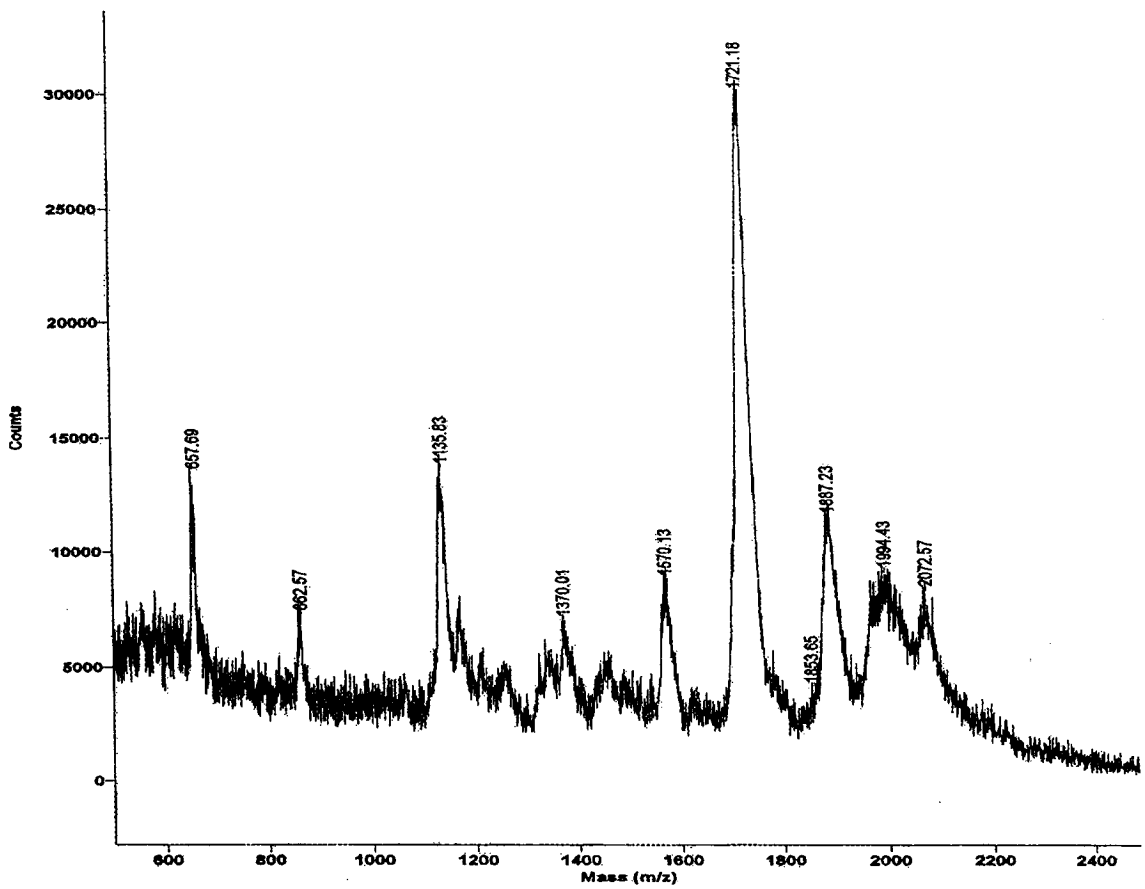

Absorbance
0.1000
0.0500
0.0000
1
2
3
4
5
6
7
8
9
10
11
5.00
10.00
20.00
30.00
40.00
50.00
55.00
Time (min)

Figure 3.7 Mass spectrum of SEC-HPLC Fraction 7 of kefir mother culture analyzed by a MALDI-TOF mass spectrometer with a laser at 337 nm and an acceleration voltage of 20.000 V.


Counts
30000
25000
20000
15000
10000
5000
0
657.69
862.57
1135.83
1370.01
1570.13
1721.18
1853.65
1887.23
1994.43
2072.57
600
800
1000
1200
1400
1600
1800
2000
2200
2400
Mass (m/z)

A preparative C4 column was utilized to obtain sufficient amounts of mother culture kefir fractions of MWCO less than 3000 Da for more extensive structure analysis and cell culture studies. The average nitrogen content of the lyophilized filtrate of MWCO less than 3000 Da was 0.58%. Five g of the lyophilized MWCO less than 3000 Da fractions was dissolved in 20 mL of water. Ten milliliters of reconstituted solution were loaded on the column, and 100 fractions were collected in 1 minute interval (Figure 3.8). The peptide concentration of each preparative HPLC fraction is shown in Figure 3.9, Fractions 28 to 58, 62 to 64, and 72 to 74 were screened for antiproliferative effects on MCF-7 cells with three concentrations of 0.4%, 2% and 10% (Figure 3.10). Fractions 29 to 41, 62 were found to have antiproliferative effects on MCF-7 cells. These fractions were further screened for antiproliferative effects with serial dilution. A dose dependent antiproliferative effect was observed when MCF-7 cells were treated with fractions 29, 30, 34, and 37, while not on HMEC cells (Figure 3.11).

Tamoxifen is a commonly used medication for breast cancer patients. In this study, only the dosage above 0.2 μM/L showed antiproliferative effects on MCF-7 cells. The effective dose reported here was higher than the value reported by Doisneau-Sixou *et al.* (2003). RP-HPLC Fraction 30 of kefir mother culture extract significantly increased MCF-7 cells' susceptibility to tamoxifen (Figure 3.12). A dose dependent antiproliferative effect was also observed.

Figure 3.8 Elution profile of MWCO less than 3000 Da fraction of kefir mother culture extract analyzed by preparative RP-HPLC. Appropriate amount of samples were loaded on a C_4 preparative column (300 Å, 5 μm, 300mm × 50mm) (Vydac Company, Herperia, CA) and separated with Waters Delta Prep 4000 HPLC system. After the column was equilibrated with buffer A at a flow rate of 13 mL/min, the fractions were eluted with a linear gradient of Buffer A (0.6% Acetic acid in water) and Buffer B (0.6% Acetic acid in acetonitrile) as follows: 0 to 70 min, 0 to 60% B; 70to 80 min, 60 to 70% B; 80 to 100 min, 70 to 80% B; 100 to 105 min, 80 to 0% B. The absorbance was measured at 210 nm. A total of 100 fractions were collected between 0 and 100 min, with samples taken at 1-min intervals.

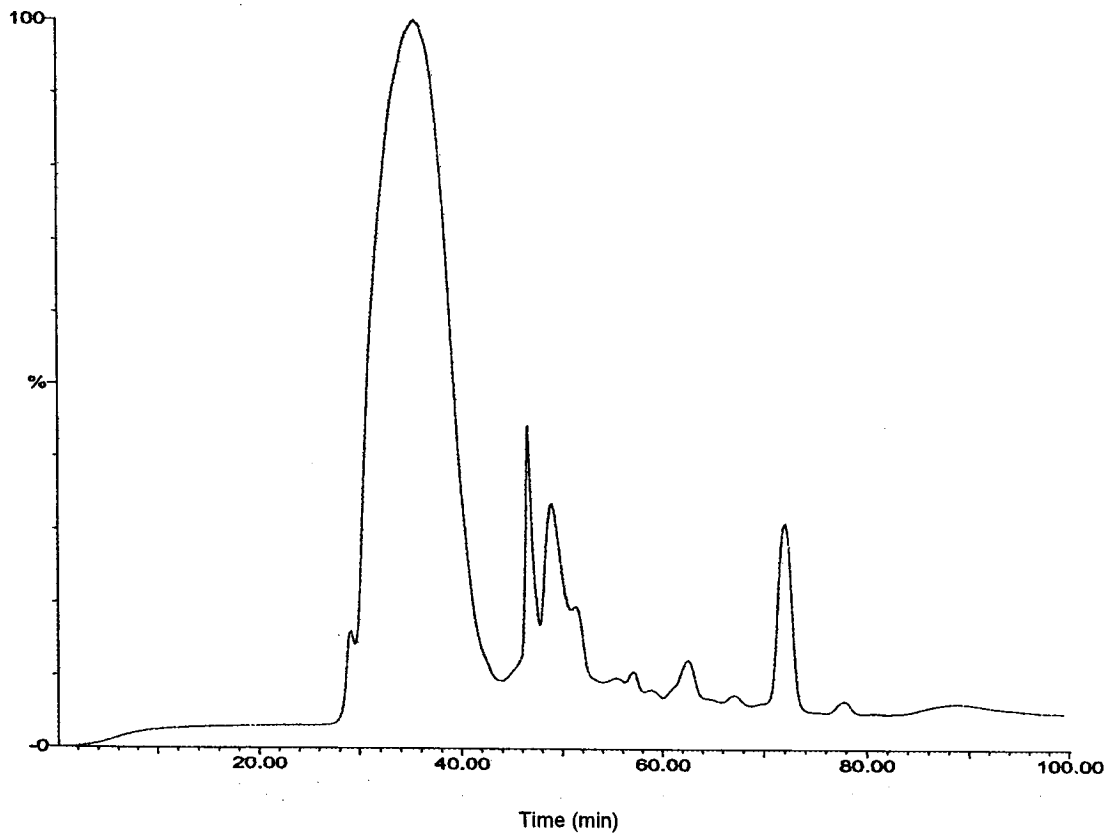

100
%
0
20.00
40.00
60.00
80.00
100.00
Time (min)

Figure 3.9 Peptide concentrations in preparative RP-HPLC fractions. One hundred fractions obtained from preparative RP-HPLC were freeze-dried and then reconstituted with nanopure water to certain concentration. Peptide concentrations were determined in triplicates by OPA method. Mean values were plotted.

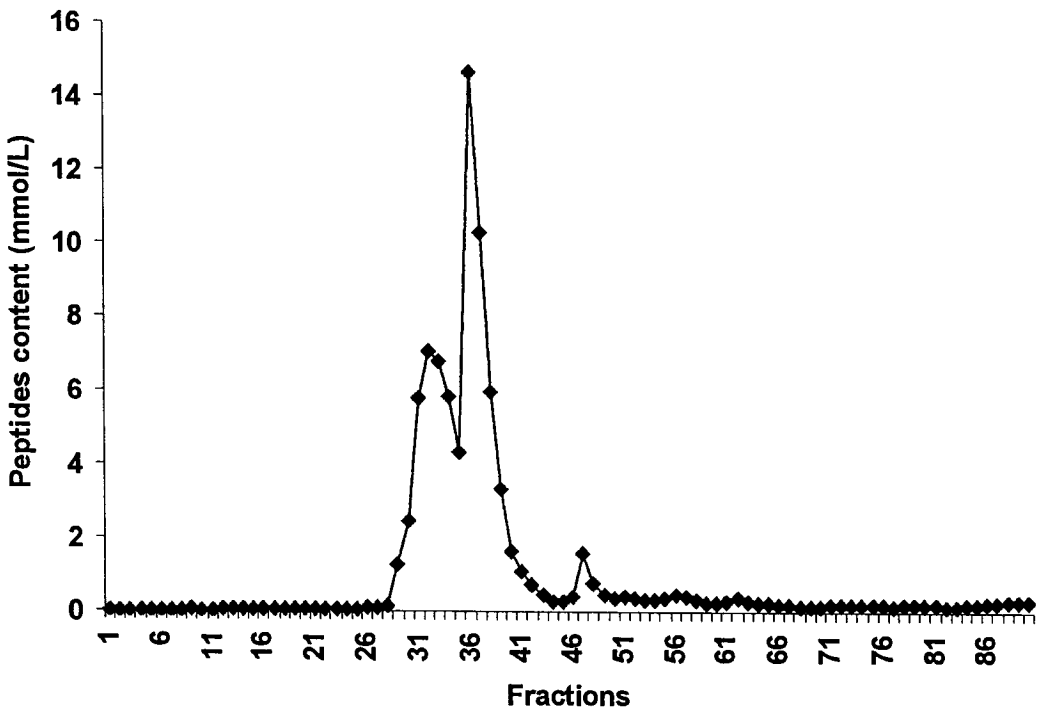

Peptides content (mmol/L)
16
14
12
10
8
6
4
2
0
1
6
11
16
21
26
31
36
41
46
51
56
61
66
71
76
81
86
Fractions

Figure 3.10 Antiproliferative effects of fractions of preparative RP-HPLC on MCF-7 cells. Cell proliferation was analyzed in 96-well plate using a CellTiter 96® Aqueous One Solution Cell Proliferation Assay kit. Each fraction was diluted at three concentrations and was run in quadruplicate. Mean values were plotted.

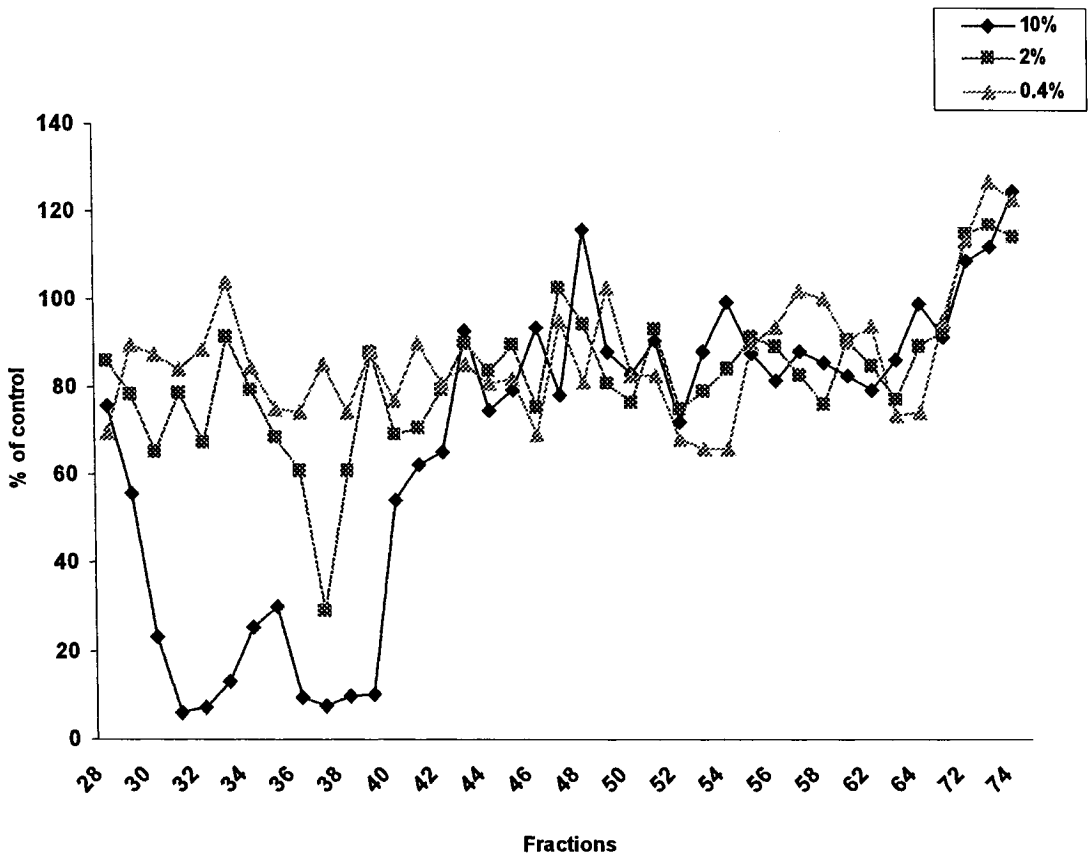

10%
2%
0.4%
140
120
100
80
60
40
20
0
% of control
28
30
32
34
36
38
40
42
44
46
48
50
52
54
56
58
62
64
72
74
Fractions

Figure 3.11 Antiproliferative effects of preparative RP-HPLC Fractions 29 (A), 30 (B), 34 (C), and 37 (D) on MCF-7 and HMEC cells. Cell proliferation was analyzed in 96-well plate using a CellTiter 96® Aqueous One Solution Cell Proliferation Assay kit. Values were graphed as Mean+SD, n=4.

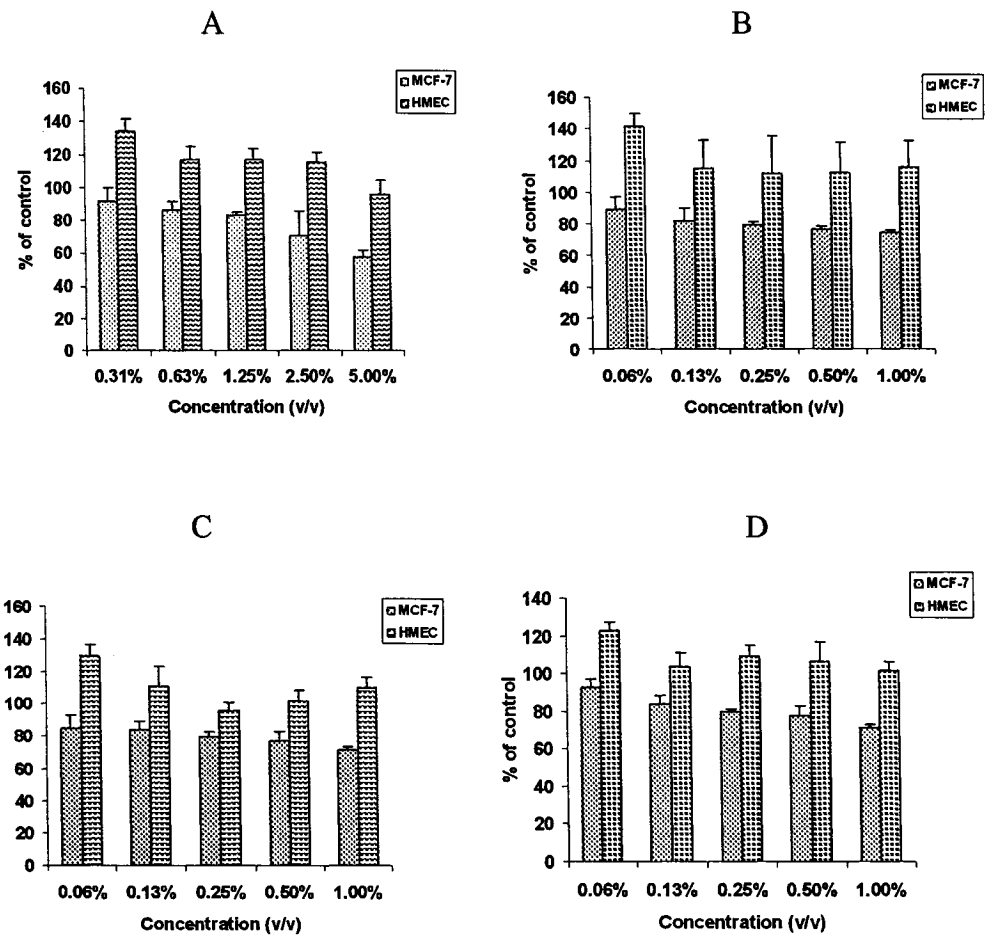
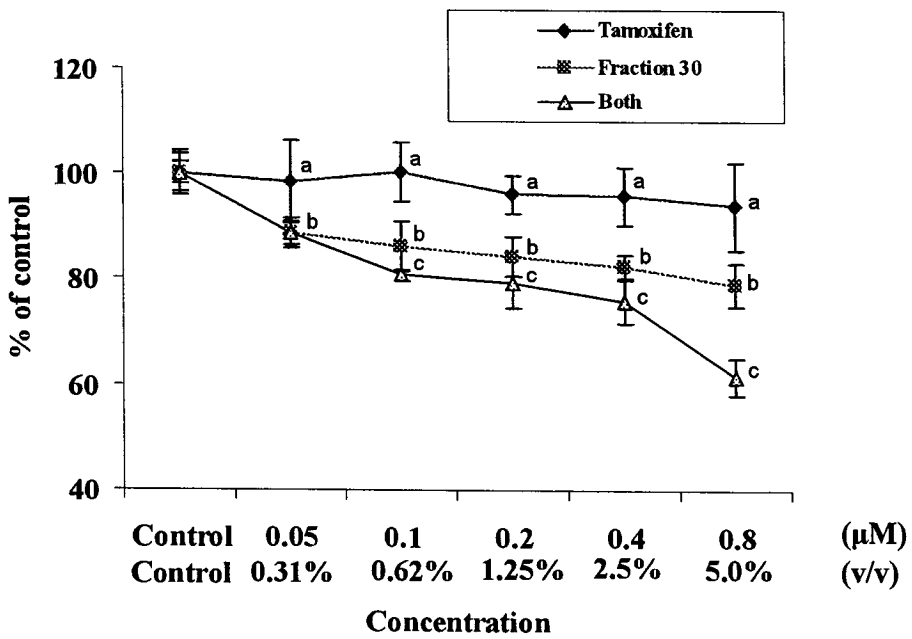

A
B
C
D
MCF-7
HMEC
% of control
Concentration (v/v)
0.31%
0.63%
1.25%
2.50%
5.00%
0.06%
0.13%
0.25%
0.50%
1.00%

Figure 3.12 Antiproliferative effect of RP-HPLC Fraction 30 of kefir alone or in combination with tamoxifen on MCF-7 cells. Means (n=4) with the same letter are not significantly different at $P < 0.05$ for comparison among treatments at the same dose level.


Tamoxifen
Fraction 30
Both
120
100
80
60
40
% of control
a
b
c
Control 0.05 0.1 0.2 0.4 0.8 (μM)
Control 0.31% 0.62% 1.25% 2.5% 5.0% (v/v)
Concentration

Electrospray mass spectrometry was used to determine the molecular weight profile of the active Fractions 29, 30, 34, and 37. Figure 3.13 is the ESI-mass spectrum of HPLC fraction 30. The spectrum represents a typical molecular ion profile of the active HPLC fractions (29, 30, 34 and 37). The similarity in the molecular ion profile of the active fractions indicated that the active component(s) is spread over HPLC Fractions 30 to 37. The spectrum showed mainly singly charged molecular ions with m/z ratios ranging from 300 to 900. The peaks appearing below m/z 300 were found to be fragments of the peaks with m/z ranging from 300 to 900.

To characterize the composition and chemical structure of the active components in the HPLC fractions, molecular ions ranging from m/z 300 to 900 were subjected to collision ion dissociation (CID) tandem mass spectrometry. Figure 3.14A, showed the CID spectrum of the molecular ion at m/z 821. Fragmentation of the molecular ion at m/z 821 produced a neutral loss of 342 mass units, which corresponds to the loss of a lactose moiety. It also produced a series of neutral loses of 60 mass units, which correspond to the loss of acetic acid moieties. The neutral loss of 60 atomic mass units may also be derived from sugar cleavage products (Figure 3.15). The CID spectrum of the molecular ion at m/z 813 (Figure 3.14B) also produced a neutral loss of 342 (lactose) and neutral losses of 120 atomic mass units, which may be derived from fragmentation of lactose (Figure 3.15). A neutral loss of 162, which corresponds to the loss of a hexose moiety (glucose or galactose) is also observed. This was also observed at m/z 759. The fragmentation patterns of the molecular ions at m/z 821 and 813 indicate that they have similar structural features.

Figure 3.13 Mass spectrum of preparative RP-HPLC Fraction 30 done by electrospray mass spectrometry. The mainly singly charged molecular ions with m/z ratios range from 300 to 900. The peaks appearing below m/z 300 were found to be fragments of the peaks with m/z ranging from 300 to 900.

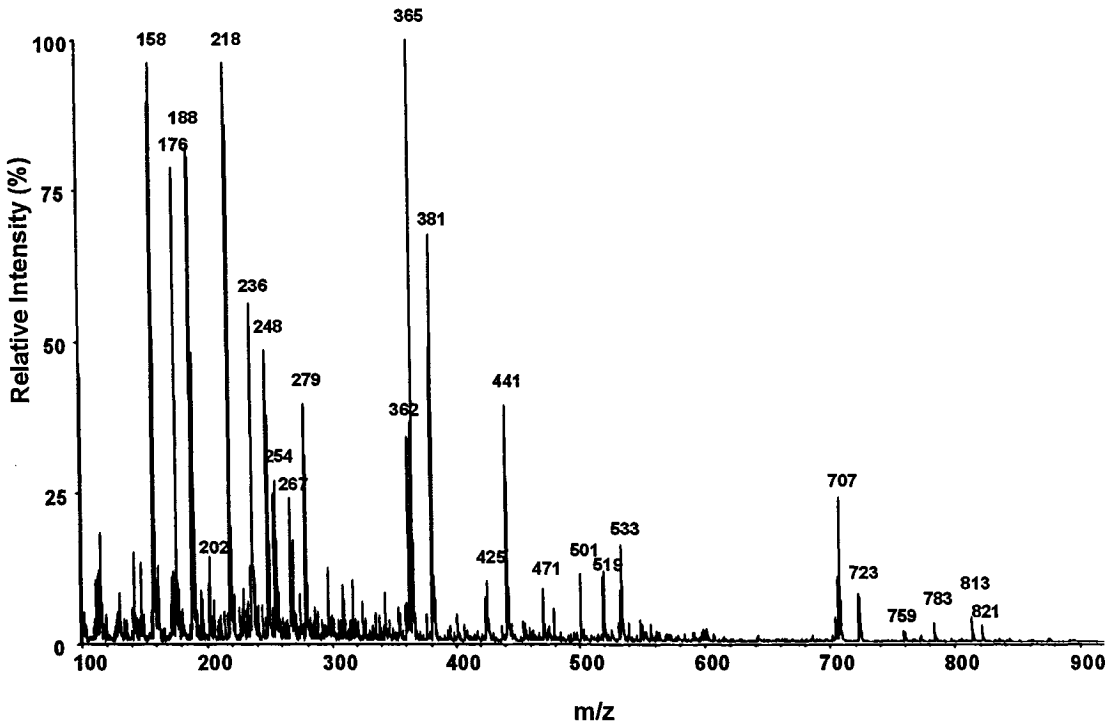

Relative Intensity (%)
100
75
50
25
0
158
218
365
188
176
381
236
248
279
362
441
254
267
707
202
425
471
501
519
533
723
759
783
813
821
100
200
300
400
500
600
700
800
900
m/z

Figure 3.14 Spectrum of molecular ion at m/z = 821 (A), m/z=813 (B), m/z= 759 (C), m/z=723 (D), and m/z=707 (E) done by collision ion dissociation (CID) tandem mass spectrometry. As indicated in figure A, fragmentation of the molecular ion at m/z 821 produced a neutral loss of 342 and 60 mass units, which corresponds to the loss of a lactose moiety and acetic acid moiety respectively. There is also a neutral loss of 98, which could not be ascribed to a known structure. Ion at m/z =261 was also observed. Fragmentation of the molecular ion at m/z 813 produced a neutral loss of 342 and 120 mass units, which might be derived from lactose and hexose moieties (glucose or galactose) respectively. Fragmentation of the molecular ion at m/z 759 produced a neutral loss of 342 and serial loss of 60 mass units. Fragmentation of the molecular ion at m/z=723 produced neutral losses of 342 and 120 atomic mass unit. The main fragment is the ion at m/z 365 with the neutral loss of 342 (lactose) from m/z 707.

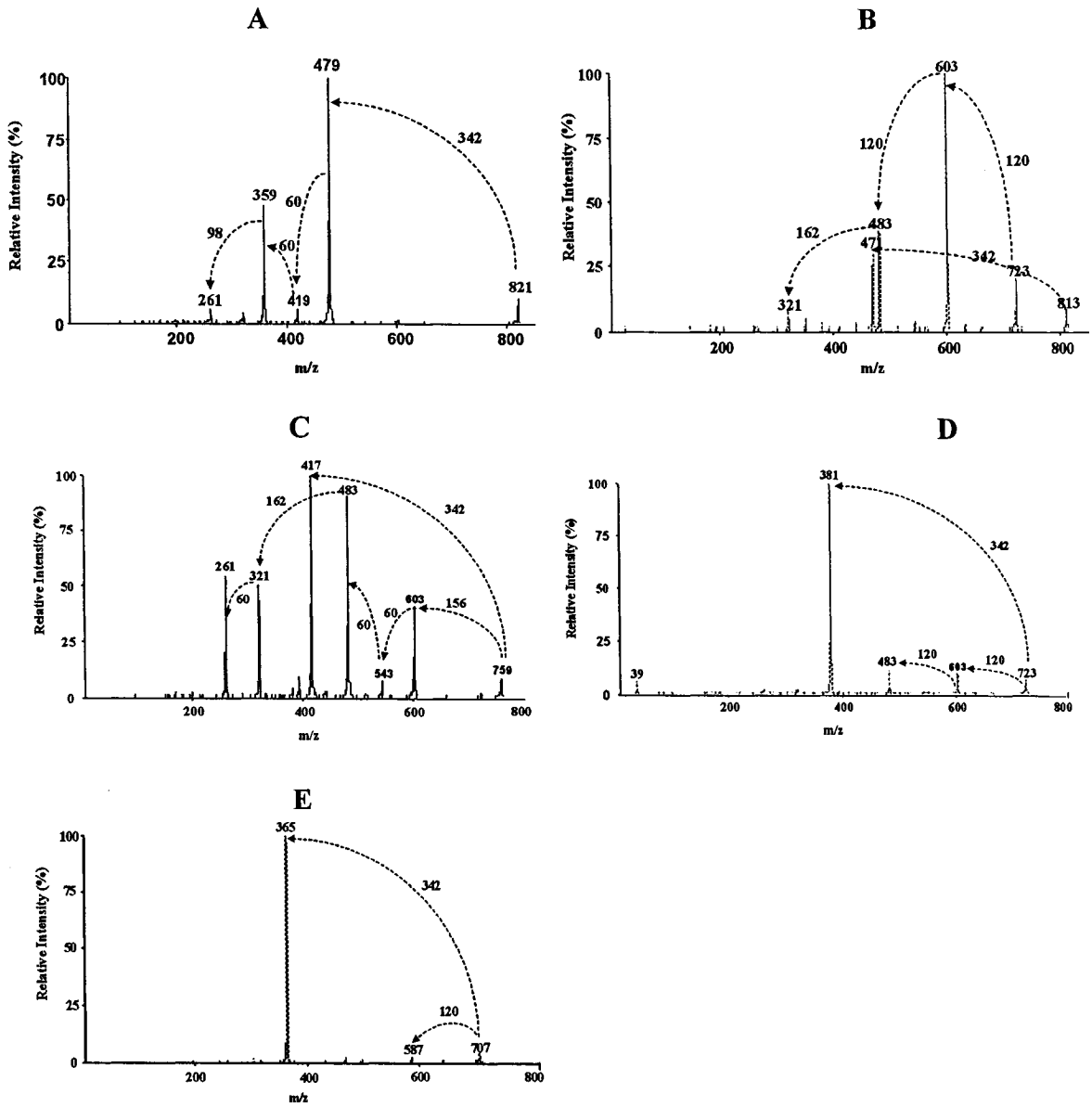

A
B
C
D
E
Relative Intensity (%)
m/z
479
342
359
60
98
60
261
419
821
603
120
120
162
483
471
342
723
321
813
417
483
162
342
261
321
60
603
156
60
60
543
759
381
342
39
483
120
603
120
723
365
342
120
587
707

Figure 3.15 Scheme 1: Generation of neutral loss of 60 and 120 atomic mass unit from fragmentation of lactose. Fragmentation at "a" and "b" will generate a neutral loss of 60 and fragmentation at "a" and "c" will generate a neutral loss of 120 atomic mass unit. They can be derived from fragmentation of acetic acid and lactose.

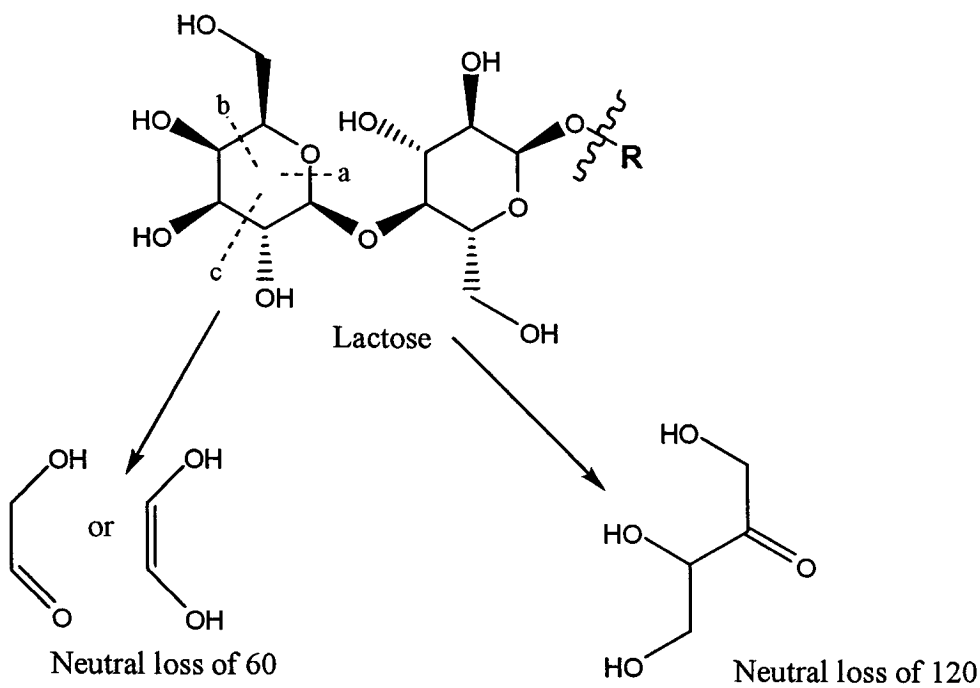
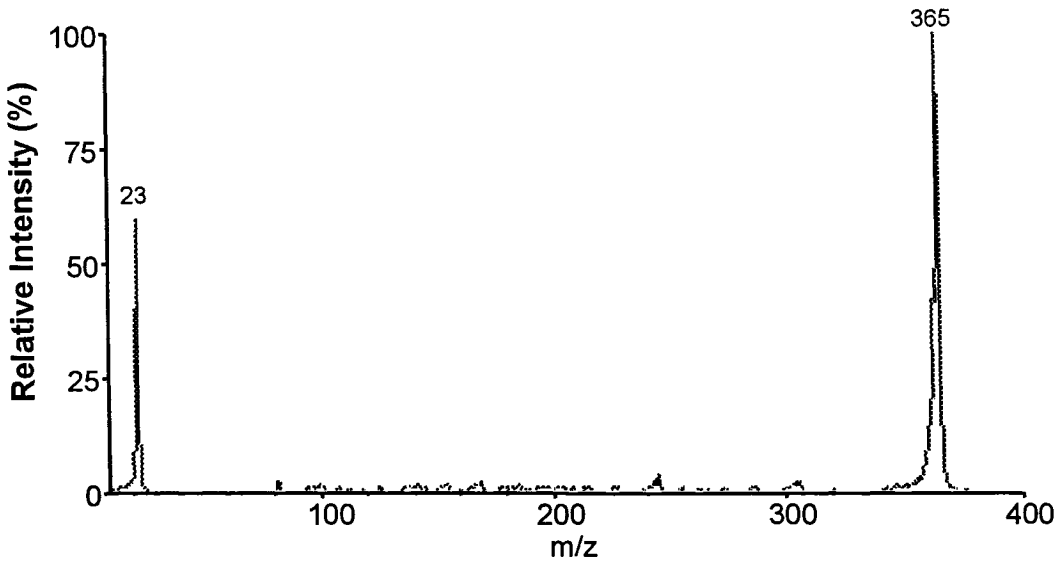

HO
b
HO
HO
c
OH
O
a
O
HO
OH
O
O
R
OH
Lactose
OH
OH
or
O
OH
Neutral loss of 60
HO
HO
O
HO
Neutral loss of 120

Figure 3.16 Spectrum of molecular ion at m/z 365 and 381 done by collision ion dissociation (CID) tandem mass spectrometry. The peaks at m/z at 365 and 381 represent the sodium and potassium ions of lactose respectively,


Relative Intensity (%)
100
75
50
25
0
23
365
100
200
300
400
m/z

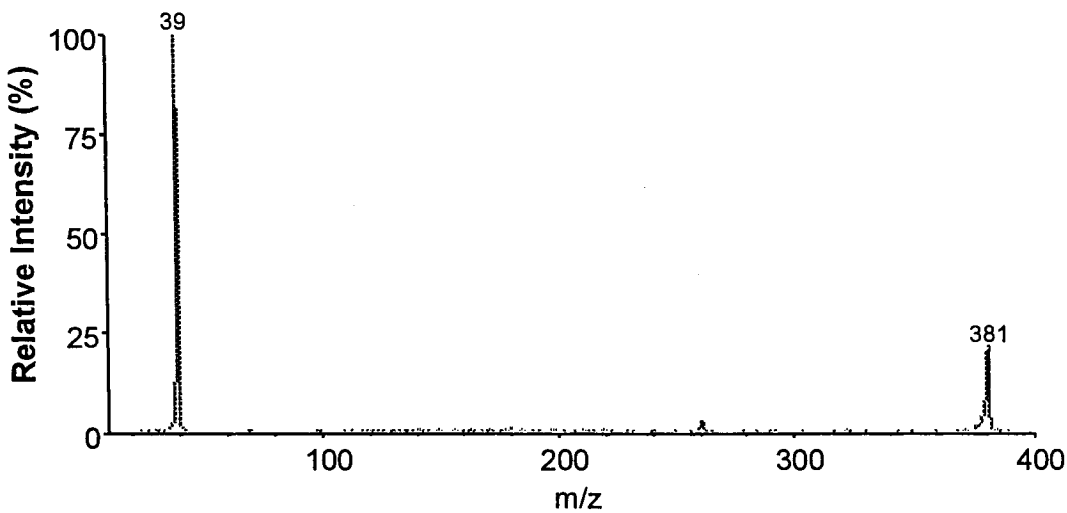

Relative Intensity (%)
100
75
50
25
0
39
381
100
200
300
400
m/z

Figure 3.17 Scheme 2: A possible interaction for the generation of the ion at m/z 759. As shown in the scheme, the interaction between the amino group of serine and the reducing end of lactose is likely a chemical reaction similar to the Maillard reaction, and the interaction between the hydroxyl groups of lactose and the carboxyl group of serine is a simple esterification reaction. The reactions, particularly the esterification reaction may also be driven biochemically by esterases.

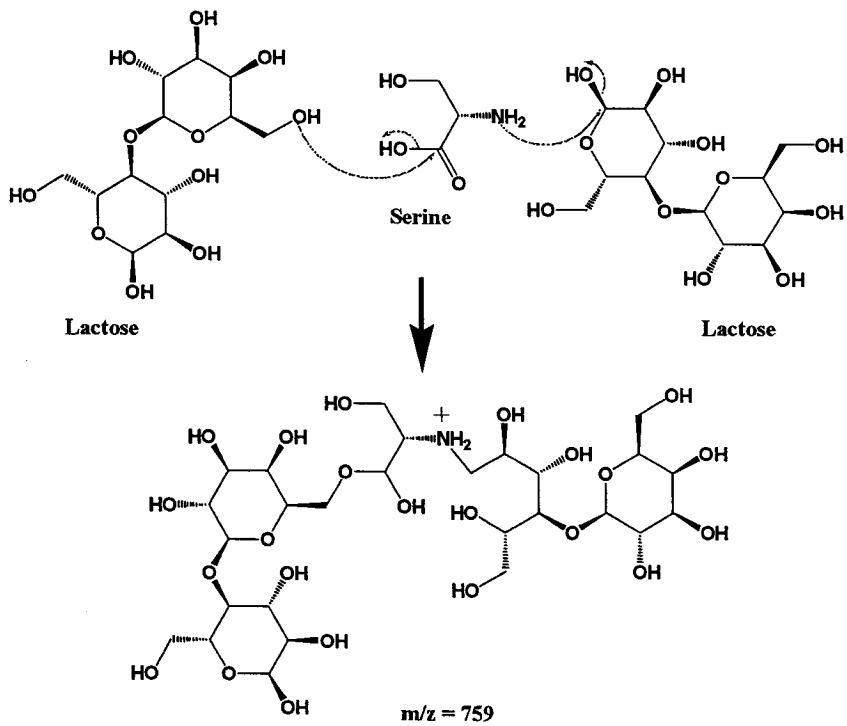
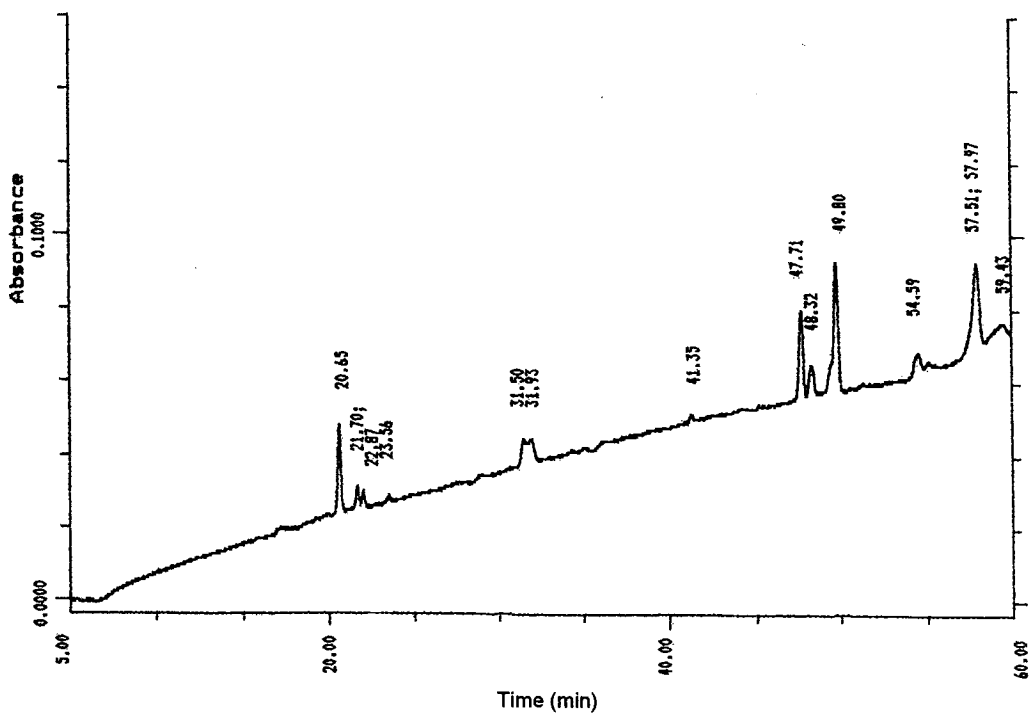

Lactose
Serine
Lactose
m/z = 759

Figure 3.18 Chromatograph of preparative HPLC Fraction 30 done by RP-HPLC. 50 µl of reconstituted preparative HPLC Fraction 30 which showed antiproliferative effect on MCF-7 cells was injected into a Prosphere 300 C4 column (5µm, 250 mm X 4.6 mm) (Alltech Associate, Inc. Deerfield, IL 60015, USA) on Beckman HPLC System and eluted with with a linear gradient of Buffer A (0.1% TFA in water) and Buffer B (60% of Acetonitrile in 0.1% TFA: 40% of 0.1% TFA in water) as follows: 0 to 60 min, 0 to 90% B; and 61 to 65 min, 90 to 0% B. Absorbance was monitored at channel A 210 nm.


Absorbance
0.1000
0.0000
20.65
21.70;
22.87
23.56
31.50
31.93
41.35
47.71
48.32
49.80
54.59
57.51; 57.97
59.45
5.00
20.00
40.00
60.00
Time (min)

The molecular ions with m/z 707 (Figure 3.14E) and 723 (Figure 3.14D) represent sodium and potassium ion dimmers of lactose, respectively. The peaks at m/z at 365 and 381 represent the sodium and potassium ions of lactose respectively (Figure 3.16).

Another interesting feature about the fragmentation pattern of with m/z ranging from 707 to 823 is that they ragmented into common fragment ions at m/z 261 and 98. Further isolation and fragmentation (MS-MS) of the ion at m/z 261 showed that its major product ion is the ion at m/z 98. Further isolation and fragmentation of the ion at m/z 98 produced two ions at m/z 54 and 39. The tandem mass spectrometric analysis of the molecular ion of the active HPLC fraction show that most of the peaks produced a stable product ion at m/z 98, but the ion at 98 could not be ascribed to known structure peptide.

Analysis of the fragmentation pattern of the molecular ion at m/z 759 (Figure 3.14C), however, reveal that the molecular ion could be derived from the interaction between serine and lactose. A possible scheme for the generation of the ion at m/z 759 is presented in Scheme 2 (Figure 3.17). The proposed structure agrees with the fragmentation pattern. This interaction may be due to chemical reactions. The interaction between the amino group of serine and the reducing end of lactose is likely a chemical reaction similar to the Maillard reaction, and the interaction between the hydroxyl groups of lactose and the carboxyl group of serine is a simple esterification reaction. The reactions, particularly the esterification reaction may also be driven biochemically by esterases.

The active fraction likely still contains a mixture of chemicals as suggested by the results of RP-HPLC (Figure 3.18). The exact molecular structure of these compounds is not known. However, results of the extensive MS analysis suggested that the compounds are possibly complex polymers that have lactose as the backbone structure. The proposed structure shown in Scheme 2 is a product with two lactose units linked by a serine. A more complex molecule with linkage with acetic acid is also possible.

The bioactive fraction we isolated showed antiproliferative effect on cancer cells while not on normal cells. As indicated in the RP-HPLC chromatograph, the

isolated fractions such as Fraction 30 are likely composed of multiple components that may be acting either singly or synergistically to exert an effect. Although the presence of peptides were suggested in the fractions as determined by the OPA methodology, the MS data did not support the presence of with known peptide structures. The failure to identify any known peptides is likely due to the limitation of the OPA method, which can only detect the presence of primary amines and thus the primary amines in peptides cannot be differentiated from other amine containing compounds. One of the main components is composed of lactose and acetic acid possibly an end-product of fermentation of the milk protein and sugar by the kefir bacteria and yeast. The lactose polymer suggests that the active component may be fragments of previously isolated polysaccharides termed as kefiran (La Riviere, 1967). Kefiran is a water-soluble polysaccharide consisted of approximately equal proportions of D-glucose and D-galactose (Kooiman, 1968; Micheli *et al.*, 1999). Kefiran has been reported to have antitumor activity (Shiomi *et al.*, 1982; Murofushi *et al.*, 1983). It was also shown to be one of the substances having few or no side effect(s) in functioning to retard tumor growth in vivo.

It is noteworthy that one of the proposed novel bioactive structures contains serine and glucose or galactose; hence, it is conceivable that this molecule could be a sphingolipid compound. The ceramide component of sphingolipids is derived from serine and sphingolipids such as cerebrosides, contain either a glucose or galactose. Sphingolipids such as gangliosides are sphingosine compounds that contain several glucose or galactose units. Also, lower molecular weight ceramide moieties have been detected in dairy gangliosides (Colarow *et al.*, 2003) and cultured dairy products have been shown to be a rich source of gangliosides (Kathleen *et al.*, 2000)

Ceramide is derived from sphingomyelin, and can act as an intracellular second messenger for tumor necrosis factor-alpha, IL-1beta, and other cytokines. Ceramide has also been implicated in the acquired drug resistance that often characterizes breast cancer cells (Liu *et al.*, 1999). C2 and C6 ceramides are cell permeable ceramide analogs that have been shown to induce cell apoptosis

(Fillet *et al.*, 2003). Bansode *et al.* (2004) reported that incubation of milk-based C6 ceramide in MCF-7 cells at 1 μM decreased cell migration, inhibited cell proliferation, caused cell death by apoptosis, and reduced the levels of angiogenesis stimulators VEGF and cathepsin D to below 50% of the control. Osada *et al.* (1993) demonstrated that kefir contains an active substance, which enhances IFN-beta secretion of a human osteosarcoma line MG-63 treated with a chemical inducer, poly I: poly C. The active substance in the fermented milk was identified to be sphingomyelin (SpM). SpM from fermented milk (F-SpM) was a mixture of four molecular species of SpMs having C21-, C22-, C23- and C24-fatty acids. F-SpM enhanced the IFN secretion 14 times, SpMs from other food sources also enhanced IFN secretion but more moderately (2-3 times). We observed a synergistic effect when MCF-7 cells were treated with ceramide analogs in the presence of extract of mother culture (data not shown). RP-HPLC fraction 30 significantly increased the susceptibility of MCF-7 cells to Tamoxifen. The putative sphingolipid component(s) in kefir that we have detected may have acted synergistically with the ceramide analogs leading to apoptosis of MCF-7 cells. In support of this concept, sphingomyelin has been shown to enhance the ceramide formation and ceramide-induced apoptosis in concert with the chemotherapeutic agent, gemcitabine, in human pancreatic cancer cells (Modrak *et al.*, 2004). Modification of ceramide metabolism also increases MCF-7 cells sensitivity to cytotoxics (Lucci *et al.*, 1999). An elevated TNF-alpha level has been observed after the MCF-7 cells were treated with kefir fractions (report in another paper). TNF-alpha in conjunction with tamoxifen produced synergistic inhibition of MCF-7 cells in vitro (Borsellino *et al.*, 1994; Saftri and Bonavida 1992; Cimoli *et al.*, 1993). The potential of using the kefir extract as co-drug for chemotherapy should be explored.

We have isolated the active components from kefir mother culture that show antitumor cell proliferative effects. The MS data indicate that the isolated compounds are likely sphingomyelin complexes such as gangliosides that might have activities that are similar to that of ceramide. The biological functions of this ceramide-like compounds warrant further studies.

CHAPTER 4

INTRODUCTION TO CHAPTER 4

In our previous study, extract of kefir mother culture was fractionated with molecular weight cut-off method, SEC-HPLC and RP-HPLC. All the fractions were screened for antiproliferative effects on MCF-7 cells. The results have shown that the fraction of MWCO less than 3000 Da, Fraction 7 of SEC-HPLC, Fractions 29, 30, 34, and 37 of RP-HPLC all showed a dose-depend antiproliferative effect on MCF-7 cells. Due to the complex mixture of components in the specific fractions and the whole kefir extracts , the role of specific components such as proteins, peptides, sphingolipids, organic acids and some small molecules, such as free amino acids, oligosaccharides and their interactions is not clear. For example, further characterization analyses showed the fraction of MWCO less than 3000 Da was still a mixture of molecules whereby Fraction 7 contains about seven components and Fraction 30 was identified to potentially contain sphingomyelin complexes such as gangliosides. Sphingomyelin complexes have been demonstrated to interact synergistically with ceramides to affect tumor cellular viability. Hence, the following study was designed to assess the impact of the combination of exogenous ceramides together with kefir fractions to further study possible mechanisms involved in the antiproliferative effect of kefir.

MANUSCRIPT C

Kefir Fractions Enhance the Antiproliferative Effects of Exogenous Ceramides and Alter Glutathione, and Tumor Necrosis Factor-alpha in MCF-7 cells

Chujian Chen, Stan Kubow and Hing Man Chan

ABSTRACT

Although kefir has been showed to have antimutagenic and antitumor properties, the mechanism(s) of action remains speculative. In the present study, an in vitro cell culture model using human breast cancer cells (MCF-7) and human mammary epithelial cells (HMEC) was used to study the antiproliferative effects of different fractions of kefir mother culture either alone or in the presence of exogenous ceramides. Three fractions containing the active components in kefir were isolated using molecular cut-off filters (<3000 Da), size-exclusion high performance liquid chromatography (SEC-HPLC) and reverse phase-HPLC. Results indicated that the whole extracts and three fractions inhibited MCF-7 growth in a dose-dependent manner. While the whole kefir extract decreased glutathione (GSH) concentration in MCF-7 cells, the three isolated fractions all increased GSH production ($P < 0.05$). There were no such effects observed on HMEC cells. On the other hand, all fractions induced tumor necrosis factor-alpha (TNF-alpha) production. Fractions of kefir also enhanced ceramides toxicity on MCF-7 cells. The observed antiproliferative effects of kefir fractions on MCF-7 cells may mainly be explained by the induction of TNF-alpha.

Key words: kefir, antiproliferative effect, ceramide, glutathioine, tumor necrosis-alpha

INTRODUCTION

Although many research studies have demonstrated the antimutagenic and antitumor properties of kefir (Cevikbas *et al.*, 1994; Biffi *et al.*, 1997;

Matar *et al.*, 1997; Furukawa *et al.*, 1990 and 2000; Nadathure *et al.*, 1994; Van't Veer *et al.*, 1989), the exact mechanism(s) are still unknown. A possible mechanism of anti-cancer action is that bioactive components present in kefir after absorption could affect target tissues or organs via different pathways, including modulation of cellular levels of glutathione (Russo *et al.*, 1986; Roberts *et al.*, 1991; Baruchel and Viau, 1996) and cytokines such as TNF-alpha (Smith *et al.*, 1994), which play an important role in apoptosis and cellular proliferation.

Glutathione (GSH) is critical in a variety of cellular defense functions including protection from toxic oxygen species and detoxification of various xenobiotics due to its participation directly in the destruction of reactive oxygen compounds and detoxification of foreign compounds (Fang *et al.*, 2002). For example, Baruchel and Viau (1996) have shown that incubation of whey protein concentrate with normal lymphocytes was associated with an increase in cell proliferation that was accompanied by an increase in cellular GSH levels. On the other hand, when whey protein concentrates were cultured with rat mammary tumor or Jurkat T tumor cells, cellular proliferation was inhibited in association with a decrease in cellular GSH concentrations. Therefore, the selective modulation of GSH concentrations in normal versus tumor cells could play a functional role in affecting cellular proliferation in normal and tumor cells.

TNF-alpha induces intracellular signals that mediate cell death. One major signal induced by TNF-alpha is activation of neutral and acdic sphingomyelinases, which catalyze the degradation of sphingomyelin to ceramide (Hannun, 1996). Ceramide has been proposed to be an important intracellular second messenger for many of the early cellular response elicited by TNF-alpha (Cai *et al.*, 1997; Schutze *et al.*, 1994; Verheij *et al.*, 1996; Westwick *et al.*, 1996). One of the most clearly illustrated targets of ceramide is cell death, and different ceramide molecules may act differently (Obeid *et al.*, 1995; Okazaki *et al.* 1998; Selzner *et al.* 2001; Fillet *et al.* 2003). Modification of ceramide metabolism increases cancer cell sensitivity of cytotoxic agents, which depends on cancer cell type (Lucci *et al.*, 1999). Lucci *et al.* (1999) demonstrated that MCF-7 adriamycin resistant cells were insensitive to ceramide, whereas MCF-7 wild-type cells were

sensitive. Blocking ceramide glycosylation potentiated cellular sensitivity to ceramide and to chemotherapeutic drugs in MCF-7 cells. Hayter *et al.* (2000) found that TNF-alpha induced a depletion of GSH, and exogenous addition of GSH blocked TNF-induced sphingomyelin hydrolysis as well as TNF-induced cell death in L929 cells. Dairy products are a rich source of sphingomyelin complexes. Sphingomyelin purified from powdered milk has been shown to reduce chemical induced colon tumors and suppress the conversion of adenomas to adenocarcinomas in mice (Dillehay *et al.*, 1994; Schmelz *et al.*, 1996). In vitro cell culture also demonstrated that induction of ceramide by hexadecylphosphocholine (HePC), a phospholipids analog, leads to cell apoptosis and membrane-permeable ceramides additively increased the apoptotic effect of HePC (Wieder *et al.*, 1998).

Components in fermented milks such as conjugated linoleic acid (CLA) (Schonberg *et al.*, 1995), sphingolipids (Dillehay *et al.*, 1994; Schmelz *et al.*, 1996), polysaccharides (Shiomi *et al.*, 1982), organic acids (Garrote *et al.*, 2000), and some proteins and peptides (Svensson *et al.*, 1999) have been proposed to be the bioactive antic-cancer compounds. Our previous work has demonstrated that whole extract and some fractions of kefir mother culture showed a dose-dependent antiproliferative effect on MCF-7 cells, but the mechanism is still unexplained. Analysis of bioactive fractions via MS-MS suggested the presence of sphingomyelin complexes such as gangliosides. In addition, Osada *et al.* (1993) has indicated that kefir contains unique sphingomyelins that enhance markedly greater secretion of the anti-proliferative cytokine, IFN-beta, in human osteosarcoma cells than sphingomyelins from other sources.

In the present study, an in vitro cell culture model using MCF-7 cells and HMEC cells was used to study the antiproliferative effects of different fractions of kefir mother culture in the presence of exogenous ceramides on concentrations of intracellular GSH and extracellular TNF-alpha.

MATERIALS AND METHODS

Kefir samples were provided by Liberte Inc. (Brossard, Canada). Three different batches were used. Upon receive of samples, they were well stirred, and centrifuge at 4 °C, 15000 rpm (32000 x g), for 60 min (Sorvall RC 5C Centrifuge, rotor ss-34, Sorvall Instruments, Wilmington, USA). The supernatant was filtrated with a 0.45 μm membrane filter followed by a 0.2 μm filter. The filtrates were stored at -80 °C for future use. Ceramide 1-phosphate, and C6 ceramide were purchased from Sigma (Sigma, St Louis, MO, USA). Ceramide I from bovine brain was obtained from Alexis Corporation (San Diego, USA).

Molecular weight cut-off fractionation (MWCO)

CENTRIPLUS Centrifugal filter devices were used to get MWCO fractions at 3000 Dalton cut-off (Millipore, Bedford, USA). Ten milliliters of whole extract were loaded to the sample reservoir and the assembled device was centrifuged at 4 °C, 3000 x g for 290 min. The filtrates were collected for further analysis.

Size exclusion High Performance Liquid Chromatography (SEC-HPLC) separation

One hundred microliter of kefir filtrate was injected into a TSK G2000SWXL 78 mm × 30 mm column (SUPELCO, Bellefonte, USA) and separated with a Shimadzu LC-6AD Liquid Chromatograph system (Shimadzu Scientific Instruments, Inc. Columbia, USA). Peaks were detected at 210 nm with UV detector. Separation buffer was 45% Acetonitrile in 0.1% Trifluorocetic acid (TFA) with a flow rate of 0.4 ml/min for 40 minutes. Nine fractions were collected for each run, and fractions from five to ten runs were pooled. The above nine fractions were concentrated with N_2 and then freeze dried, stored in -80°C for further cell culture testing. Only the fraction 7 (SEC-HPLC Fraction 7), which showed a dose dependent antiproliferative effect on MCF-7 cells (results reported in separate paper) was used for exploration of mechanisms of antiproliferative action in this study.

Preparative High Performance Liquid Chromatography (Pre-HPLC) fractionation

Three batches of extracts of kefir were pooled and fractionated with MWCO 3000 Da device. The fraction of MWCO less than 3000 Da was freeze-dried by

using a FLEXI-DRY MP Freeze dryer (FTS Systems, Inc., Stone Ridge, USA). Five g of the lyophilized MWCO fractions were dissolved in 20 mL of water. Ten milliliters of reconstituted solution were loaded on a C_4 preparative column (300 mm × 50 mm, 300 Å, 5 μm) (Vydac Company, Herperia, CA) and separated with a Water Delta Prep 4000 HPLC system (Waters Corporation, Milford, USA). After the column was equilibrated with buffer A (0.6% Acetic acid in water) at a flow rate of 13 mL/min, the fractions were eluted with a linear gradient of Buffer A and Buffer B (0.6% Acetic acid in acetonitrile) as follows: 0 to 70 min, 0 to 60% B; 70 to 80 min, 60 to 70% B; 80 to 100 min, 70 to 80% B; 100 to 105 min, 80 to 0% B.

A total of 100 fractions were collected between 0 and 100 min, with samples taken at 1-min intervals. The fractions were then lyophilized and kept at −80°C for further cell culture and analysis. The fractions were reconstituted by adding water. Peptide and protein concentrations in the reconstituted fractions were determined by the method of Church *et al.* (1983) before cell culture. Only the fraction 30 (RP-HPLC Fraction 30) which showed a dose dependent antiproliferative effect on MCF-7 cells (results reported in separate paper) was used for exploration of antiproliferative mechanisms of action in the present study.

Screening for antiproliferative effects

MCF-7 cells were purchased from ATCC (ATCC, Manassas, USA). Cells were routinely propagated as a monolayer culture in Dulbcco's Modified Eagle Medium (DMEM) (Gibco, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (HI-FBS) (Gibco, Grand Island, USA), in a 75-cm^2 plastic dish at 37 °C in a humidified atmosphere with 5% CO_2, and passaged every 3-4 d. A normal human mammary epithelial cell line (HMEC) was kindly provided by Dr. M. R. Stampfer (Lawrence Berkeley National Laboratory, Berkeley, USA). Cells were routinely propagated as a monolayer culture in Mammary Epithelial Growth Media (MEGM, Clonetics, San Diego, USA) supplemented with 10% HI-FBS, in a 75-cm^2 plastic dish at 37°C in a humidified atmosphere with 5% CO_2, and passage every week. For the

experiments, both cells were harvested from the dish using 0.25% trypsin-EDTA solution (Sigma, St Louis, USA).

Cell proliferation experiments in 24-well plates

Cells previously harvested were seeded in 24-well plates, i.e., 10,000 cells for MCF-7 per well in DMEM supplemented with 10% FBS and 5,000 cells for HMEC per well in MEGM supplemented with 10% FBS. The cells were allowed to attach for 24 h. After that period, old media were removed and fresh media containing different dosages of fractions of kefir, ceramides (Ceramide I, ceramide 1-phosphate, and C6 ceramide), and the combination of fraction and ceramide respectively were added to each treatment well. Control wells were incubated with the culture medium with the dosing vehicle Dulbecco's Phosphate Buffered Saline (PBS) or medium containing 0.001% (v/v) ethanol. The final concentrations for mother culture fraction in cell culture medium were 5%, 2.5%, 1.3%, 0.6%, and 0.3% (vol/vol) respectively; for ceramide I were 5, 10, 20, 40, 80 μM; ceramide 1-phosphate were 0.1, 0.5, 1, 5, 10 μM; C6 ceramide were 2 μM, 4 μM, 6 μM, 8 μM, 10 μM respectively. Quadruplicates were done for each dose. To standardize results, each plate had its own control. PBS buffer was added to the culture media to keep pH between 7.0 and 7.4. Cells were incubated at 37°C in a humidified atmosphere with 5% CO_2. Six days later, old media were collected, centrifuged at 4 °C, 4000 rpm for 10 min and the supernatant were stored at -80°C for TNF-alpha analysis. Cells were rinsed with 1 mL cool PBS containing 1 mM EDTA (Gibco BRL, Grand Island, NY) twice, and then harvested with a rubber policeman. Cells from the same treatment were combined. The final volume for each treatment was 2 mL. The cells were counted using the trypan blue method. For each treatment, the antiproliferative effects were expressed as a percentage of control.

The cell samples were sonicated with a sonicator, and then centrifuged at 10,000 × g for 15 min at 4°C. The supernatant was collected and stored on ice. A equal volume of MPA reagent (dissolve 5 g of metaphosphoric acid in 50 mL water) was added to the sample and the mixture was vortexed. The the mixture was allowed to stand at room temperature for 5 min before centrifuged at

1750 × g for 10 min. The supernatant was carefully collected without disturbing the precipitate, and then stored at –80°C for GSH analysis.

TNF-alpha was determined with a High Sensitivity TNF-Alpha assay kit (Amersham Biosciences, Buckinghamshire, UK). GSH was analyzed using a assay Kit obtained from Cayman Chemical (Cayman Chemical Company, Ann Arbor, USA).

Cell proliferation experiments in 96-well plates

Cells previously harvested were seeded in a 96-well plates at 1,000 cells per well for MCF-7 in DMEM supplemented with 10% FBS and for HMEC in MEGM supplemented with 10% FBS. The cells were allowed to attach for 24 hr. After that period, old media were removed and fresh media and different treatments were added to each well. Serial dilutions of each test fractions and ceramides treatments (same as for 24-well plate) were made to study the dose-response. PBS was added to the culture media to keep the final pH between 7.0 and 7.4. Cells were incubated at 37°C in a humidified atmosphere with 5% CO_2 for 6 days and the cell numbers in each well were determined by using a CellTiter 96® Aqueous One Solution Cell Proliferation Assay kit (Promega, Madison, USA). Each sample was run in quadruplicate. Control cells were incubated with the culture medium with the dosing vehicle (PBS). To standardize results, each plate had its own control and cell proliferation was expressed as the percent of control.

Statistical analysis

The antiproliferative effects of fractions of kefir or fractions in combination with ceramide were determined by counting cell numbers after treatment and expressing results as a percentage of control from different treatments and doses. A two-way ANOVA was used to analyze the effects of treatments and doses. Differences among doses and treatments were determined by the Student-Newman-Keuls (SNK) multiple comparison test. Correlations among GSH, TNF-alpha and cell number after MCF-7 and HMEC cells were treated with different fractions of kefir were also analyzed. Statistical significance was considered at

$P < 0.05$. All statistics test were performed using SAS 8.2 for PC (SAS, Cary, NC).

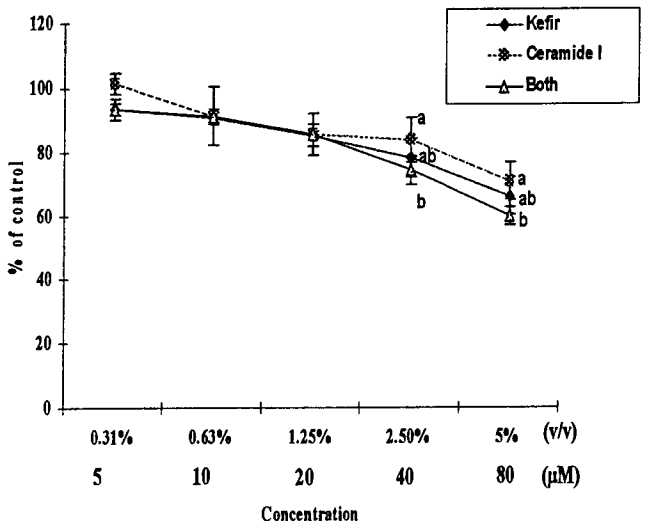
RESULTS

MCF-7 cells were treated with different fractions of kefir alone or in the presence of ceramide I or ceramide 1-phosphate or C6 ceramide. Extracts of kefir, the MWCO less than 3000 Da fraction of kefir extract, ceramide I from bovine brain and C6 ceramide all independently showed dose dependent antiproliferative effects on MCF-7 cells (Fig. 4.1, 4.2 and 4.3). In combination with both kefir extract and the MWCO less than 3000 Da fraction of kefir extract with ceramide I showed an combined additive antiproliferative effect on MCF-7 cells at a concentration of 2.5% and 5% (v/v) (Figure 4.1). No dose dependent antiproliferative effect was observed when MCF-7 was treated with ceramide 1-phosphate (Figure 4.2). In the presence of ceramide 1-phosphate and kefir extract or MWCO fraction of kefir extract, an enhanced dose dependent antiproliferative effects was observed (Figure 4.2). As shown in Figure 4.3, C6 ceramide had a strong antiproliferative effect on MCF-7 cells. Only about 40% of MCF-7 cells survived after 6 d following incubation with 8 μM/L C6 ceramide. In combination with both the kefir extract and the MWCO less than 3000 Da fraction of kefir extract with C6 ceramide showed an combined additive antiproliferative effect on MCF-7 cells only at the lower doses of 2 and 4μM/L (Figure 4.3). On the other hand, SEC-HPLC Fraction 7 did not enhance the antiproliferative effect of C6 ceramide on MCF-7 cells (Figure 4.4A).

C6 ceramide also depressed the growth of HMEC (Figure 4.4B). Even at the lowest concentration at 2 μM/L, only about 75% of cells survived after 6 d. Fraction 7 significantly reduced the C6 ceramide toxicity on HMEC cells ($P < 0.05$).

Figure 4.1 Antiproliferative effects of extract of kefir (A) and MWCO less than 3000 Da (B) alone or in combination with ceramide I on MCF-7 cells. Values were expressed as Mean±SD, n=4. Means with the same letter are not significantly different for comparison among treatments at the same dose level.

A


Kefir
Ceramide I
Both
% of control
120
100
80
60
40
20
0
a
ab
b
a
ab
b
0.31%
0.63%
1.25%
2.50%
5%
(v/v)
5
10
20
40
80
(μM)
Concentration

B

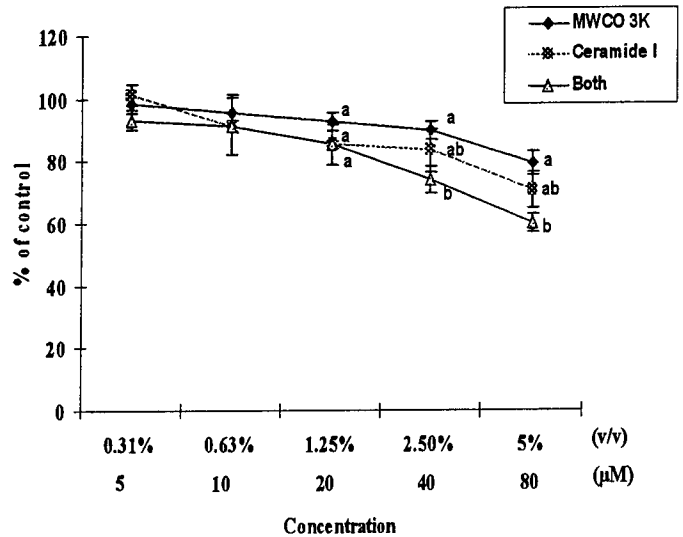
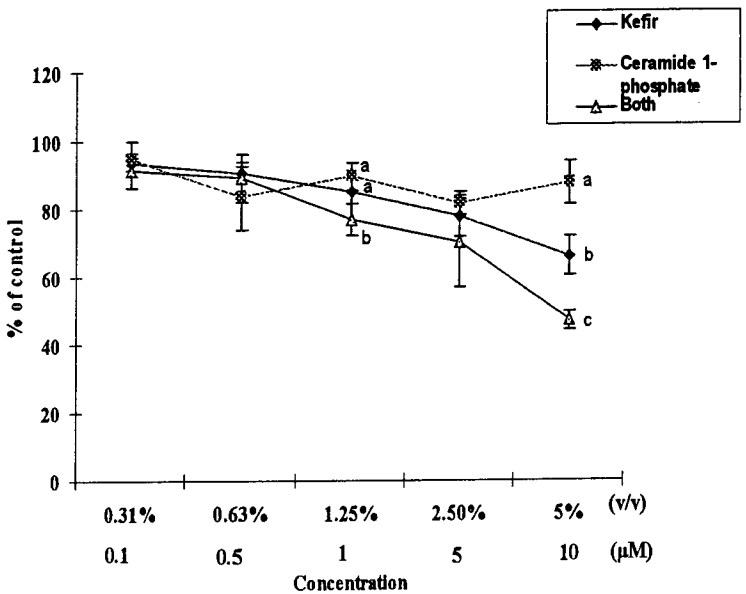

MWCO 3K
Ceramide I
Both
% of control
120
100
80
60
40
20
0
a
a
a
a
ab
b
a
ab
b
0.31%
0.63%
1.25%
2.50%
5%
(v/v)
5
10
20
40
80
(μM)
Concentration

Figure 4. 2 Antiproliferative effects of extract of kefir (A) and fraction of MWCO less than 3000 Da (B) alone or in combination with ceramide 1-phosphate on MCF-7 cells. Values were expressed as Mean±SD, n=4. Means with the same letter are not significantly different at $P < 0.05$ for comparison among treatments at the same dose level.

A


Kefir
Ceramide 1-phosphate
Both
% of control
120
100
80
60
40
20
0
a
a
b
a
b
c
0.31%
0.63%
1.25%
2.50%
5%
(v/v)
0.1
0.5
1
5
10
(μM)
Concentration

B

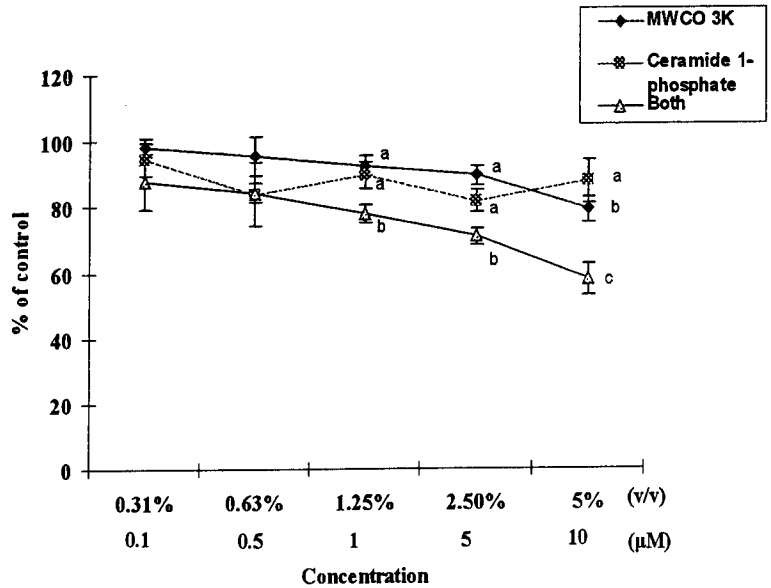
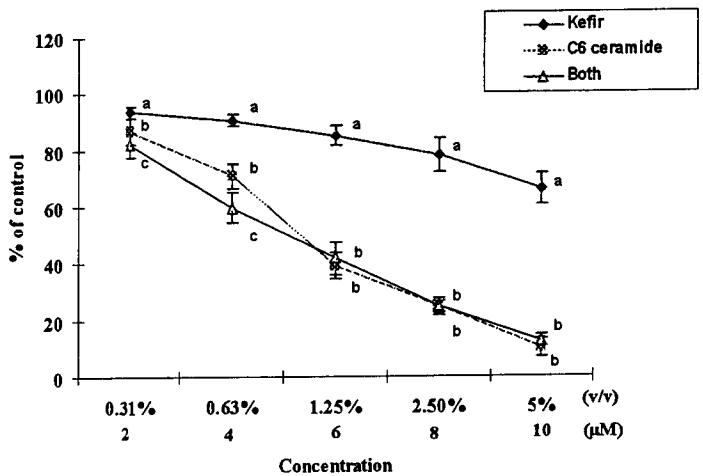

MWCO 3K
Ceramide 1-phosphate
Both
% of control
120
100
80
60
40
20
0
a
a
b
a
a
b
b
c
0.31%
0.63%
1.25%
2.50%
5%
(v/v)
0.1
0.5
1
5
10
(μM)
Concentration

Figure 4.3 Antiproliferative effects of extract of kefir (A) and fraction of MWCO less than 3000 Da (B) alone or in combination with C6 ceramide on MCF-7 cells. Values were expressed as Mean±SD, n=4. Means with the same letter are not significantly different at $P < 0.05$ for comparison among treatments at the same dose level.

A


Kefir
C6 ceramide
Both
% of control
120
100
80
60
40
20
0
a
b
c
0.31%
0.63%
1.25%
2.50%
5%
(v/v)
2
4
6
8
10
(μM)
Concentration

B

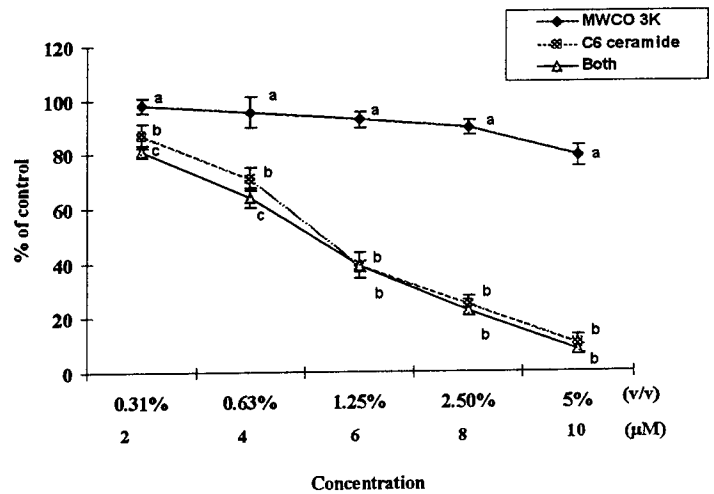
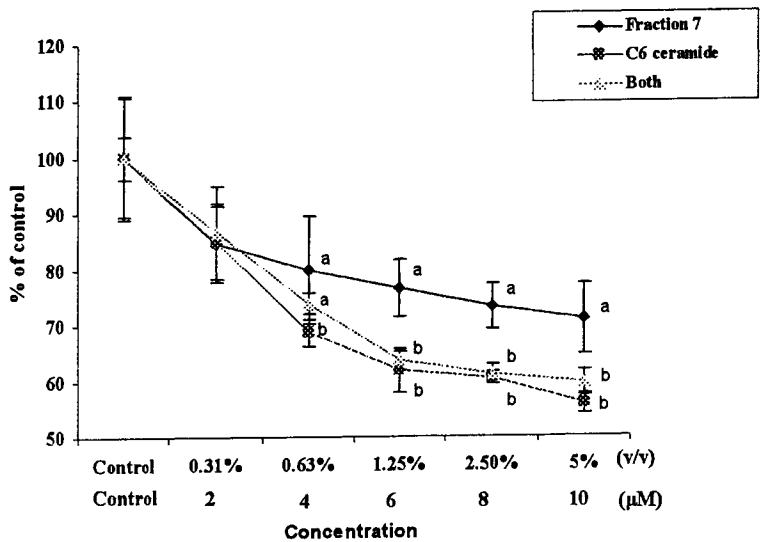

MWCO 3K
C6 ceramide
Both
% of control
120
100
80
60
40
20
0
a
b
c
0.31%
0.63%
1.25%
2.50%
5%
(v/v)
2
4
6
8
10
(μM)
Concentration

Figure 4.4 Antiproliferative effects of SEC-HPLC Fraction 7 of kefir alone or in combination with C6 ceramide on MCF-7 cells (A) and HMEC cells (B). Values were expressed as Mean±SD, n=4. Means with the same letter are not significantly different at $P < 0.05$ for comparison among treatments at the same dose level.

A


Fraction 7
C6 ceramide
Both
% of control
120
110
100
90
80
70
60
50
a
b
Control
0.31%
0.63%
1.25%
2.50%
5%
(v/v)
Control
2
4
6
8
10
(µM)
Concentration

B

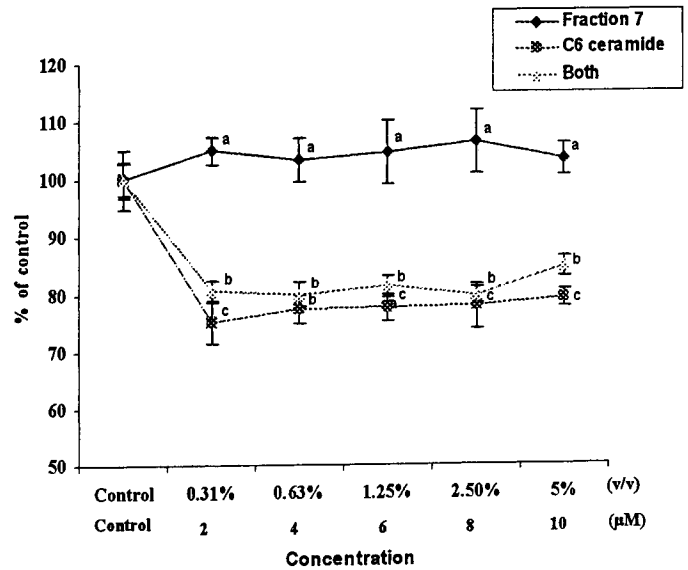
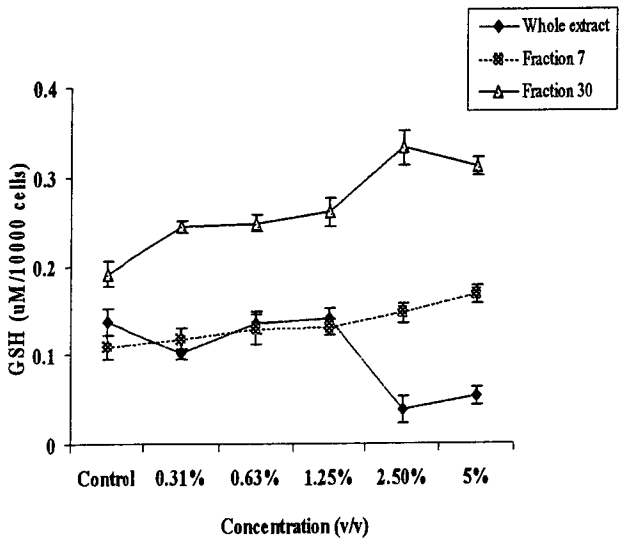

Fraction 7
C6 ceramide
Both
% of control
120
110
100
90
80
70
60
50
a
b
c
Control
0.31%
0.63%
1.25%
2.50%
5%
(v/v)
Control
2
4
6
8
10
(µM)
Concentration

Figure 4.5 Comparison of intracellular GSH concentration in MCF-7 cells (A) and HMEC cells (B) treated with whole extract of kefir, SEC-HPLC Fraction 7 of kefir extract, and preparative HPLC fraction 30 of kefir extract. Values were plotted as means±SD, n=3.

A


Whole extract
Fraction 7
Fraction 30
GSH (uM/10000 cells)
0.4
0.3
0.2
0.1
0
Control
0.31%
0.63%
1.25%
2.50%
5%
Concentration (v/v)

B

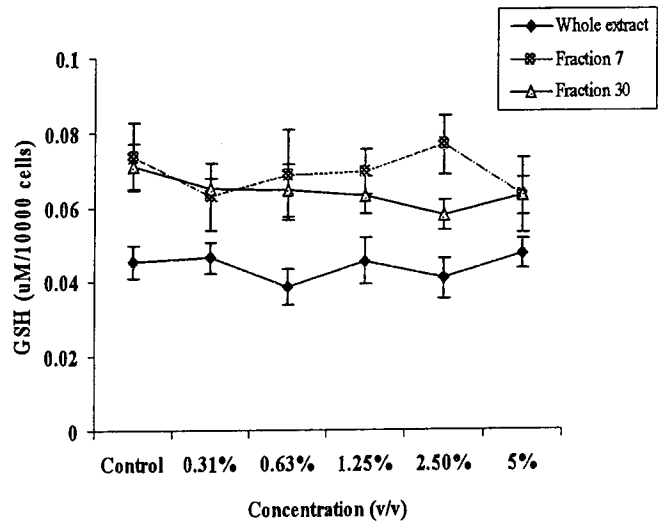
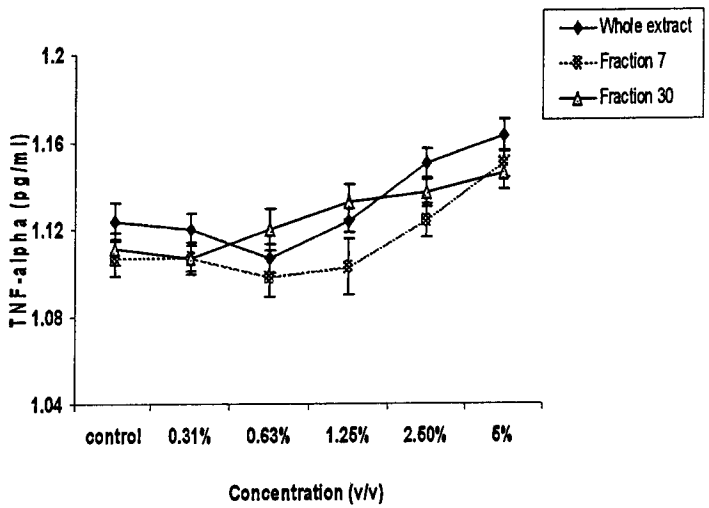

Whole extract
Fraction 7
Fraction 30
GSH (uM/10000 cells)
0.1
0.08
0.06
0.04
0.02
0
Control
0.31%
0.63%
1.25%
2.50%
5%
Concentration (v/v)

Figure 4.6 Comparison of TNF-alpha concentration in cell culture medium after MCF-7 cells (A) and HMEC cells (B) were treated with whole extract of kefir, SEC-HPLC Fraction 7 of kefir extract, and preparative HPLC Fraction 30 of kefir extract. Values were plotted as means±SD, n=3.

A


Whole extract
Fraction 7
Fraction 30
1.2
1.16
1.12
1.08
1.04
TNF-alpha (pg/ml)
control
0.31%
0.63%
1.25%
2.50%
5%
Concentration (v/v)

B

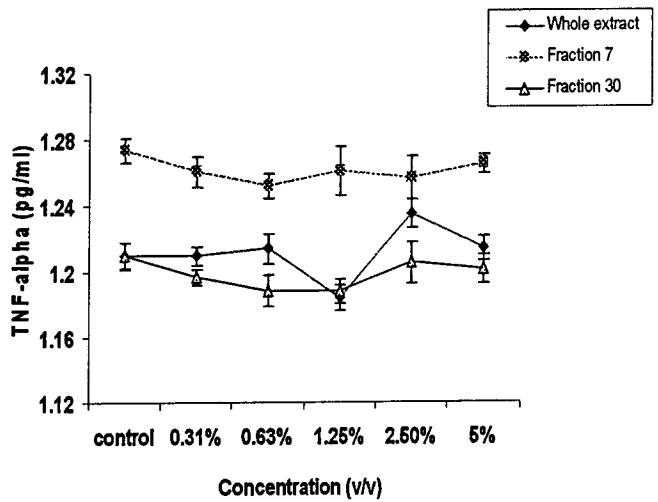
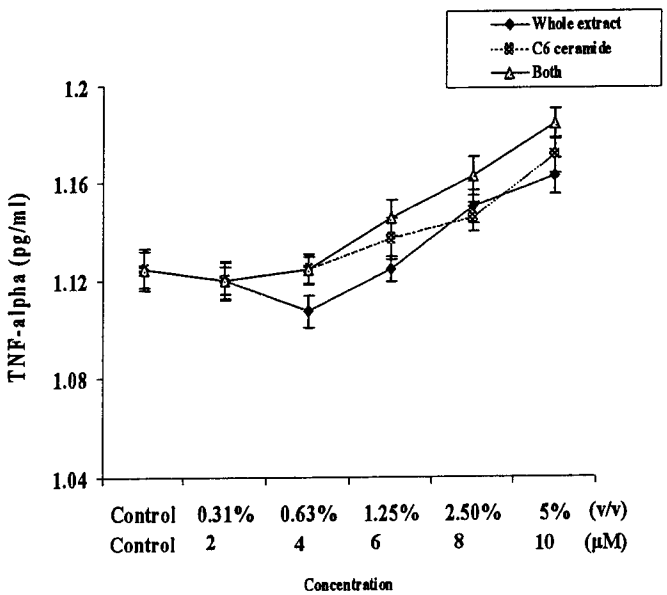

Whole extract
Fraction 7
Fraction 30
1.32
1.28
1.24
1.2
1.16
1.12
TNF-alpha (pg/ml)
control
0.31%
0.63%
1.25%
2.50%
5%
Concentration (v/v)

Figure 4.7 Comparison of TNF-alpha concentration in cell culture medium after MCF-7 cells (A) and HMEC cells (B) were treated whole extract of kefir, C6 ceramide and both. Values were plotted as means±SD, n=3.

A


Whole extract
C6 ceramide
Both
TNF-alpha (pg/ml)
1.2
1.16
1.12
1.08
1.04
Control 0.31% 0.63% 1.25% 2.50% 5% (v/v)
Control 2 4 6 8 10 (µM)
Concentration

B

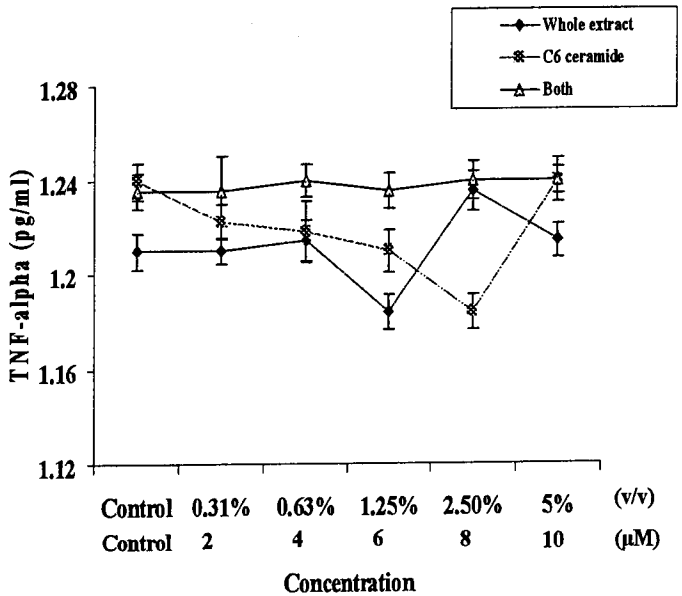
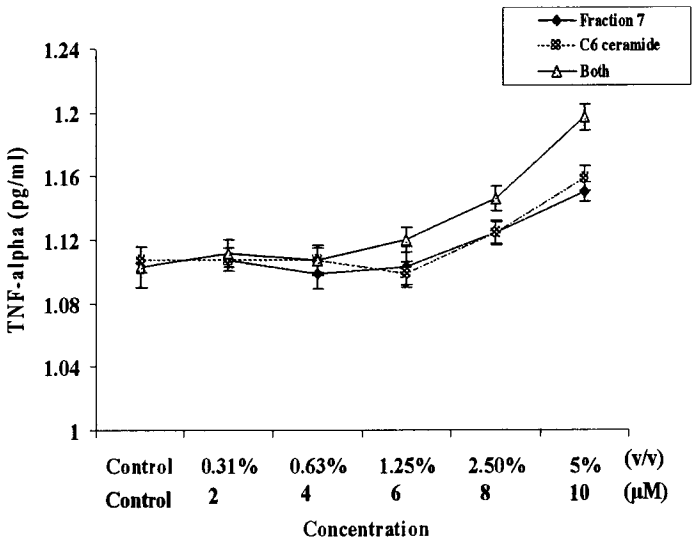
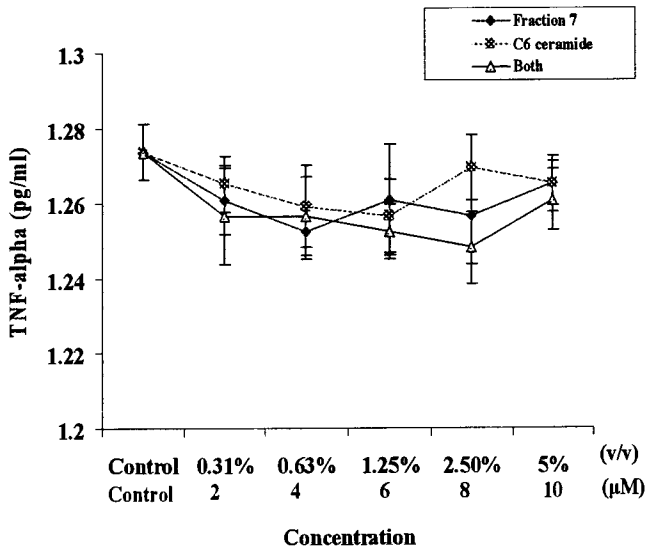

Whole extract
C6 ceramide
Both
TNF-alpha (pg/ml)
1.28
1.24
1.2
1.16
1.12
Control 0.31% 0.63% 1.25% 2.50% 5% (v/v)
Control 2 4 6 8 10 (µM)
Concentration

Figure 4.8 Comparison of TNF-alpha concentrations in cell culture medium after MCF-7 cells (A) and HMEC cells (B) were treated SEC-HPLC fraction 7 of whole extract of kefir, C6 ceramide and both. Values were plotted as means±SD, n=3.

A


Fraction 7
C6 ceramide
Both
TNF-alpha (pg/ml)
1.24
1.2
1.16
1.12
1.08
1.04
1
Control 0.31% 0.63% 1.25% 2.50% 5% (v/v)
Control 2 4 6 8 10 (μM)
Concentration

B


Fraction 7
C6 ceramide
Both
TNF-alpha (pg/ml)
1.3
1.28
1.26
1.24
1.22
1.2
Control 0.31% 0.63% 1.25% 2.50% 5% (v/v)
Control 2 4 6 8 10 (μM)
Concentration

The changes of intracellular GSH concentrations in MCF-7 cells treated with fractions of kefir were shown in Figure 4.5A. After MCF-7 cells were treated with whole extract of kefir mother culture, GSH was significantly reduced with the two higher doses (2.5% and 5%). Fractions 7 and 30 were associated with significantly higher intracellular GSH concentrations ($P < 0.05$). There was no significant change of intracellular GSH concentrations in HMEC cells treated with the different fractions of kefir relative to control concentrations (Figure 4.5B).

Analyses of TNF-alpha in cell culture media collected 6 d post treatment showed that after MCF-7 cells were treated with kefir extract, Fraction 7, or Fraction 30, a dose dependent increase of TNF-alpha in cell culture media was observed (Figure 4.6, 4.7, and 4.8). After MCF-7 cells were treated with C6 ceramide together with Fraction 7, higher concentrations of TNF-alpha were observed in the cell culture media as compared to the concentrations detected following incubations of either C6 ceramide or Fraction 7 (Figure 4.8A). No effect on TNF-alpha concentrations was observed with HMEC cells following incubations of either C6 ceramide, Fraction 7 or the combined incubation of C6 ceramide and Fraction 7 (Figure 4.8B). Correlation analyses, as shown in Table 4.1, further indicated that the impact of kefir fractions on GSH and TNF-alpha concentrations in cancer cells and normal cells differed. A positive correlation was observed between the intracellular concentration of GSH and the number of live cells after MCF-7 cells were treated with kefir whole extract ($r=0.76$, $P < 0.05$), in contrast to a negative correlation when MCF-7 cells were treated with Fraction 7 and Fraction 30 ($r= -0.86$, $P < 0.05$; $r=-0.84$, $P < 0.05$ respectivly). High concentration of intracellular GSH was associated with low concentration of TNF-alpha with treatment of kefir whole extract on MCF-7 cells while high concentration of intracellular GSH was associated with high TNF-alpha concentration with treatments of Fraction 7 and Fraction 30.

Table 4.1 Correlation analyses of GSH and TNF-alpha in MCF-7 and HMEC cells treated with different fractions of kefir

		GSH vs cell number	TNF-α vs cell number	GSH vs TNF-α
Whole extract	MCF-7	0.76*	-0.85*	-0.86*
	HMEC	0.13	0.22	0.34
Fraction 7	MCF-7	-0.86*	-0.82*	0.85*
	HMEC	-0.08	-0.31	0.06
Fraction 30	MCF-7	-0.84*	-0.85*	0.83*
	HMEC	-0.45	-0.35	0.20

* Significance test of correlation coefficient, $P < 0.05$

DISCUSSION

Research on the involvement of sphingolipids in the signal transduction pathways that mediate cell growth, differentiation, multiple cell functions and cell death discovered that ceramide can be an intracellular second messenger for tumor necrosis factor-alpha, IL-1beta, and other cytokines; sphingosine, sphingosine 1-phosphate and other sphingolipid metabolites modulate cellular calcium homeostasis and cell proliferation (Merrill *et al.*, 1997). Recent evidence has suggested a role for aberrantly low levels of cellular ceramide in the pathogenesis and chemoresistance of cancer thereby indicating that enhancement of tumor ceramide levels may be a useful strategy for breast cancer treatment. In this regard, ceramide analogs have shown considerable potential in the induction of antiproliferative effects in breast cancer cells depending on the ceramide compound used (Struckhoff *et al.*, 2004). In the present study, ceramide I (C2-ceramide analog) and C6 ceramide showed a strong dose dependent antiproliferative effect on MCF-7 cells, while ceramide 1-phosphate (C18 ceramide analog) from bovine brain did not. This result is similar to the findings of Fillet *et al.* (2003) who observed that C2 and C6 ceramides, as cell permeable ceramide analogs, induced apoptosis in HCT116 and OVCAR-3 cancer cells whereas C16-ceramide, which is impermeable, showed no effect. The ceramide analogs were indicated to induce apoptosis via nuclear factor-κBDNA-binding, caspase-3 activation, poly(ADP-ribose) polymerase degradation, and mitochondrial cytochrome c release, indicating that apoptosis occurs through the caspase cascade and the mitochondrial pathways. The ceramide analog, C6-ceramide, also induced a double block in G1 and G2, thus emptying the S phase (Fillet *et al.*, 2003).

The present findings showed that the combination of fractions of kefir extract with ceramide compounds enhanced their cytotoxicity in MCF-7 cells but not in HMEC cells. As we have reported previously (Manuscript B), MS-MS spectroscopy analysis of RP-HPLC Fraction 30 indicated that the bioactive compounds in the Fraction might be sphingomyelin complexes. Fraction 30

significantly increased the susceptibility of MCF-7 cells to tamoxifen. Lucci *et al.* (1999) demonstrated that modification of ceramide metabolism increased cancer cell sensitivity to cytotoxics. The addition of tamoxifen to the C6 ceramide treatment regimen reduced MCF-7-AdrR cells (adriamycin resistant) viability to 42% and elicited apoptosis (Lucci *et al.*, 1999). Sphingomyelin complexes, which have also have been detected in kefir by other workers (Osada *et al.*, 1993), could play a role in the enhancement of antiproliferative effects of ceramide compounds and tamoxifen. For example, sphingomyelin has been shown to enhance the ceramide formation and ceramide-induced apoptosis induced by the chemotherapeutic agent, gemcitabine, in human pancreatic cancer cells (Modrak *et al.*, 2004). Moreover, a ganglioside complex induced by treatment of the retinoic acid derivative, fenretinide, has been indicated to induce apoptosis and the generation of reactive oxygen species in neuroblastoma cells (Lovat *et al.*, 2004). Hence, it is conceivable that the gangliosides present in kefir Fraction 30 played a role in the greater potency of anti-proliferative effects via the combination with either ceramide or tamoxifen.

Modulation of cellular GSH concentrations has been indicated to play a crucial role in cell proliferation and tumor resistance. Numerous studies have demonstrated that cellular concentrations of GSH respond differentially to GSH-modulating treatments in normal versus tumor cells lines, which also show major differential outcomes from these treatments in terms of cellular viability and proliferation (Russo *et al.*, 1986; Roberts *et al.*, 1991; Baruchel and Viau, 1996). Surprisingly, the results of the present work showed that although the whole extract of kefir, SEC-HPLC Fraction 7, and RP-HPLC Fraction 30 demonstrated antiproliferative effects on MCF-7 cells, differential GSH responses were noted in the different fractions. The whole extract of kefir was associated with significantly lower intracellular GSH concentrations ($P < 0.05$), while treatment of MCF-7 cells with the sub-fractions of the whole kefir extract, i.e., SEC-HPLC Fraction 7 and RP-HPLC Fraction 30, was associated with significantly higher cellular GSH concentrations ($P < 0.05$). Notably, neither

whole kefir extract nor the whole kefir sub-fractions was associated with changes in cellular GSH concentrations in HMEC cells.

The selective depletion of intracellular GSH in MCF-7 cells associated with whole kefir extract treatment is a plausible mechanism by which whole kefir extract exerts anti-proliferative effects on cancer cells either singly or in combination with either tamoxifen or ceramides. Cellular GSH-depletion results in a reduced rate of cell proliferation in human lung (Kang and Enger, 1990) and colon carcinoma cells (Benard and Balasubramanian, 1997). Similar findings in terms of selective effects on the proliferation and cellular GSH levels of tumor cells have been reported with whey protein concentrates (WPC) and isolates. Whey protein concentrate treatment has been shown to inhibit rat mammary tumor or Jurkat T tumor cell proliferation (Baruchel and Viau, 1996). The authors speculated that the selective modulation of GSH in normal versus tumor cells was a possible mechanism explaining the antiproliferative effect of WPC since GSH levels were decreased with WPC treatment only in tumor cells. Animal studies have shown that whey protein, in comparison to red meat, soy bean meal and/or casein, is more protective against carcinogen-induced colon tumor expression (Bounous *et al.*, 1988; Papenburg *et al.*,1990; McIntosh *et al.*, 1995; Hakkak *et al.*, 2001). The chemotherapeutic potential of depletion of tumor cell GSH involving WPC has been suggested by a phase II clinical trial involving daily intake of WPC in patients with metastatic carcinoma of the breast, pancreas and liver. The patients initially showed above normal blood lymphocyte GSH levels reflecting high tumour GSH levels. Patients that responded with lowered GSH levels following WPC treatment, however, showed stabilization of the tumour growth (Kennedy *et al.*, 1995). Another study, however, showed no significant differences in survival rates in human hepatoma HepG2 cells treated with a whey protein isolate despite a reduction of cellular GSH concentrations by 20% (Tsai *et al.*, 2000). However, the combination of the whey protein isolate with the anticancer drug, baicalein, enhanced significantly the cytotoxic action of the drug on HepG2 cells. The depletion of GSH by whole kefir extract that is specific to MCF-7 tumor cells could thus be a therapeutic approach as well as a useful

adjunct to chemotherapy since elevated GSH levels in tumor tissue are associated with resistance to chemotherapy (Schröder *et al.*, 1996). Hence, the observed similarities between WPC and kefir extract treatments in terms of the selective response of tumor cells versus normal cells with respect to both anti-proliferative effects and depletion of cellular GSH indicates that similar mechanisms of action play a role in their anti-proliferative effects. Although whey proteins were isolated in the milk extract, no anti-cancer activities were noted as opposed to the kefir-fermented milk extract. It is likely that the dose of whey proteins provided by the milk extract was too low to induce anti-proliferative action. Treatment of MCF-7 cells via fermented milk in the form of yogurt extracts showed significantly lower anti-proliferative potency relative to the kefir extract. Moreover, anti-proliferative effects were observed in normal mammary cells following yogurt extract treatment in contrast to kefir extract treatment. In contrast, the same dose of kefir fermented milk induced potent anti-proliferative effects on MCF-7 cells but not in normal mammary epithelial, as has been previously observed with WPC treatment of tumor cell lines. Thus these findings support the idea that similar physiological mechanisms are involved with kefir extract and WPC treatments, which is not observed with milk or yogurt extracts. These findings support a therapeutic role of kefir extracts, in view of the supportive animal and clinical evidence for the positive therapeutic effects of WPC in cancer treatment and prevention.

In addition to GSH, there are other factors involved in tumor cell proliferation such as TNF-alpha. One of the prominent effects of TNF-alpha is the induction of apoptosis in a variety of tumor cells (Ware *et al.*, 1996). Since cellular depletion of glutathione is associated with a greater sensitivity to TNF-alpha-induced apoptosis (Kaipia *et al.*, 1996), TNF-alpha concentrations were assessed in association with the kefir treatments. All the fractions tested induced the production of TNF-alpha in MCF-7 cells but no effect on TNF-alpha was observed in HMEC cells. The antiproliferative effect of kefir on MCF-7 cells may thus be explained by TNF-alpha induced apoptosis, which has been noted in cancer cells (Davis *et al.*, 2000; Donato and Klostergaard, 2004; Liu *et al.*, 1998). Interestingly, both the lowered and increased cellular GSH concentrations that

were observed in association whole kefir extract and kefir sub-fraction treatments, respectively, showed enhanced TNF-alpha concentrations in MCF-7 cells. The inverse relationship observed between TNF-alpha and GSH concentrations is plausible since as cytokines such as TNF-alpha act as mediators of oxidative stress and they have been indicated to decrease GSH concentrations by affecting GSH shuttling and recycling (Chen *et al.*, 1998). Likewise, induction of TNF-alpha has been associated with depletion of cellular GSH in a variety of tumor cell types such as L929 cells (Hayter *et al.*, 2000). Conversely, GSH precursors (Pena *et al.*, 1999; Jeannin *et al.*, 1995) have been shown to down-regulate cytokine synthesis, activation, and downstream processes. The discordance between concentrations of GSH and TNF-alpha between the whole kefir extract and extracts of kefir sub-fractions is surprising. This observation requires further study to examine the possible mechanisms of action; however, it appears that induction of TNF-alpha is a likely mechanism of action for both extracts of whole kefir as well as the kefir sub-fractions.

It is conceivable that whole kefir extract and extracts of kefir sub-fractions exert their anti-proliferative effects on MCF-7 cells via different bioactive components and mechanisms of action. As discussed above, whole kefir extracts could represent a complex mixture of low molecular weight peptides; bacteria and yeast metabolites, together with other unknown components, i.e. sphingomyelins complexes, that are generated after kefir fermentation of bovine milk with kefir grains. Hence, the observed antiproliferative effects of the whole kefir extract may be an outcome of a synergy among a variety of bioactive components in kefir. On the other hand, extracts of kefir sub-fractions appear to contain isolates that likely contain primarily sphingolipid complexes.

Our previous findings showed that after kefir fermentation, proteins with high molecular weight were broken down resulting in the generation of more peptides (results reported in separate paper). The observed dose-dependent antiproliferative effect of the whole kefir extract on MCF-7 cells might thus be due to bioactive peptides originating from either hydrolysis of protein or metabolites of bacteria and yeast. The absence of antiproliferative responses in the

unfermented isolated milk fraction suggests production of certain bioactive compounds from the kefir fermentation. Hence, although no peptides were identified in the RP-HPLC Fraction 30, peptides may still contribute to the observed antiproliferative effects of the whole kefir extract and the MWCO kefir fraction. Matar *et al.* (1997) showed that protein fractions of milk fermented by *Lactobacillus helveticus* L89 significantly inhibited mutagenesis induced by 4-nitroquinoline-N'-oxide in the Ames test. One of the effective protein fractions that was further purified and sequenced was shown to contain four peptides derived primarily from beta-casein. Likewise, Ganjam *et al.* (1997) reported that isolated yogurt fraction containing 100-500 Da fractions of yogurt decreased intestinal epithelial cell IEC-6 and CaCo-2 cell proliferation, while isolated milk fraction of the same molecular weight range or lactic acid solutions showed no effect. In this study, the whole kefir extract may have modulated GSH synthesis via peptide action as peptides including those generated from milk fermentation have been suggested to modulate GSH concentrations (Zommara *et al.*, 1996). whereas the GSH-modulating peptides are unlikely to present in the RP-HPLC fractions, which are likely acting via different mechanisms to inhibit tumor cell proliferation, i.e. stimulation of TNF-alpha. Other components of fermented milk such as conjugated linoleoic acid (CLA) (Schonberg *et al.*, 1995), sphingolipids (Dillehay *et al.*, 1994; Schmelz *et al.*, 1996), polysaccharides (Shiomi *et al.*, 1982), and organic acids (Garrote *et al.*, 2000) have been suggested to protect against carcinogenesis.

In summary, the antiproliferative effect of whole kefir extracts and extracts of kefir sub-fractions on MCF-7 cells appear to be explained by an induction of TNF-alpha, which likely induced an apoptotic process. In addition, a potential chemotherapeutic impact of kefir fractions was exhibited via an enhancement of ceramide cytotoxicity on MCF-7 cells while showing no adverse effects on HMEC cell proliferation.

CHAPTER 5

INTRODUCTION TO CHAPTER 5

Our previous study demonstrated that extracts from kefir and yogurt exerted antiproliferative effects on MCF-7 cells, while extracts from milk showed no effect. We proposed that small molecular weight proteins and/or peptides generated by kefir fermentation may be the main bioactive components. Capillary zone electrophoresis (CZE) analysis of extracts of kefir and milk showed that more peaks were generated after kefir fermentation, which suggests the presence of relatively higher amounts of bioactive peptides. As there is likely further generation of bioactive peptides following gastrointestinal (GI) digestion, it would be of interest to explore the further compositional changes in kefir after in vitro digestion. The major objective of this study was a comparative analysis of the compositional changes of bovine milk before and after kefir fermentation, compositional changes of kefir before and after in vitro enzymatic digestion in terms of protein and peptide profiles using RP-HPLC and CZE. The results of this study provided further information in terms of the impact of fermentation and in vitro digestion on the generation of bioactive peptides from milk protein.

MANUSCRIPT D

Comparative Analysis of the Effects of Kefir Fermentation and In Vitro Enzymatic Digestion on the Protein and Peptide Profile of Bovine Milk

Chujian Chen, Stan Kubow and Hing Man Chan

ABSTRACT

Although kefir fractions have been studied in cell culture and whole animal models, the differential changes in compositional of bovine milk following kefir fermentation and further gastrointestinal digestion have not been characterized. In this study, protein and peptide profiles obtained using reverse phase high performance liquid chromatography (RP-HPLC) and capillary zone electrophoresis (CZE) were compared following kefir fermentation and in vitro enzymatic digestion of kefir, milk and whey protein isolates. Although there was significant peptide production and protein breakdown via microflora fermentation and in vitro enzymatic digestion, some novel peptides appear to be released only by kefir fermentation, which were not observed via in vitro digestion.

Key words: microflora fermentation, enzymatic digestion, protein, peptide, kefir

INTRODUCTION

Bovine milk is a rich source of calcium and protein. Several studies have demonstrated that both in vitro enzymatic digestion and microflora fermentation of milk may produce bioactive hydrolysates or peptides (Ganjam *et al.*, 1997; Laffineur *et al.*, 1996; Pihlanto-Leppala *et al.*, 2000; Rokka *et al.*, 1997). Kefir is a widely consumed fermented milk product (FM) in former Soviet Union and some European countries. It is produced with a unique start culture, kefir grains.

In general, kefir grains consist of polysaccharides, lactic acid bacteria, acetic acid bacteria and yeasts. In the presence of live bacteria and yeasts, pasteurized milk is fermented. Those bacteria and yeasts consume nutrients such as lactose and proteins in the milk and release their metabolites into kefir. Comparison of amino acid profiles of milk, yogurt and Turkish kefir indicated that only very slight differences in amino acid profiles exist among the three different products (Guzel-Seydim *et al.*, 2003). Research aiming at bioactive components indicated that polysaccharides from kefir grain (Shiomi *et al.*, 1982), some organic acids (Garrote *et al.*, 2000), and some proteins and peptides in FM (Matar *et al.*, 1997; Rokka *et al.*, 1997; Tsuda *et al.*, 2000) were shown to have certain health benefits. In vivo animal studies (Furukawa *et al.*, 1990) and human epidemiological studies (Veer *et al.*, 1989; Ronco *et al.*, 2002) all showed consumption of fermented milk, including kefir, exerted protective effects towards cancer.

Although some bioactive components in kefir have been studied in cell culture and animal models (Shiomi *et al.*, 1982; Rokka et al., 1997; Cevikbas 1994), the content of components such as peptides following gastrointestinal digestion are largely unknown. The impact of fermentative process and *in vivo* digestion on the generation of peptides from kefir has not been studied. However, it is likely that there may be additional peptides generated in the digestive process.

The objective of this study is to compare water soluble protein and peptide content in kefir, milk powder and whey protein isolates, and the impact of in vitro digestion on the protein and peptides profiles in these three products via analysis using reverse phase high performance liquid chromatography (RP-HPLC) and capillary zone electrophoresis (CZE).

MATERIALS AND METHODS

Materials

Kefir, milk and milk powder are kindly provided by Liberte Inc. (Brossard, Canada). Three different batches of kefir fermented on three different dates were used for analysis. Whey protein isolate (WPI) was obtained from Inovatech USA Inc. (Bellingham, USA). The peptide calibration kit was purchased from Sigma

Chemical Co. (Sigma, St Louis, USA). All the reagents used in the study were HPLC grade or higher.

Preparation of extracts

Kefir samples (whole kefir) were well stirred, and centrifuged at 4°C, 32,000 x g, for 60 min (Sorvall RC 5C Centrifuge, rotor ss-34, Sorvall Instruments, Wilmington, USA). The supernatant was filtered with a 0.45 μm membrane filter followed by a second filtration using a 0.2 μm filter. The filtrates (extracts of kefir) were stored at -80 °C for later in vitro digestion and future analysis. Some whole kefir samples were also stored for later in vitro digestion assays and analytical analyses.

Protein analysis

A LECO FP-428 Nitrogen Determination System (LECO Corporation, St. Joseph, USA) was used to determine nitrogen content in triplicate for freeze-dried samples of kefir, yogurt and milk. Crude protein content was calculated using a conversion factor of 6.25. Water soluble protein in extracts and digests were determined with Bio-Rad assay kit (Bio-Rad Laboratories, Hercules, USA). Peptide concentrations in solution were analyzed by the method of Church *et al.* (1983).

In vitro digestion method I

Satterlee's method (1982) was adopted for the *in vitro* digestion of protein, using the following enzymes (Sigma Chemical Co., St Louis, USA): porcine pancreatic trypsin (type IX), porcine intestinal peptidase (Grade I), bovinepancreatic α-chymotrypsin (Type II) and bacterial protease. Two solutions were prepared: *solution A*: Dissolve 227040 BAEE units of trypsin + 1860 BAEE units of α-chymotrypsin + 0.520 L-leucine β-naphthylamide units of peptidase in 10 mL nanopure water. *Solution B:* Dissolve 65 casein units of bacterial protease in 10 mL nanopure water. Store both solutions on ice.

Appropriate amount of the whole kefir or kefir extract or milk powder or whey protein isolates (2 mL liquid sample or 50 mg freeze-dried samples) were placed in 30 mL vials containing magnetic stirring bar and pH was adjusted to

8.00 ± 0.03 at 37°C under constant stirring. To each vial, 1 mL of enzyme solution A was added while stirring. Sixty min after the addition of solution A, 1 mL of enzyme B was added, and the temperature was increased to 55°C. After 60 min of digestion, pH was recorded at 37°C. The digest was filtered with 0.2 μm membrane filter, and stored at -80 °C if not analyzed within two days.

In vitro digestion method II

This method was developed by R. Vilela from our research group (data has not been published). Whey protein isolates (WPI) (0.3%) was dissolved in distilled H_2O in 30 mL vials containing magnetic stirring bar, and pH was adjusted to 1.5 with 1 M HCl and 1M NaOH under constant stirring. After equilibration in water bath at 37 °C for 30 min, proper amount of pepsin (5mg/mL in 0.01N HCl, 6.5 μl/ per mL of WPI solution) (Sigma, St Louis, USA) was introduced to protein solution. After 30 min, the pH was adjusted to 7.8, and proper amount of pancreatin (Sigma, St Louis, USA) (5mg/mL in pH 7.5 phosphate buffer, 200μl/per mL WPI solution) was added. The solution was transferred to 40°C water bath for 60 min. At the end of this final incubation, a stop solution (0.1 M Na_2CO_3) was added and the digest was filtered with 0.2 μm membrane filter, and stored at -80°C if not analyzed within two days.

Molecular weight cut-off fractionation (MWCO)

A CENTRIPLUS Centrifugal filter devices were used to obtain MWCO fractions of 3000 and 1000 Da (Millipore, Bedford, USA). Ten milliliters of samples were loaded to the sample reservoir and the assembled device was centrifuged at 4°C, 3000 x g for 290 min. The filtrates were collected for further analysis.

Characterization of extracts and digests by using CE and RP-HPLC

Capillary zone electrophoresis (CZE) was performed using P/ACE ™ 2200 HPAC instrument controlled by System Gold software (Beckman, Fullerton, CA, USA) coupled to IBM PC 486 computer (IBM Corp., Portmouth, England) for data acquisition and analysis. A neutral uncoated fuse silica capillary column (57 cm × 50 μm, the length from intake to detector is 50 cm)(Polymicro

Technologies, Phoenix, Arizona USA) was assembled in the P/ACE cartridge for capillary separations. Injection volume, buffer concentration and running voltage were optimized to achieve the best resolution with the shortest running time.

The CZE capillary column was conditioned by running solutions of 1 M sodium hydroxide, nanopure water, 0.1 M of sodium hydroxide, 1 M hydrochloric acid, and again nanopure water, each for 5 min at a pressure of 40 psi. The capillary column was then conditioned for additional 30 min with a commercially available peptides separation buffer (Sigma Chemical Co., St Louis, USA). Peptides standard (Sigma Chemical Co., St Louis, USA) and sample injections were carried out at the anode end of the capillary using N_2 pressure (0.5 psi) for 5 sec, and the separation occurred at a constant temperature of 20°C and 20 kV. In between each sample or standard separation, the capillary column was purged with nanopure water (1 min), 0.1 M NaOH (1 min), nanopure water (1 min), and separation buffer (2 min). After the completion of each run, the following purge cycles were done: 1 min with nanopure water, 1 min with 0.1 M NaOH, 1 min with nanopure water. On-line detection was performed using an UV detector at 200 nm.

Reverse Phase HPLC (RP-HPLC) was performed on a Beckman Gold HPLC System (Beckman Coulter, Fullerton, USA) with a Prosphere 300 C4 column (5μm, 250 mm X 4.6 mm) (Altech Associate, Inc. Deerfield, USA). After the column was equilibrated with buffer A (0.1% TFA in water) at a flow rate of 1 mL per minute, 50 μl of properly diluted sample solutions were injected and eluted with a linear gradient of Buffer A (0.1% TFA in water) and Buffer B (60% of Acetonitrile in 0.1% TFA : 40% of 0.1% TFA in water) as follows: 0 to 60 min, 0 to 90% B; and 61 to 65 min, 90 to 0% B. Dual channel absorbance was monitored at 210 nm (channel A) and 280 nm (channel B).

All RP-HPLC elution profiles and capillary electropherograms of kefir, milk and whey protein isolates and their digests were compared manually.

Statistic analysis

The concentrations of protein and peptide in kefir fractions were compared by using Student T test. Statistical significance was considered at $P < 0.05$. All statistics test were performed using SAS 8.2 for PC (SAS, Cary, NC).

RESULTS

The RP-HPLC elution profiles of extract of milk (Fig. 5.1A) and whey protein isolate (Fig. 5.1B) were similar. The number of peaks was increased after milk was fermented with kefir grains for 24 h (Fig. 5.1C). These differences in protein and peptide profiles among extracts of kefir, milk, and whey protein isolates were confirmed by capillary electrophoresis (Fig. 5.2). For example, 36 peaks were observed in the extract of kefir as compared to 16 peaks in the extract of milk. Hence, this result suggests that the kefir fermentation process generates increased numbers of small proteins and peptides.

After the digestion of the kefir extract with trypsin, peptidase, α-chymotrypsin, and protease, most peaks with retention time (RT) of greater than 40 min disappeared, but one new peak appeared (RT=10.2 min), and five peaks (RT=10.54, 21.82, 25.53, 35.88, 36.86 min) increased in relative intensity (Fig. 5.3). Protein and peptide analyses showed that protein content in the extract of the kefir digests decreased significantly from 152.9 μg/ml to 3.3 μg/ml ($P < 0.05$), while peptide concentration increased significantly from 5.5 mmol/L to 6.1 mmol/L ($P < 0.05$). The same in vitro digestion method was also used to hydrolyze whole kefir and study the effect of microflora fermentation and in vitro enzymatic digestion on protein and peptide profiles using RP-HPLC and CZE (Fig. 5.3 and 5.4). In comparison to the extract of kefir, three peaks increased in relative peak areas (RT= 5.36, 8.82, and 12.17 min respectively) and two new peaks (RT=11.61, 38.81 min respectively) were found in the whole kefir digest (Fig. 5.3). The electropherograms showed that the numbers of peaks detected in the extract of kefir, kefir extract digest and the whole kefir digest were 36, 80, and 92, respectively (Fig. 5.4). All the peaks in the kefir extract digest were also observed in the whole kefir digest despite differences in the overall CZE profiles.

The protein and peptide profile of the whole kefir digest was also compared to the whole milk digest. The former sample can be considered as the combination of microflora fermentation and in vitro digestion, whereas later sample demonstrated the protein and peptide profile resulting from in vitro digestion only. As shown in Figures 5.5 (RT = 12.29, 28.69 min) and 5.6 (RT=13.27, 15.96 min), there were several new peaks observed in the whole kefir digest in comparison to the whole milk digest.

The two enzymatic approaches also yielded different outcomes (Figure 5.7). Method I generated 88 peaks while Method II yielded 69 peaks. Seven new peaks (RT= 5, 5.44, 5.85, 22.55, 23.23, 24.3, 27.45 min respectively) were observed in the digest with method I, while one new peak (RT=21.18 min) was found in digest with method II.

A molecular weight cut-off method was used to study the differences of different molecular weight fractions with the two in vitro digestion methods. No observed difference was found among different MWCO fractions (Fig. 5.8 and 5.9).

Figure 5.1 Elution profiles of extract of milk, native whey protein, and extract of mother culture using RP-HPLC. A: Extract of milk; B: WPI solution; C: Extract of mother culture.

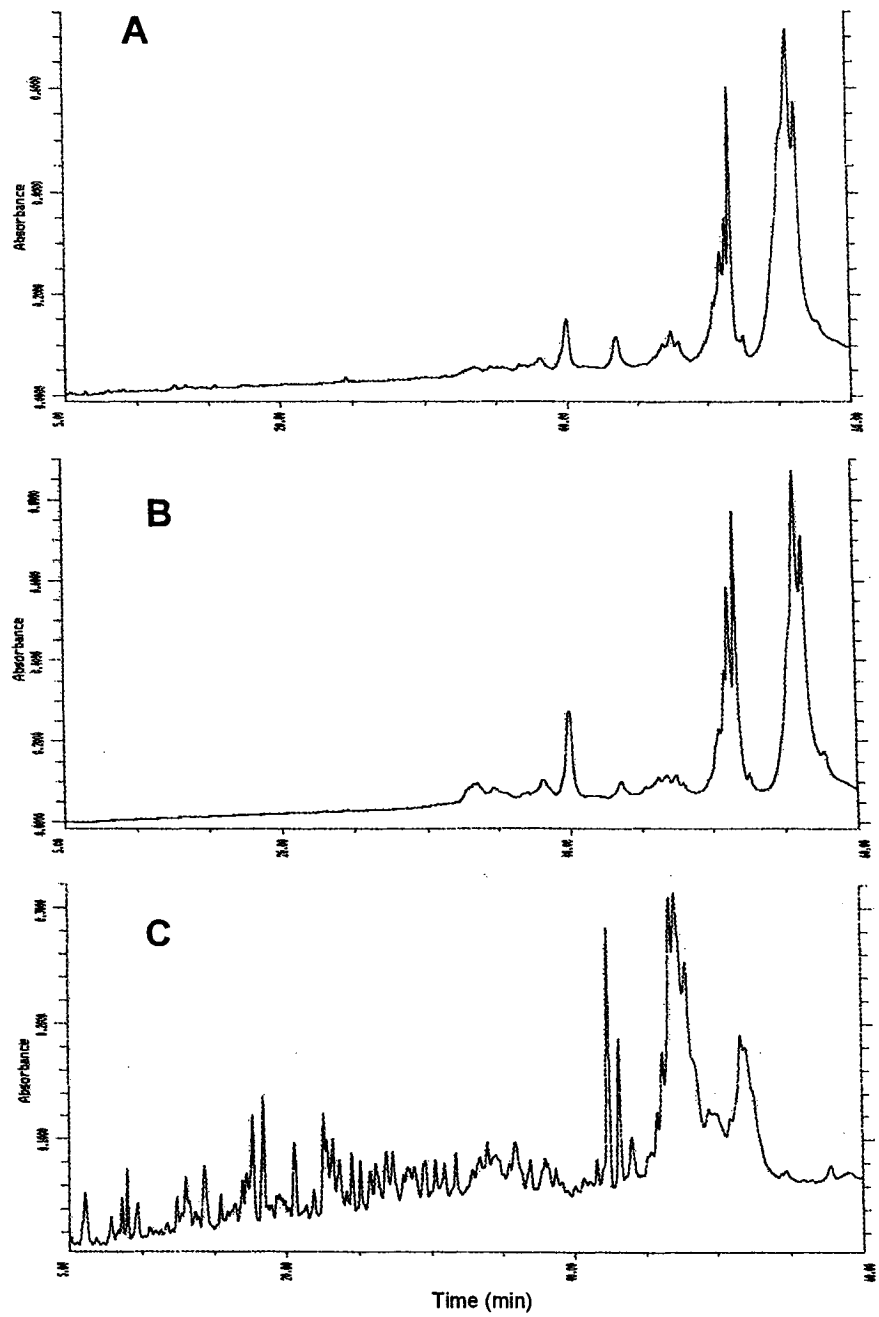

A
B
C
Absorbance
Time (min)

Figure 5.2 Capillary electropherograms of extract of milk, whey protein isolates solution, and extract of mother culture. A: Extract of milk; B: WPI solution; C: Extract of mother culture.

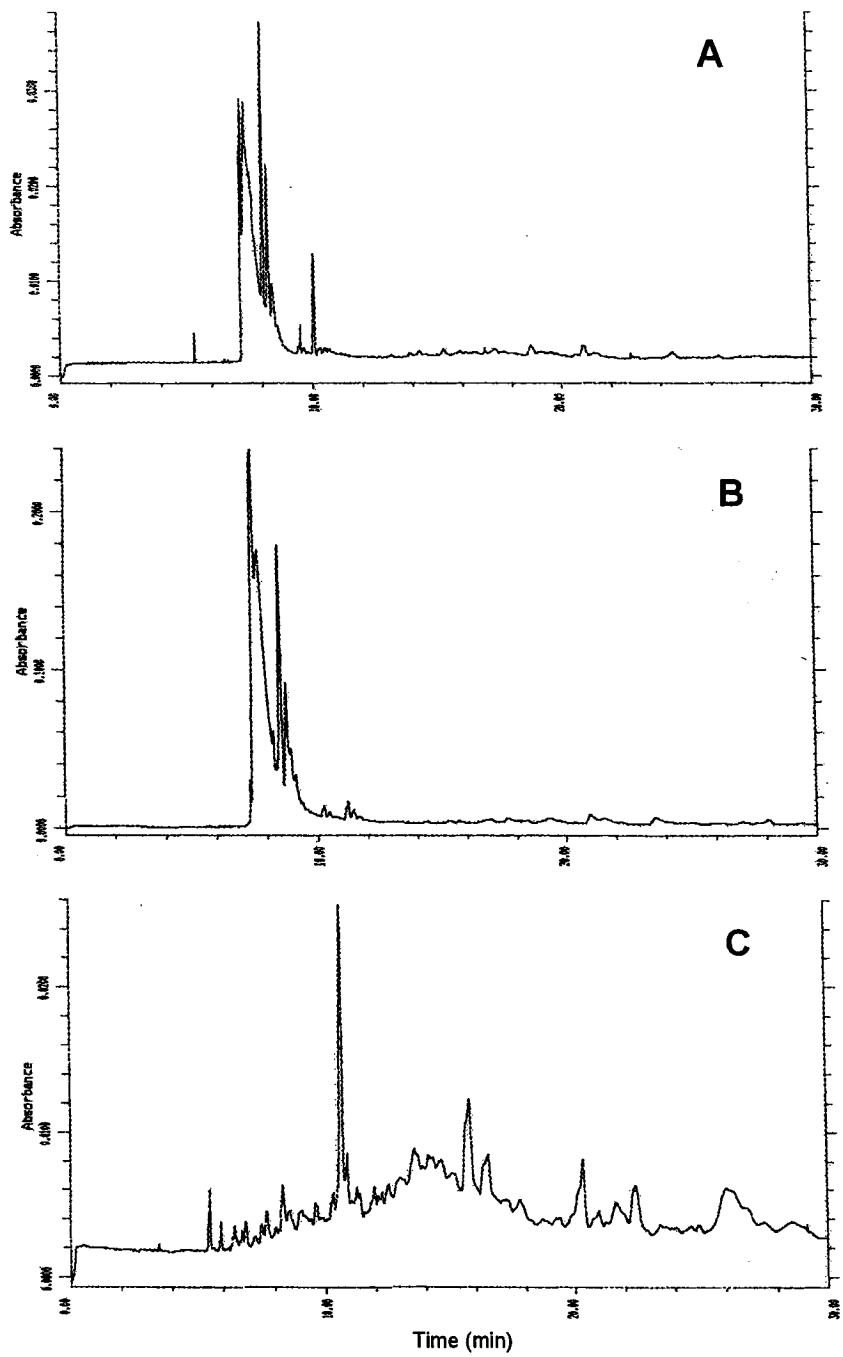

A
B
C
Absorbance
Absorbance
Absorbance
Time (min)

Figure 5.3 Elution profiles of extract of kefir, kefir extract digest and the whole kefir digest obtained with in vitro digestion method I. A: Non-digested extract of mother culture; B: Kefir extract digest; C: Whole kefir digest. Peak ratio was calculated using peak areas. Profile comparison was made to non-digested extract of kefir. ↓ relatively decreased peaks; ↑ relatively increased peaks; × disappeared peaks.

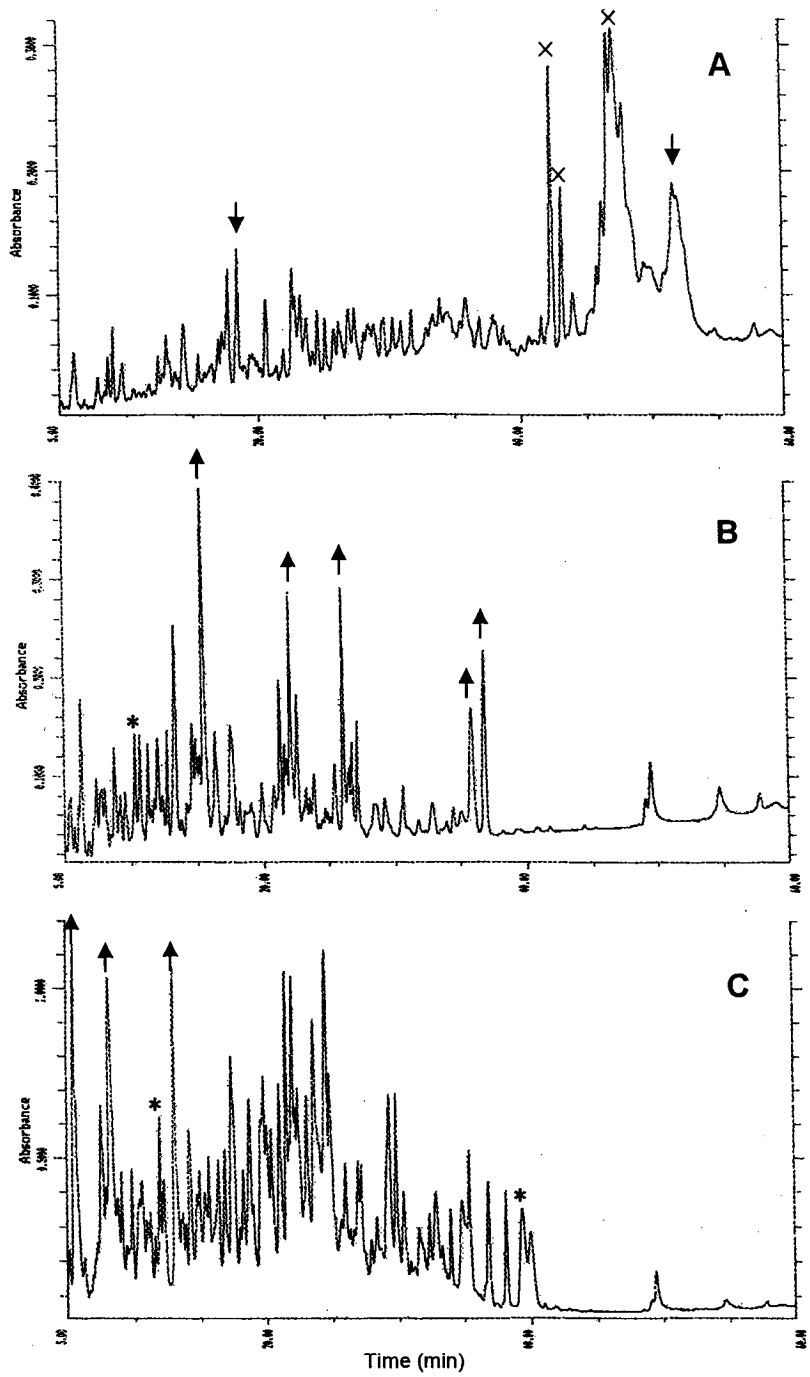

A
B
C
Absorbance
Absorbance
Absorbance
Time (min)

Figure 5.4 Capillary electropherograms of non-digested extract of kefir, kefir extract digest and the whole kefir digest with in vitro digestion method I. A: Non-digested extract of mother culture; B: Kefir extract digest; C: Whole kefir digest using in vitro digestion method I. Profile comparison was made to non-digested extract of mother culture. * new peaks. × disappeared peak.

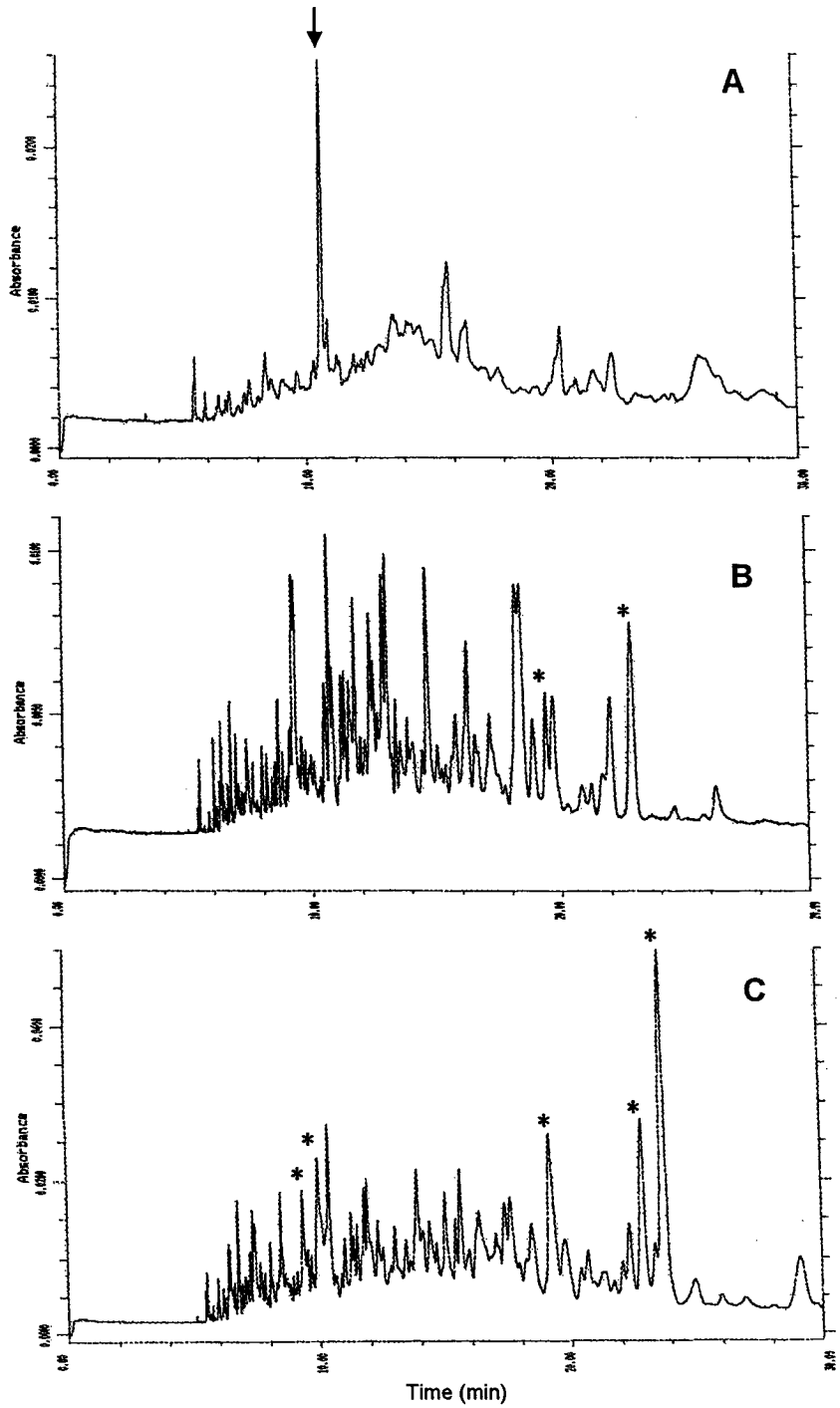

A
B
C
Absorbance
Time (min)

Figure 5.5 Elution profiles of milk powder digest (in vitro digestion) and whole kefir digest (bacteria and in vitro digestion) using digestion method I. A: Milk powder digests. B: Whole kefir digest.

* new peaks.

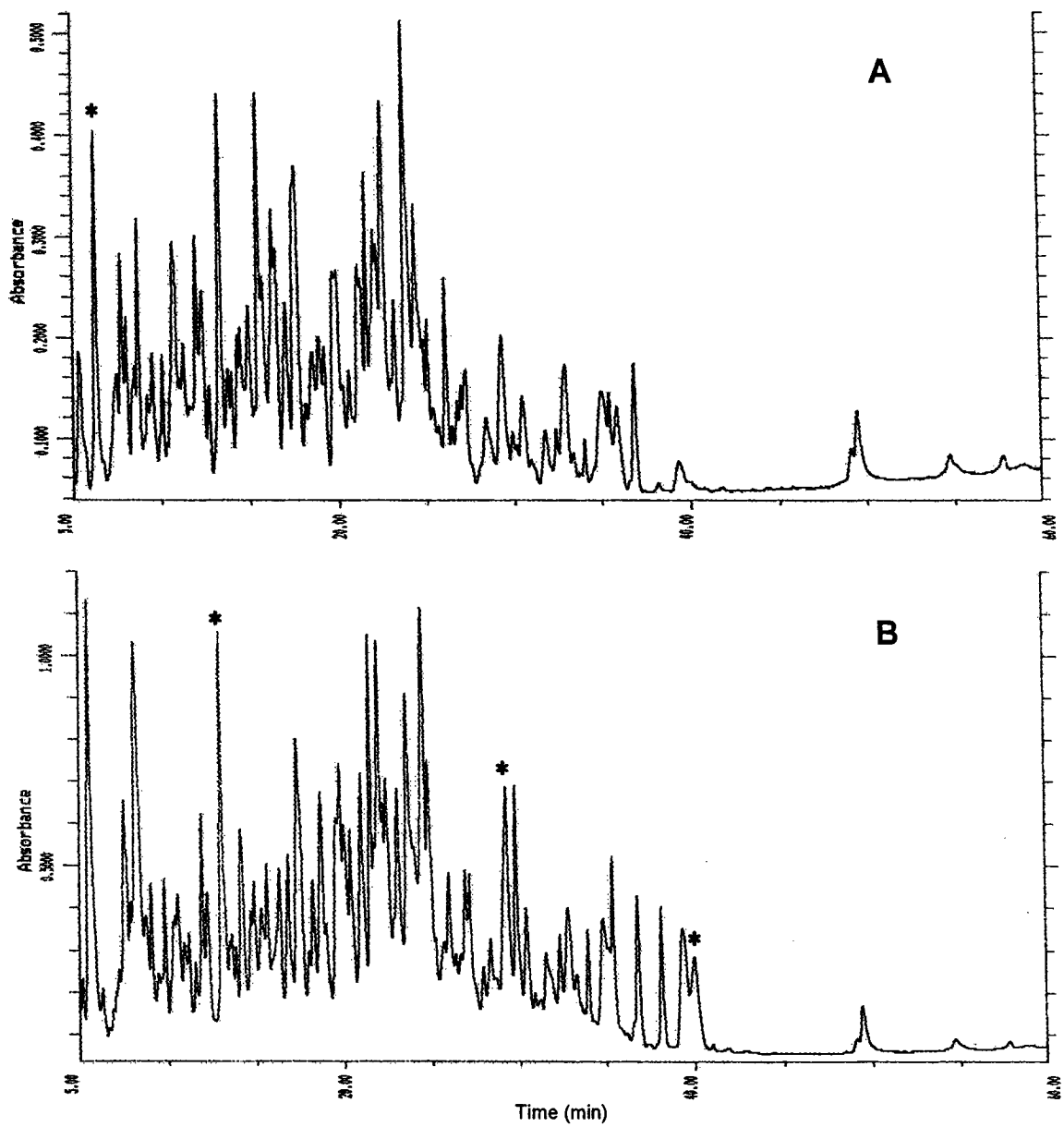

A
B
Absorbance
Time (min)

Figure 5.6 Capillary electropherograms of milk powder digest and whole kefir digest. A: Milk powder digest; B: Whole kefir digest. * new peaks.

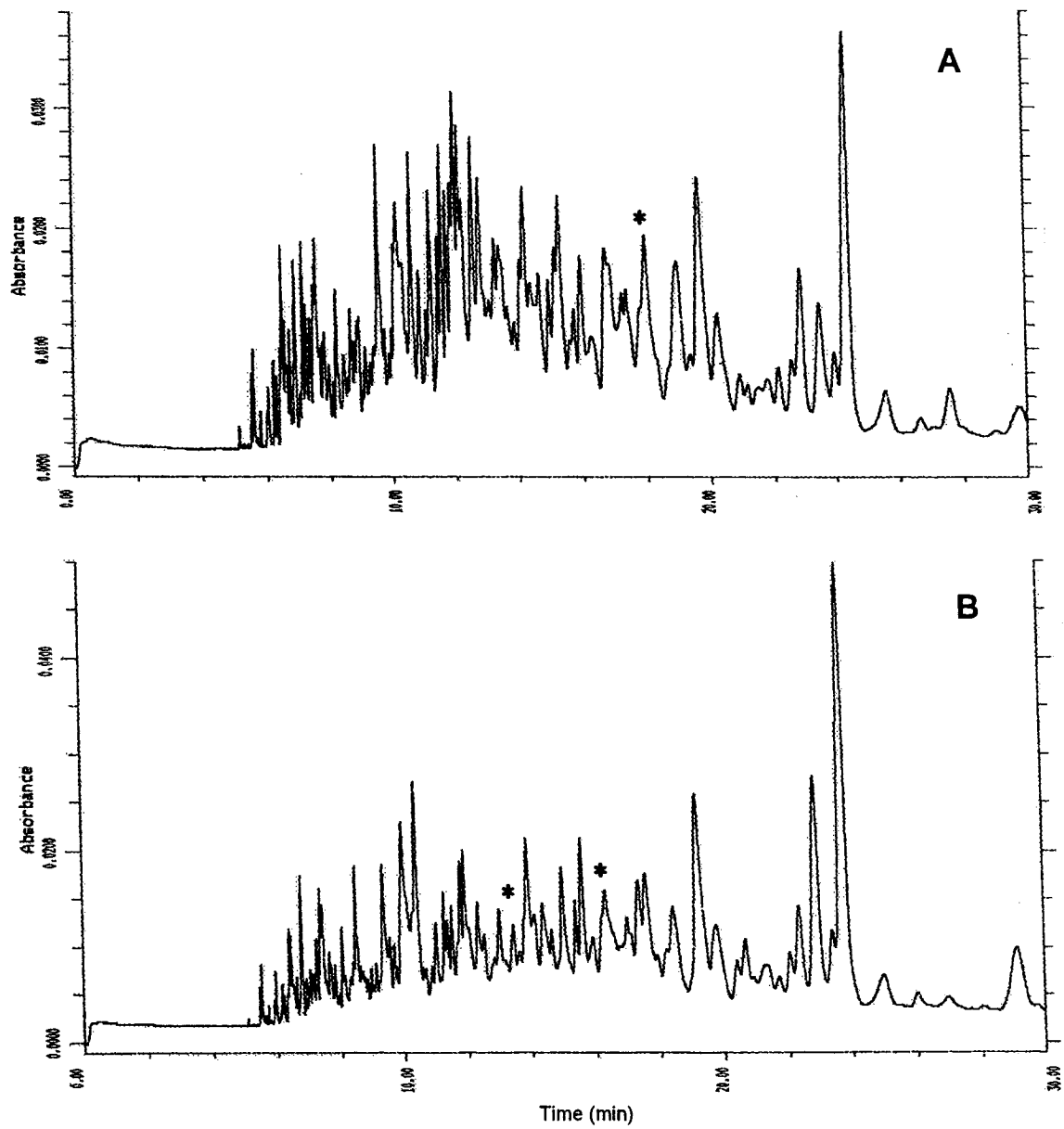

A
B
Absorbance
Absorbance
Time (min)

Figure 5.7 Capillary electropherograms of WPI digests with different in vitro digestion methods. A: WPI was digested with enzymes trypsin, peptidase, α-chymotrypsin, and protease. B: WPI was digested with enzymes pepsin and pancreatin.

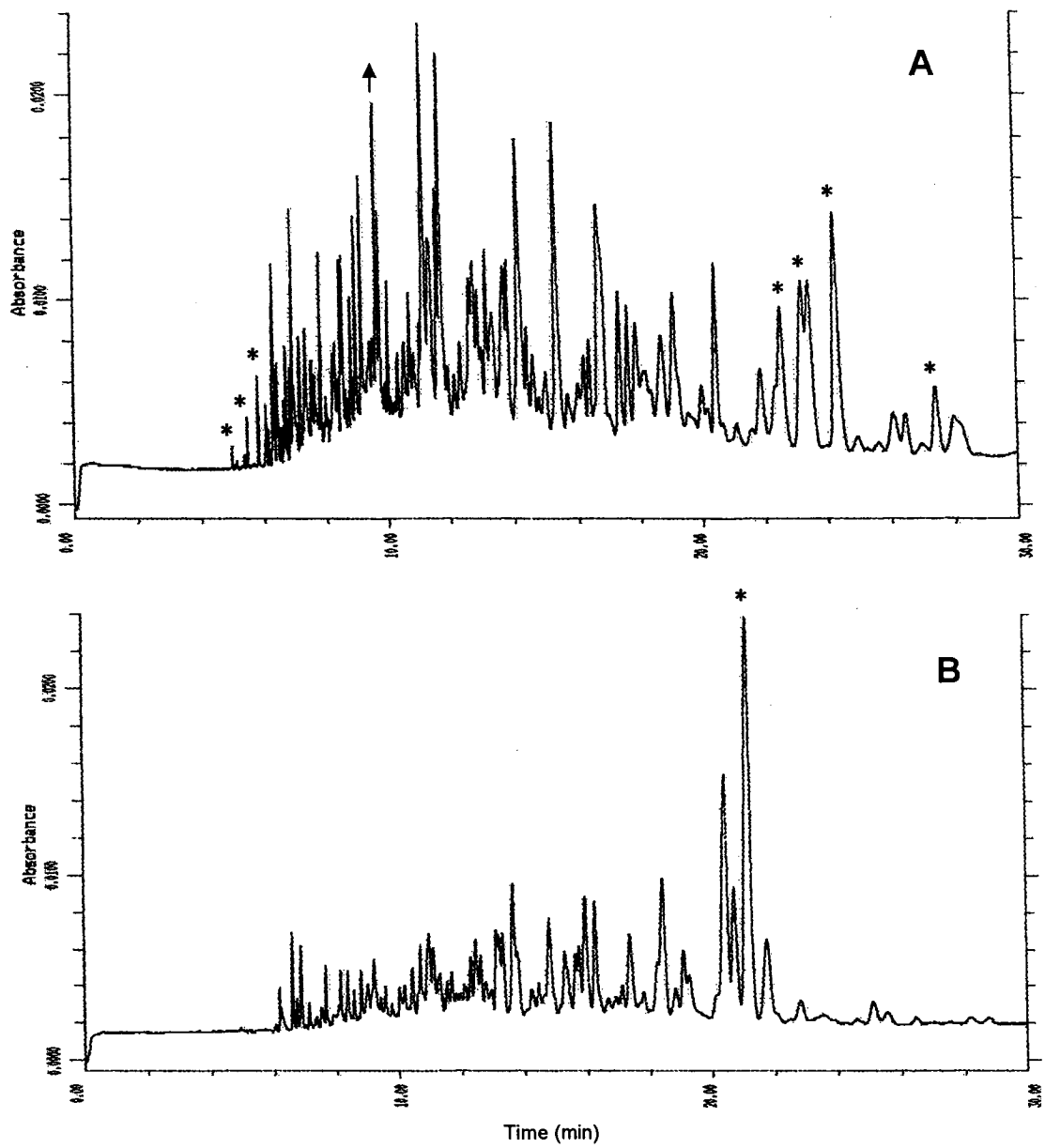

A
B
Absorbance
Absorbance
Time (min)

Figure 5.8 Capillary electropherograms of kefir extract digest using digestion method I and its fraction of MWCO less than 3000 Dalton. A: Kefir extract digest; B: Fraction of MWCO less than 3000 Dalton of kefir extract digest.

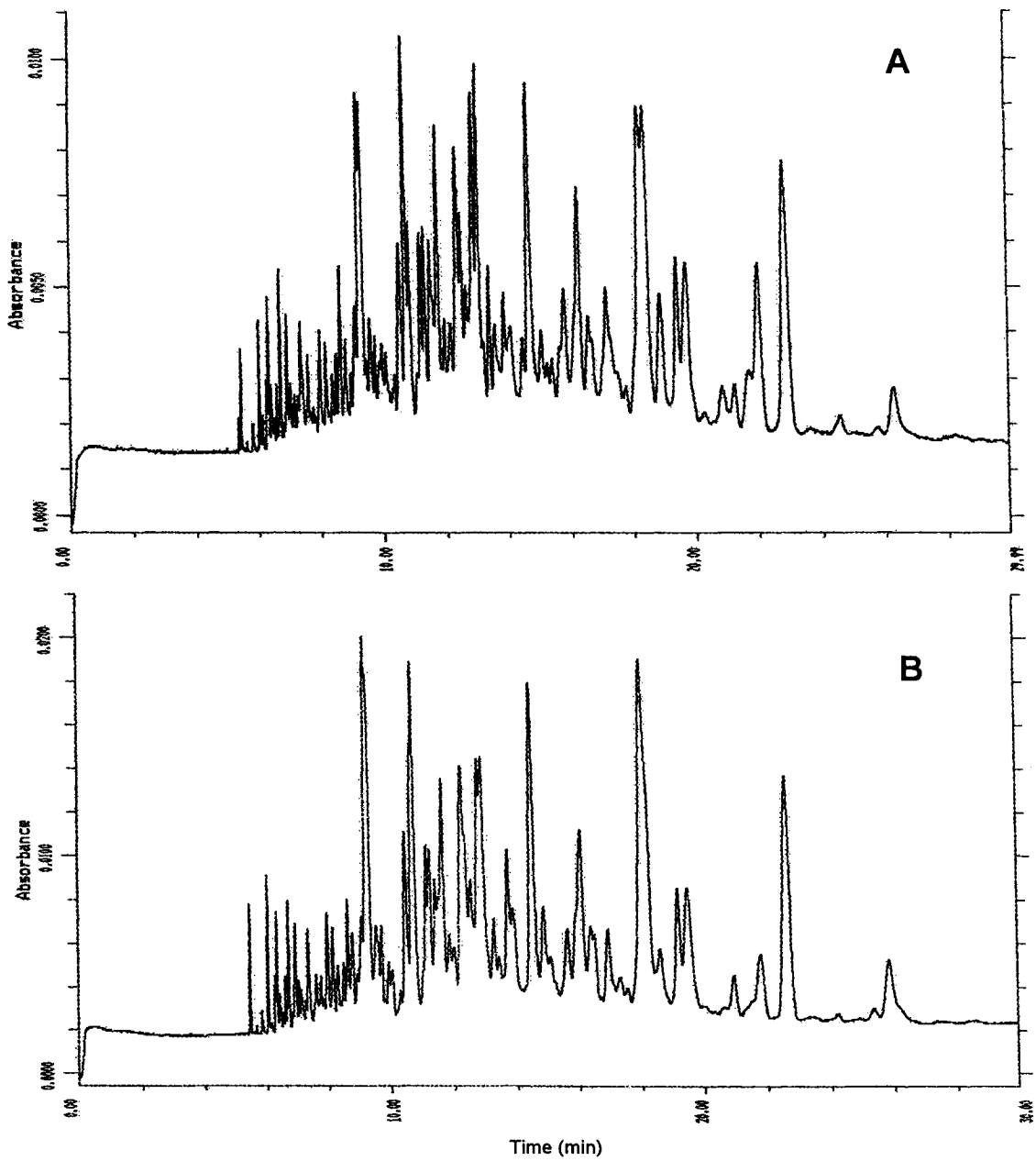
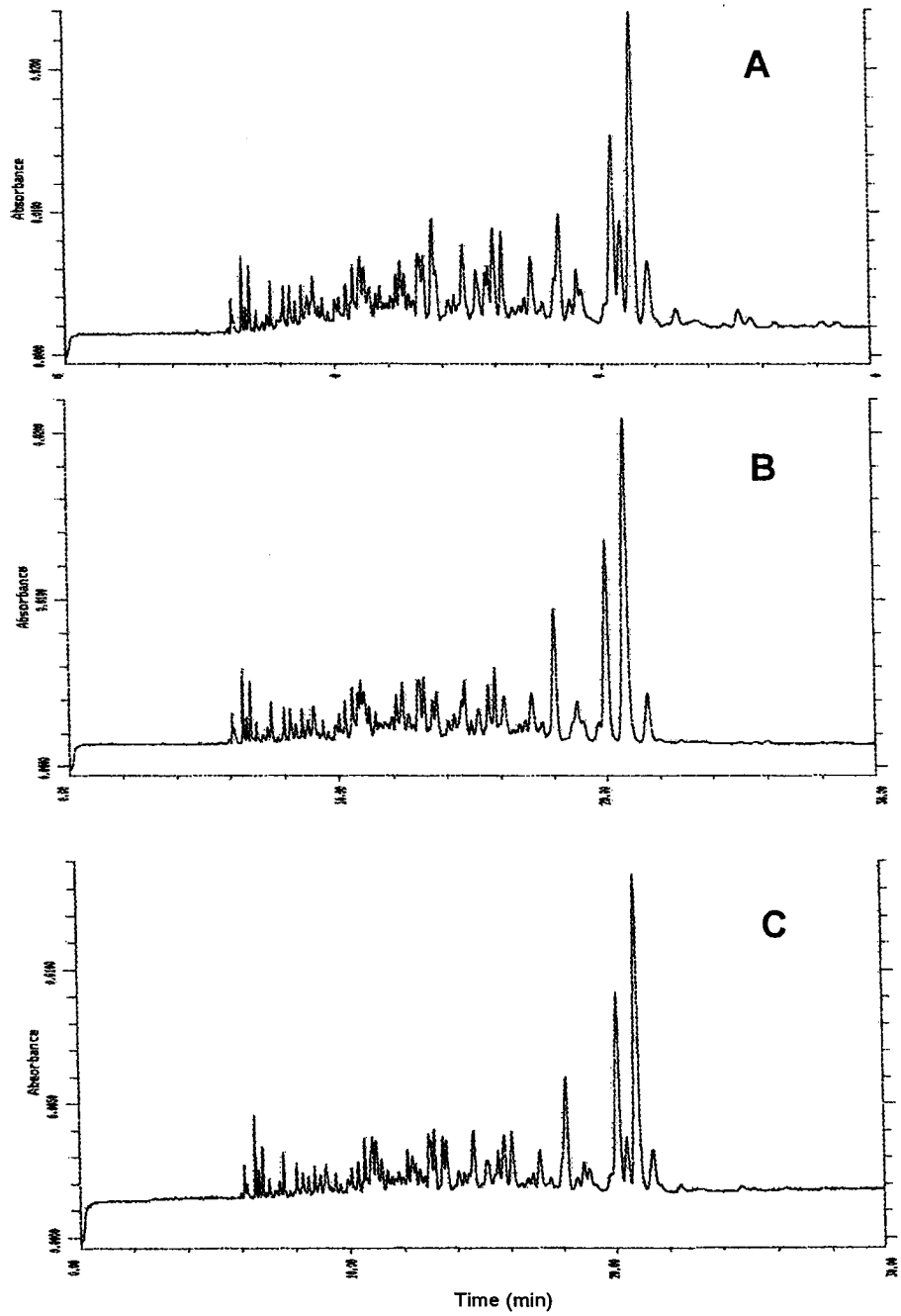

A
B
Absorbance
Absorbance
Time (min)

Figure 5.9 Capillary electropherograms of fractions of whey protein isolate digest (method II) using 3000 and 1000 Dalton MWCO. A: whole WPI digest; B: Fraction of MWCO less than 3000 Dalton; C: Fraction of MWCO less than 1000 Dalton.


A
B
C
Absorbance
Absorbance
Absorbance
Time (min)

DICUSSION

RP-HPLC separation of proteins and peptides is generally achieved via interactions between specific non-polar animo acid residues and the column stationary phase, so that factors other than side-chain residues may affect the separation. CZE separations depend on peptide solvent interactions, where physical characteristics such as charge-to-mass ratios are important. CZE may thus separate closely related peptides which may coelute in the RP-HPLC mode (Rudnick *et al.*, 1994). Our results indicated that CZE can be a complementary tool to RP-HPLC in comparison protein and peptide profiles.

Our previous work has demonstrated that extracts from kefir and yogurt exert antiproliferative effects on MCF-7 cells, whereas milk extracts showed no effect in this regard (Chen *et al.*, submitted, 2005). We proposed that small molecular weight proteins and/or peptides generated by kefir fermentation may be the primary bioactive components involved in the anti-proliferative effects. Guzel-Seydim *et al.* (2003) have shown that there are only very slight differences in amino acid profiles among milk, yogurt and Turkish kefir as determined by in vitro hydrolysis methods. Kefir showed higher amounts of threonine, serine, alanine, lysine and ammonia than either milk or yogurt. Hence, thesedifferences in amino acid could also reflect the different protein or peptide profiles obtained in the present study via RP-HPLC and CZE.

Many bioactive peptides from milk proteins are believed to exist as an inactive state in a precursor form (Schanbacher *et al*, 1998; Rokka *et al.*, 1997). Milk protein is usually digested in the stomach by pepsin, further hydrolyzed in the intestine, and then absorbed through the intestinal wall. Different digestive enzymes have their specific cleavage sites within a protein or peptide. It has also been shown that bioactive peptides may be released in proteolysis induced by microflora fermentation, which could have important functional effects. For example, Zommara et al. (1998) have demonstrated that the antioxidative effect of the whey proteins are enhanced by bacterial fermentation, possibly via the release of bioactive peptides associated with the fermentative process (Zommara *et al.*, 1998). Matar *et al.* (1997) investigated the antimutagenic effects of whey, acetone

extracts, and protein fractions isolated from milk that had been fermented *Lactobacillus helveticus* L89 using the mutagen 4-nitro-quinoline-N'-oxide (4NQO) in the Ames test. They found that fermented milk significantly inhibited mutagenesis induced by 4NQO, while milk fermented by a nonproteolytic variant of the same strain showed no inhibitory effects. This indicates that antimutagenic compounds were produced in milk during fermentation by *L. helveticus*, and that fermentation via different bacterial strains may induce the release different peptides.

Kefir grains used for kefir fermentation have a mass of several different strains of bacteria and yeasts (Angulo et al., 1993; Bottazzi, 1994; Koroleva, 1988). Capillary electrophoresis provided a better resolution than HPLC in separating the peptides as the separation is based on both charge and size. Therefore, the CZE results showed greater differences in peptide profiles between kefir and the other milk samples tested. As shown in Figures 5.1 and 5.2, more peaks were observed in elution profile of extract of kefir mother culture than observed in either unfermented milk or whey protein isolates. This suggested greater protein breakdown and peptide release after milk protein fermentation with bacteria and yeasts. Similar to digestive processes in the GI tract, small molecular proteins and peptides are produced from milk proteins by the enzymes of bacteria and yeast during kefir fermentation. Consequently, following consumption of kefir, it is likely that some of the small molecular proteins and peptides formed during the fermentation process can be directly absorbed or further digested and absorbed to exert biofunctional effects. Our results also showed that, with further in vitro enzymatic digestion, digests of whole kefir had the highest number of peaks relative to either whole milk or whey protein isolates. This finding indicates that the combination of microflora fermentation and enzymatic digestion leads to a greater peptide production as compared to enzymatic digestion alone.

Research has indicated that only those peptides of molecular weight less than 1000 Da are absorbed from the gastrointestinal tract (Webb, 1986). Despite using different MWCO of 3000 and 1000 Da, the electrophoretic profiles looked similar

following digestion. This finding suggests that, after in vitro digestion, molecular weights of most peptide fragments are less than 1000 Da. For further in vivo study, there is no need to do molecular weight cut-off for the digests

In summary, both microflora fermentation and in vitro enzymatic digestion showed a significant impact on the molecular protein and peptide profiles as determined by RP-HPLC and CZE. Small molecular weight proteins and peptides were detected after both microflora fermentation and in vitro digestion and some peptides were found to be associated with kefir fermentation, which were not found in vitro digestion. Moreover, kefir fermentation and digestive enzyme breakdown exerted a synergistic effect in generating additional peptide peaks. Further experiments are needed to study the possible functional effects of these peptides and whether they can resist proteolysis and reach the blood or specific receptors.

ACKNOWLEDGEMENT

I would like to thank R. Vilela for kindly providing me the in vitro digestion method II and Razan El-Ramahi for providing me the WPI digests for comparison.

FINAL CONCLUSION AND SUMMARY

Breast cancer is the most commonly diagnosed cancers in women. Resistance to therapy is the major reason for failure of current cancer treatment. Epidemiological studies have indicated that fermented milk consumption is associated with a lower incidence of breast cancer (Van't Veer *et al.*, 1989; Ronco *et al.*, 2002). Hence, it is critical to identify new agents that can either help prevent the occurrence of breast cancer and/or increase the susceptibility of breast cancer cells to chemotherapeutic drugs, which would improve patient outcome, prevent relapse, and prolong patient survival.

Kefir is a widely consumed traditional fermented milk, which has been suggested to provide important health benefits. Research on the putative health benefits of kefir has shown that beyond basic nutrition, kefir may have blood cholesterol-lowering, immune system enhancement, antioxidant, antibacterial, antifungal, antimutagenic and antitumor activities (Cevikbas *et al.*, 1994; Biffi *et al.*, 1997; Matar *et al.*, 1997; Furukawa *et al.*, 1990 and 2000; Nadathure *et al.*, 1994; Sanders, 2000; Zacconi *et al.*, 1995). In addition, some components derived from kefir are manufactured as health foods for the prevention and control of obesity (Tokumaru *et al.*, 1999), dietary supplements for the prevention of osteoporosis (Weissmahr *et al.*, 2000). However, the bioactive components in kefir and the mechanisms involved in the various biofunctional effects of kefir are still not well characterized, particularly in terms of anti-cancer activities.

A major achievement of this thesis was successful development of a cell culture model using both breast cancer cells and normal mammary epithelial cells, which demonstrated potent antiproliferative effects of kefir and kefir components that were specific to tumor cells. The development of the in vitro screening system allowed the comparison of milk, yogurt and kefir fractions to test for their antiproliferative effects on MCF-7 and HMEC cells, which were related to compositional differences among these three milk products. The extracts of kefir, kefir mother culture and yogurt all showed a dose-dependent antiproliferative

effects on MCF-7 cells; however, the kefir mother culture fraction clearly showed the most potency whereas milk extracts exerted no effect. Significantly, the antiproliferative effect of kefir extracts was not observed in HMEC cells whereas the yogurt extracts exerted anti-proliferative effects at higher doses. This latter result was indicative of unique component(s) present in kefir that were not present in either yogurt or milk that were exerting antiproliferative effects specific to tumor cells. Peptide analyses and capillary electrophoretic profiles showed that kefir-mediated milk fermentation led to an increase in peptide concentrations and a change in peptide profiles in comparison to milk or yogurt. The fermentation process of kefir that was mediated by both bacteria and yeast clearly induced the production of active ingredients that inhibits the growth of the estrogen-positive breast cancer cells with little cytotoxicity as shown in the lack of effects in the normal cells. Whether the same inhibitory effects will apply to the estrogen-negative cells is yet to be tested. However, since about 60% of the breast cancer cells are estrogen receptor positive, the kefir extract should have a wide application to substitute or co-administer with other cancer treatment therapy such as tamoxifen.

The main achievement of the thesis was the demonstration that an HPLC fraction (RP-HPLC Fraction 30) possessed dose-dependent antiproliferative effect on MCF-7 cells. This latter fraction was isolated and identified with the help of protein separation and purification methods (i.e. high speed centrifugation, molecular weight cut-off filtration, SEC-HPLC and RP-HPLC) and the cell culture screening model. Although RP-HPLC analysis indicated that the Fraction 30 was still a mixture of complex molecules, characterization of the bioactive fraction using mass spectrometry (MS-MS) indicated the main components in the fraction are likely fragments of kefiran and/or ceramide containing compounds such as gangliosides. We observed that kefir-mediated milk fermentation led to an increase in peptide concentrations and a change in peptide profiles in comparison to milk or yogurt. The presence of peptides was also suggested in Fraction 30 as determined by the methodology to detect peptide content using *o*-phthaldialdehyde (OPA) methodology. Surprsingly,

however, the MS data did not support the presence of peptide in the active fractions. Research on antitumor properties of fermented milk including kefir indicated that a variety of components present in fermented milk, such as conjugated linoleic acid (Schonberg *et al.*, 1995), sphingolipids (Dillehay *et al.*, 1994; Schmelz *et al.*, 1996), polysaccharides (Shiomi *et al.*, 1982), organic acids (Garrote *et al.*, 2000), and some proteins and peptides (Svensson *et al.*, 1999) might be responsible for the observed antimutagenic and antitumor effects. On this basis, it is likely that bioactive components in kefir could act both independently and synergistically to exert antitumor effects. Instead of searching for single compound in kefir, a mixture or a combination of components may be the best approach to have the highest potency for antitumor properties. In fact, Fraction 30 increased significantly the susceptibility of MCF-7 cells to tamoxifen, a commonly used anti-breast cancer drug. These results thus provide the rationale for future research to explore the potential of using kefir extracts as co-drugs for breast cancer chemotherapy and/or a functional food used for prevention of breast cancer.

The third achievement of the thesis was the investigation of possible mechanisms that might involve in the observed antiproliferative effects. A variety of kefir fractions including the whole extract as well as MWCO SEC-HPLC and RP-HPLC fractions that represent different combinations of bioactive components were tested for their antiproliferative effects in order to identify the bioactive molecules. The results indicated that different fractions from kefir could depress tumor cell growth via different pathways. Hence, whole extracts of kefir depleted glutathione (GSH) in MCF-7 cells, while the SEC-HPLC Fraction 7 and the RP-HPLC Fraction 30 induced GSH productions in MCF-7 cells. On the other hand, all the isolated fractions induced the production of TNF-alpha. Although we did not measure apoptosis in the cell lines, the antiproliferative effect on MCF-7 cells may likely be explained by the TNF-alpha induced apoptosis. The relationship between GSH production and TNF-alpha induction, however, remains to be elucidated.

The final chapter of the thesis explored the effects of in vitro digestion on kefir and compared that to the process of fermentation. Comparison of RP-HPLC elusion profiles and CE electrograms among kefir, milk, whey protein isolates, and their digests indicated that both microflora fermentation and in vitro enzymatic digestion have similar effects on protein and peptide profiles. However, the cleavage site on the protein may be different resulting in different HPLC profiles and electrograms between the two processes. In future studies, it is recommended to digest the kefir products to mimic the effects of GI tract digestion as kefir products are usually orally consumed. The resulting products will likely be more representative of possible bioactive compounds that will be bioavailable in vivo.

The overall results suggested that kefir fermentation resulted in the compositional changes of cow's milk and generation of novel bioactive components. More than one single compound in the kefir may be responsible for the observed antitumor properties of kefir. Kefir extract and its sub-fractions stand for different combinations of bioactive compounds which acted via different pathways. Whole kefir extract contains proteins, peptides, polysaccharides, sphingolipids, and unknown bioactive compounds; MWCO and SEC-HPLC fractions contain certain molecular weight of above mentioned compounds; while the main component of Fraction 30 may include sphingolipids containing ceramide analogs or ganglosides. Previous studies have shown that fermented whey proteins depleted cellular GSH (Baruchel andViau, 1996); sphingolipids (i.e. sphingomyelin) enhanced ceramide production leading to an increase of cellular GSH (Modrak, 2004). Furthermore, Hayter *et al.* (2001) reported that TNF-alpha induced a depletion of GSH, and exogenous addition GSH blocked TNF-alpha-induced sphingomyelin (SM) hydrolysis which leads to an increase of cellular ceramide as well as TNF-alpha-induced cell death. Overall, the cellular GSH was depleted when MCF-7 cells were treated with whole kefir extract. For fraction 30, the observed increase of GSH and TNF-alpha may be mainly explained by ceramide pathway. Addition of kefir fraction (i.e. fraction 30) means addition of certain kinds of sphingolipids. This leads to the accumulation of ceramide in the

cells. Hannun *et al.* (2000) demonstrated that ceramide induced reactive oxygen species in mitochondria, which in turn induced cellular GSH. In addition, in our study, antiproliferative effect of tamoxifen was observed at higher doses than levels used by other researchers. This indicated somewhat tamoxifen resistance in the current MCF-7 cells we used. Combination of kefir fraction 30 and tamoxifen enhanced antiproliferative effect on MCF-7 cells. This is much similar as reported by Lucci *et al.* (1999). Drug resistant cells had an increased capacity to convert ceramide to glucosylceramide. Blocking the conversion of ceramide to glucosylceramide increases MCF-7 AdrR (adriamycin resistant) cells sensitivity to ceramide as well as to antitumor agents. Treatment of MCF-7 cells with adriamycin elicited a 5-fold increase in ceramide, and caused oligonucleosomal fragmentation, characteristic to apoptosis. In MCF-7 AdrR cells, neither C6-ceramide nor tamoxifen was cytotoxic. However, the addition of tamoxifen to the C6-ceramide treatment regimen reduced cell viability to 42% and elicited apoptosis. The result of Lucci's study supported our findings.

In conclusion, the cell culture screening model can be useful tool to explore bioactive component in other fermented milk or similar products. The data presented in this thesis provided scientific evidence for possible health benefits of kefir and provide valuable information regarding possible mechanisms and bioactive components that could be involved in the antitumor effects of kefir.

The present thesis thus provides encouraging findings for further work in (1) further characterization of bioactive compounds in kefir with tools such as chemical synthesis of proposed molecules, and/or nuclear magnetic resonance (NMR) spectrometer analysis; (2) study of combination effects of different fractions of kefir; (3) analysis of cellular ceramide level to confirm the proposed ceramide pathway; (4) the kefir fractions showing antiproliferative effects on MCF-7 cells may also be tested with other cancer cell; (5) use of animal model or human clinic trials to study antitumor properties of kefir.

REFERENCES

Abdelali, H., Cassand, P., Soussotte, V., Bocabeille, B.K. and Narbonne, J.F. (1995). Antimutagenicity of components of dairy products. *Mutation Res.* 331: 133-141.

Abdelali, H., Cassand, P., Soussotte, V., Daubeze, M., Bouley, C. and Narbonne, J.F. (1995). Effect of dairy products on initiation of precursor lesions of colon cancer in rats. *Nutr. Cancer.* 24: 121 – 132.

Agerbaek, M., Gerdes, L.V., and Richelsen, B. (1995). Hypocholesterolaemic effect of a new fermented milk product in healthy middle-aged men. *Eur. J. Clin. Nutr.* 49: 246-352.

Alm, L. (1983). Survival rate of *Salmonella* and *Shigella* in fermented milk products with and without added human gastric juics: an in vitro study. *Prog. Food Nutr. Sci.* 7: 19-26.

Alm, L. (1981). Kefir: characterization of a fermented milk. *J. Sci. Food Agric.* 32: 1247 - 1251.

Angulo, L., Lopez, E., and Lema, C. (1993). Microflora present in kefir grains of the Galician Region (North-West of Spain). *J. Dairy Res.* 60: 263-267.

Baricault, L., Denariaz, G., Houri, J.J., Bouley, C., Sapin, C. and Trugnan, G. (1995). Use of HT-29, a cultured human colon cancer cell line, to study the effect of fermented milks on colon cancer cell growth and differentiation. *Carcinogenesis.* 16(2): 245-252.

Baruchel, S. and Viau, G. (1996). *In vitro* selective modulation of cellular glutathione by a humanized native milk protein isolate in normal cells and rat mammary carcinoma model. *Anticancer Research.* 16: 1095-1100.

Baruchel, S., Wang, T., Farah, R., Jamali, M. and Batist, G. (1995). In vivo selective modulation of tissue glutathione in rat mammary carcinoma model. *Biochemical Pharmacology.* 10 (9): 1505-1508.

Batinkov, E.L. (1971). Use of milk and kefir in peptic ulcer of the stomach and duodenum. *Vopr. Pitan.* 30: 89-91. (English abstract)

Beena, A. and Prasad, V. (1997). Effect of yogurt and bifidus yogurt fortified with skim milk powder, condensed whey and lactose-hydrolyzed condensed whey on serum cholesterol and triacylglycerol concentrations in rats. *J. Dairy Res.* 64: 453-457.

Belobrajdic, D.P. and McIntodh, G.H. (2000). Dietary butyrate inhibits NMU-induced mammary cancer in rats. *Nutr. Cancer.* 36(2): 217-223.

Benard, O. and Balasubramanian, K.A. (1997). Modulation of glutathione level during butyrate-induced differentiation in human colon derived HT-29 cells. *Mol. Cell Biochem.* 170: 109-114.

Benno, Y. and Mitsuoka, T. (1992). Impact of *Bifidobacterium longum* on human fecal microflora. *Microbiol. Immunol.* 36: 683-694.

Bezault, J., Bhimani, R., Wiprovnick, J. and Furmanski, P. (1994). Human lactoferrin inhibits growth of solid tumors and development of experimental metastases in mice. *Cancer Res.* 54: 2310-2312.

Biffi, A., Coradini, D., Larsen, R., Riva, L. and Di Fronzo, G. (1997). Antiproliferative effect of fermented milk on the growth of a human breast cancer cell line. *Nutr. Cancer.* 28 (1): 93-99.

Bodana, A.R. and Rao, D.R. (1990). Antimutagenic activity of milk fermented by *Streptococcus thermophilus* and *Lactobacillus bulgaricus. J. Dairy Sci.* 73: 3379-3384.

Borek, C. (1993). Molecular mechanisms in cancer induction and prevention. *Environ. Health Perspcet.* 101 (Suppl. 3): 237-245

Borek, C., Ong, A., Stevens, V. L., Wang E. and Merrill, A. H. (1991). Long chain (sphingoid) bases inhibit multistage carcinogenesis in mouse C3H/10T1/2 cells treated with radiation and phorbol 12-myristate 13-acetate. *Proc. Natl. Acad. Sci.* USA 88: 1953-1957.

Borsellino, N., Crescimanno, M., Flandina, C., Flugy, A. and Alessandro, N. (1994). Combined activity of interleukin-1alpha or TNF-alpha and doxorubin on multidrug resistant cell lines: evidence that TNF and DXR have synergistic antitumor and differentiation-inducing effects. *Anticancer Research.* 14: 2643-2648.

Bosselaers, I.E.M., Caessens, P.W., Van Boekel, M.A.J.S. and Alink G.M. (1994). Differential effects of milk proteins, BSA and soy protein on 4NQO or MNNG-induced SCEs in V79 cells. *Food Chem. Toxicol.* 32: 905-909.

Bottazzi, V., Zacconi, C., Sarra, P.G., Dallavalle, P. and Parisi M.G. (1994). Kefir: microbiologica, chimica e tecnologia. Industr. *Latte.* 30: 41-62.

Bouhnik, Y., Flourie, B., Andrieux, C., Bisetti, N., Briet, F. and Ranbaud, J.C. (1996). Effects of *Bifidobacterium sp* fermented milk ingested with or without inulin on colonic bifidobacteria and enzymatic activities in healthy humans. *Eur. J. Clin. Nutr.* 50: 269-273.

Bounous, G., Papenburg, R., Kongshavn, P.A.L. and Gold, P. (1988). Dietary whey protein inhibits the development of dimethylhydrazine induced malignancies. *Clin. Invest. Med.* 11: 213-217.

Bounous, G. (2000). Whey protein concentration (WPC) and glutathione modulation in cancer treatment. *Anticancer Research.* 20: 4785-4792.

Bounous, G., Batist, G. and Gold P. (1991). Whey proteins in cancer prevention. *Cancer Lett.* 57: 91-94

Bourtourault, M., Buleon, R., Samperez, S. and Jouan, P. (1991). Effet des protein du lactoserum bovin sur la multiplicaton de cellules cancereuse humaines. *C.R. Soc. Biol.* 185: 319-323. (English abstract)

Bourlioux, P. and Pochart, P. (1988). Nutritional and health properties of yogurt. *World Review of Nutrition and Dietetics.* 56: 217-258.

Brialy, C., Rivalland, P., Coiffard, L. and De Roeck Holtzhauer, Y. (1995). Microbiological study of lyophilized dairy kefir. *Folia Microbiol.* 40: 198-200.

Cai, Z., Bettaieb, A., Mahdani, N. E., Legres, L. G., Stancou, R., Masliah, J., and Chouaib, S. (1997). Alteration of the sphingomyelin/ceramide pathway is associated with resistance of human breast carcinoma MCF-7 cells to tumor necrosis factor-alpha-mediated cytotoxicity. *J. Biol. Chem.* 272: 6918–6926.

Cassand, P., Abdelali, H., Bouley, C., Denariaz, G. and Narbonne, J.F. (1994). Inhibitory effect of dairy products on the mutagenicities of chemicals and dietary mutagens. *J. Dairy Res.* 61: 545-552.

Cevikbas, A., Yemni, E., Ezzedenn, F.W. and Yardimici, T. (1994). Antitumoural, antibacterial and antifungal activities of kefir and kefir grain. *Phytother. Res.* 8: 78-82.

Chan, J.M., Giovannucci, E., Anderson, S.O., Yuen, J., Adami, H.O. and Wolk, A. (1998). Dairy products, calcium, phosphorous, vitamin D, and risk of prostate cancer (Sweden). *Cancer Causes Control.* 9: 559-566.

Chen, C.Y., Huang, Y.L. and Lin, T.H. (1998). Association between oxidative stress and cytokine production in nickel-treated rats. *Arch. Biochem. Biophys.* 356: 127-132.

Chen, X. and Batist, G. (1998). Sentization effect of L-2 oxothiazolidine-4-carboxylate on tumor cells to melphalan and the role of 5-oxo-Lprolinase in glutathione modulation in tumor cells. *Biochem. Pharmacol.* 56 (6): 743-749.

Church, F.C., Swaisgood, H.E., Porter, D.H. and Catinani, G.L. (1983) Spectrophotometric assay using o-phthaldialdehyde for determination of proteolysis in milk and isolation milk proteins. *J. Dairy Sci.* 66: 1219-1227.

Cimoli, G., Valenti, M., Venturini, M., Cafiero, F. and Russo, P. (1993). Reversion of at-MDR by tumr necrosis factor on ovarian cancer cell line. *Proc. Am. Assoc. Cancer Res.* 34: 328-335.

Colarow, L., Turini, M., Teneberg, S. and Berger A. (2003). Characterization and biological activity of gangliosides in buffalo milk. *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids.* 1631: 94-106.

Daniel, H., Vohwinkel, H. and Rehner, G. (1993). Effects of casein and β-casomorphins on gastrointestinal motility in rats. *J. Nutr.* 120: 252-257.

Davis, M. A., Flaws, J. A., Young, M., Collins, K. and Colburn, N. H. (2000). Effect of ceramide on intracellular glutathione determines apoptotic or necrotic cell death of JB6 tumor cells. *Toxicol. Sci.* 53: 48-55.

De Simmone, C., Ciardi, A., Drassi, A., Lambert-Gardini, S., Tzantzoglou, S., Trinchieri, V., Moreti, S. and Jirillo, E. (1992). Effect of Bifidobacterium bifidum and Lactobacillus acidophilus on gut mucosa and peripheral blood B lymphocytes. *Immunopharmacol. Immunotoxicol.* 14: 331-340.

Trickler, D., Shklar, G. and Schwartz, J. (1993). Inhibition of oral carcinogenesis by glutathione. *Nutr. Cancer.* 20: 139-144.

Dillehay, D. L., Webb, S. J., Schmelz, E.-M. and Merrill, A. H., Jr. (1994). Dietary sphingomyelin inhibits 1,2-dimethylhydrazine-induced colon cancer in CF1mice. *J. Nutr.* 124: 615–620.

Doisneau-Sixou, S.F., Cestac, P., Faye, J.C., Favre, G. and Sutherland R. (2003). Additive effects of tamoxifen and the farnesyl transferase inhibitor FTI-277 on inhibition of MCF-7 breast cancer cell-cycle progression. *Int. J. Cancer.* 106: 789-798.

Donato, N.J. and Klostergaard, J. (2004). Distinct stress and cell destruction pathways are engaged by TNF and ceramide during apoptosis of MCF-7 cells. *Experimental Cell Research.* 294: 523-533.

Donenko, F.V., Sitdikova, S.M., Kabieva, A.O., Polotskii, B.E., Machaladze, Z.O., Davydov, M.I. and Moroz LV. (1995). Use of glutathione inhibitors and glutathione transferases to overcome tumor resistance to cytostatics in vivo. *Bulletin of Experimental Biology and Medicine.* 119(2): 212-214.

Donnet-Hughes, A, Rochat, F., Serrant, P., Aeschkimann, J.M. and Schiffrin, E.J. (1999). Modulation of nonspecific mechanisms of defence by lactic acid bacteria: effect dose. *J. Dairy Sci.* 82: 863-869.

Duitschaever, C.L., Kemp, N. and Emmons, D. (1988). Comparative evaluation of five procedures for making kefir. *Milchwiss.* 43: 343-345.

Fang, Y.Z., Yang, S. and Wu, G. (2002). Free radicals, antioxidants, and nutrition. *Nutrition.* 18:872-879.

Farnworth, E.R. (1999). Kefir: from folklore to regulatory approval. *Journal of Nutraceuticals, Functional & Medical Foods.* 1(4): 57-68.

Fillet, M., Bentires-Alj, M., Deregowski, V., Greimers, R., Gielen, J., Piette, J., Bours, V. and Merville, M.P. (2003). Mechanisms involved in esopenous C2- and C6-ceramide-induced cancer cell toxicity. *Biochemical Pharmacology.* 65: 1633-1642.

Fox, P.F. and Flynn, A. (1992). Biological properties of milk proteins. In Fox P.F. (Ed.) *Advances in Dairy Chemistry.* Vol. 1. 2nd Edn. Chapman and Hall, London, p. 255-284.

France-Lanord, V., Brugg, B., Michel, P. P., Agid, Y. and Ruberg M. (1997). Mitochondrial free radical signal in ceramide-dependent apoptosis : a putative mechanism for neuronal death in Parkinson's disease. *J. Neurochem.* 69: 1612-1621.

Furukawa, N., Matsuoka, A. and Yamanaka, Y. (1990). Effects of orally administered yogurt and kefir on tumor growth in mice. *J. Japan. Soc. Nutr. Food Sci.* 43: 450-453. (English abstract)

Furukawa, N., Matsuoka, A., Takahashi, T. and Yamanaka, Y. (1991). Effects of fermented milk on the delayed-type hypersensitivity response and survival day in mice bearing Meth-A. *Anim. Sci .Technol.* 62: 579-585. (English abstract)

Furukawa, N., Matsuoka, A., Takahashi, T. and Yamanaka, Y. (2000). Anti-metastatic effect of kefir grain components on Lewis lung carcinoma and highly metastatic B16 melanoma in mice. *Tokyo Nogyo Daigaku Nogaku Shuho.* 45(1): 62-70.

Ganjam, L.S., Thornton, W.H., Marshal, R.T., Marshall, R.T. and MacDonald, R.S. (1997). Antiproliferative effects of yogurt fractions obtained by membrane dialysis on cultured mammalian intestinal cells. *J. Dairy Sci.* 80 (10): 2325-2329.

Ganjam, L.S., Thornton, W.H. Jr, Marshall, R.T. and Macdonald, R.S. (1997). Antiproliferative effects of yogurt fractions obtained by membrane dialysis on cultured mammalian intestinal cells. *J. Dairy Sci.* 80(10): 2325-2329.

Garcia-Ruiz, C., Colell, A., Mari, M., Morales, A. and Fernandez-Checa, J. C. (1997). Direct effect of ceramide on the mitochondrial electron transport chain leads to generation of reactive oxygen species. *J. Biol. Chem.* 272: 11369-11377.

García-Ruiz, C., Colell, A., Marí, M., Morales, A., Calvo, M., Enrich, C. and Fernández-Checa, J. C. (2003). Defective TNF-alpha–mediated hepatocellular apoptosis and liver damage in acidic sphingomyelinase knockout mice. *The Journal of Clinical Investigation.* 111(2): 197-209.

Garrote, G.L., Abraham, A.G. and Antoni, G.L. (2000). Inhibitory power of kefir; the role of organic acids. *Journal of Food Protection.* 63(3): 364-369.

Gerschenson, L.E. and Rotello, R.J. (1992). Apoptosis: a different type of cell death. *FASEB J.* 6: 2450-2455.

Giovannucci, E. (1998). Dietary influences of 1,25-$(OH)_2$ vitamin D in relation to prostate cancer: a hypothesis. *Cancer Causes Control.* 9: 567-582.

Glinghammar, B., Venturi, M, Rowland, I.R. and Rafter, J.J. (1997). Shift from a dairy product-rich to a dairy product-free diet: influence on cytotoxicity and genotoxicity of fecal water-potential risk factors for colon cancer. *Am. J. Clin. Nutr.* 66: 1277-1282.

Goldin, B.R., Gorbach, S.L. (1980). Effect of lactobacillus acidophilus dietary supplements on 1,2-dimethylhydrazine dihydrochloride-induced intestinal cancer in rats. *JNCI.* 64: 263-265.

Goldin, B.R., Gorbach, S.L., Saxelin, M., Barakat, S., Gualtiere, L. and Salminen, S. (1992). Survival of Lactobacillus species (strain GG) in human gastrointestinal tract. *Dig. Dis. Sci.* 37: 121-128.

Goldkorn, T., Balaban, N., Shannon, M., Chea, V., Matsukuma, K., Gilchrist, D., Wang, H. and Chan, C. (1998). H_2O_2 acts on cellular membranes to generate ceramide signaling and initiate apoptosis in tracheobronchial epithelial cells. *J. Cell. Sci.* 111(Pt. 21): 3209-3220.

Gorbach, S.L. (1984). Estrogens, breast cancer, and intestinal flora. *Rev. Infect. Dis.* 6: S85-90.

Gourley, G.R., Kreamer, B.L. and Cohnen, M. (1997). Inhibition of beta-glucuronidase by casein hydrolysate formula. *Journal of pediatric gastroenterology and nutrition*. 25(3): 267-272.

Gudz, T.I., Tseng, K-Y. and Hoppel, C.L. (1997). Direct inhibition of mitochondrial respiratory chain complex III by cell-permeable ceramide. *J. Biol. Chem.* 272: 24154-24158.

Guo, X., Chan, H.M., Guevremont, R. and Siu, K.W.M. (1999). Analysis of metallothioneins by means of capillary electrophoresis coupled to electrospray mass spectrometry with shealthless interfacing. *Rapid Commun. Mass Spectrom.* 13: 500-507.

Guzel-Seydim, Z.B., Seydim, A.C. and Greene, A.K. (2003). Comparison of amino acid profiles of milk, yogurt and Turkish kefir. *Milchwissenschaft.* 58(3/4): 158-160.

Hague, A. and Paraskeva C. (1995). The short-chain fatty acid butyrate induces apoptosis in colorectal tumor cell line. *Eur J. Cancer Prev.* 4: 359-364.

Hakimuddin, F., Paliyath, G. and Meckling, K. (2004). Selective cytotoxicity of a red grape wine flavonoid fraction against MCF-7 cells. *Breast Cancer Res. Treat.* 85: 65-79.

Hakkak, R., Korourian, S., Ronis, M.J.J., Johnston J.M. and Badger, T.M. (2001). Dietary whey protein protects against azoxymethane-induced colon tumors in male rats. *Cancer Epidemiol. Biomark. Prev.* 10: 555-558.

Hakkak, R., Korourian, S., Shelnutt, S.R., Lensing, S., Ronis, M.J.J., and Badger, T.M. (2000). Diets containing whey protein or soy protein isolate protect against 7,12-dimethylbenz(a)anthracene-induced mammary tumors in female rats. *Cancer Epidemiol. Biomark. Prev.* 9: 113-117.

Hall, A.G. (1999). The role of glutathione in the regulation of apoptosis. *European Journal of clinical investigation.* 29: 238-245.

Hallé, C, Leroi, F, Dousset, X. and Pidoux, M. (1994). Les kéfirs : des associations bactéries lactiques - levures. In Bactéries lactiques: Aspects fondamentaux et technologiques. Vol. 2, ed. H de Roissart, FM Luquet. Uriage, France: Lorica, p.169-182.

Hannun, Y. A. (1996). Functions of ceramide in coordinating cellular responses to stress. *Science.* 274: 1855-1859.

Hannun, Y.A. (1994). The sphingomyelin cycle and the second messenger function of cetamide. *J. Biol. Chem.* 269: 3125-3128.

Hannun, Y.A., and Luberto, C. (2000). Ceramide in the eukaryotic stress response. *Trends Cell Biol.* 10: 73-80.

Hata, Y., Yamamoto, M., Ohni, M., Nakajima, K., Nakamura, Y. and Takano T. (1996). A placebo controlled study of the effect of sour milk on blood pressure in hypertensive subjects. *Am J. Clin. Nutr.* 64: 767-771.

Hayter, H.L., Pettus, B.J., Ito, F., Obeid, L.M. and Hannun, Y.A. (2000). TNF-alpha-induced glutathione depletion lies downstream of $cPLA_2$ in L929 cells. *FEBS Letters.* 507: 151-156.

Hertzler, S.R. and Clancy, S.M. (2003). Kefir improves lactose digestion and tolerance in adults with lactose maldigestion. *J. Am. Diet. Assoc.* 103: 582-587.

Holt, P.R., Atillasoy, E.O., Gilman, J., Guss, J., Moss, S.F., Newmark, H., Yang, K. and Lipkin, M. (1998). Modulation of abnormal colonic epithelial cell proliferation and differentiation by low-fat dairy foods: a randomized controlled trial. *JAMA.* 280: 1074-1079.

Hosono, A., Tanabe, T. and Otani, H. (1990). Binding properties of lactic acid bacteria isolated from kefir milk with mutagenic amino acid pyrolyzates. *Milchwiss.* 45: 647-651.

Hosono, A., Wardojo, R. and Otani, H. (1990). Inhibitory effects of lactic acid bacteria from fermented milk on the mutagenicities of volatile nitrosamines. *Agri. Biol. Chem.* 54: 1639-1643.

Hosono, A., Shashikanth, K.N. and Otani, H. (1988). Antimutagenic activity of whole casein on the pepper-induced mutagenicity to streptomycin-dependant strain SD 510 of salmonela typhimmurium TA 98. *J Dairy Res.* 55: 435-442.

Huang, C., Ma, W., Ding, M., Bowden, G. T., and Dong, Z. (1997). Direct evidence for an important role of sphingomyelinase in ultraviolet-induced activation of c-Jun N-terminal kinase. *J. Biol. Chem.* 272: 27753-27757.

Hungarian Democratic Republic (1981): *Kefir. Standard MSZ.* 122, p.5.

Hursting, S.D., Slaga, T.J., Fischer, S.M., DiGiovanni, J. and Phang, J.M. (1999). Mechanism-based cancer prevention approaches: targets, examples, and the use of transgenic mice. *Journal of the National Cancer Institute.* 91: 215-225.

Ip, C., Singh, M., Thompson, H. and Scimeca, J.A (1994). Conjugated linoleic acid suppresses mammary carcinogenesis and proliferative activity of the mammary gland in the rat. *Cancer Res.* 54: 1212-1215.

Iwai, K., Kondo, T., Watanabe, M., Yabu, T., Kitano, T., Taguchi, Y., Umehara, H., Takahashi, A., Uchiyama, T. and Okazaki, T. (2003). Ceramide increases oxidative damage due to inhibition of catalase by caspase-3-dependent proteolysis in HL-60 cell apoptosis. *J. Biol. Chem.* 278(11): 9813-9822.

Jacobson, M.D., Burne, J.F., King, M.P., Miyashita, T., Reed, J.C. and Raff, M.C. (1993). Bcl blocks apoptosis in cells lacking mitochondrial DNA. *Nature.* 361: 365-369.

James, S. J.and Muskhelishvili, L. (1994). Rates of apoptosis and proliferation vary with caloric intake and may influcence incidence of spontaneous hepatoma in C57BL/6 C3H F1 mice. *Cancer Research.* 54(21): 5508-5510.

Jeannin, P., Delneste, Y., Lecoanet-Henchoz, S., Gauchat, J.F., Life, P., Holmes, D. and Bonnefoy, J.Y. (1995). Thiols decrease human interleukin (IL)-4 production and IL-4-induced immunoglobulin synthesis. *J. Exp. Med.* 182: 1785-1792.

Ji, S.T. (1997). Studies on the antigenotoxic and anticarcinogenic effects of lactic acid bacteria: characterization of bacterial cell fractions on detoxifying food relevant carcinogens. *Medizinische Fakultat der Universitat.* (in German, English abstract).

Judith Butriss. (1997). Nutritional properties of fermented milk products. *International Journal of Dairy Technology.* 50: 21-27.

Kaipia, A., Chun, S.Y., Eisenhauer, K. and Hsueh, A.J. (1996). Tumor necrosis factor-alpha and its second messenger, ceramide, stimulate apoptosis in cultured ovarian follicles. *Endocrinology.* 137: 4864-4870.

Kang, Y.J. and Enger, M.D. (1990). Glutathione content and growth in A549 human lung carcinoma cells. *Exp. Cell Res.* 187: 177-79.

Karine, T. and Douglas, L.S. (2001). Kefir milk enhances intestinal immunity in young but not old rats. *J. Nutr.* 131: 807-812.

Kathleen, H. Moore A., Ettinger, C. and Melvin T. Y. (2000) Variation in Ganglioside Content of Bovine Dairy Products. *Journal of Food Composition and Analysis.* 13: 783-790.

Kayer, H. and Meisel, H. (1996). Stimulation of human peripheral blood lymphcytes by bioactive peptides derived from milk protein. *FEBS letters.* 383: 18-20.

Kennedy, R.S., Konok, G.P., Bounous, G., Baruchel, S. and Lee, T.D. (1995). The use of a whey protein concentrate in the treatment of patients with metastatic carcinoma: a phase I-II clinical study. *Anticancer Res.* 15: 2643-2649.

Kerr, J.F., Winterford, C.M. and Harmon, B.V. (1994). Apoptosis: its significance in cancer and cancer therapy. *Cancer.* 73: 2013-2026.

Kneifel, W. and Mayer, H.K. (1991). Vitamin profiles of kefirs made from milks of different species. *Int. J. Food Sci. Tech.* 26: 423-428.

Kohler, C., Hakansson, A., Svanborg, C., Orrenius, S. and Zhivotovsky, B. (1999). Protease activation in apoptosis induced by MAL. *Experimental Cell Research.* 249(2): 260-268.

Kohwi, Y., Imai, K., Tamura, Z. and Hashimoto Y. (1978). Antitumor effect of bifidobacterium infantis in mice. *Gann.* 69: 613-618.

Komai, M. and Nanno, M. (1992). Intestinal microflora and longevity. In: Functions of fermented milk, Nakazawa Y and Hosono.A, eds. London, Elsevier Applied Science, p.343-346.

Kondo, T., Matsuda, T., Tashima, M., Umehara, H., Domae, N., Yokoyama, K., Uchiyama, T. and Okazaki, T. (2000). Suppression of Heat Shock Protein-70 by Ceramide in Heat Shock-induced HL-60 Cell Apoptosis. *J. Biol. Chem.* 275: 8872-8879.

Kooiman, P. (1968). The chemical structure of kefiran, the water-soluble polysaccharide of the kefir grain. *Carbohyd. Res.* 7: 200-211.

Korneva, V.V., Nabukhotnyi, T.K., Cherevko, S.A., Kravets, A.A. and Sidorchuk, I.I. (1979). Use of propiono-acidophylus milk in the complex treatment of intestinal disbacteriosis in infants with taphylococcal infections and sepsis. *Vopr. Pitan.* 5: 37-40.

Koroleva, N.S. (1988). Technology of kefir and kumy. *IDF Bull.* 227: 96-100.

Koroleva, N.S. (1988). Starters for fermented milks. Section 4: Kefir and kumys starters. *IDF Bull.* 227: 35-40.

Kosikowski, F. (1982). Cheese and fermented milk foods. 2nd ed. Kosikowki and Associates, New York.

Kroger, M. (1993) Kefir. *Cult. Dairy. Prod. J.* 28(2): 26-29.

Kubo, M., Odani, T., Nakamura, S., Toumaru, S. and Matsuda, H. (1992). Pharmacological study on kefir- a fermented milk product in Caucasus I. On antitumor activity. *Yakugaku Zasshi.* 112: 489-495. (English abstract)

Kubow, S. and Fotouhinia, M. PCT Int. Appl., 2001.

Kuchan, M.J., and Milner, J.A. (1992). Influence of intra-cellular glutathione on selenite-mediated growth inhibition of canine mammary tumor cells. *Cancer Res.* 52: 1091-1095.

Kuo Ching-Yun and Lin Chin-Wen. (1999). Taiwanese kefir grains: their growth. Microbial and chemical composition of fermented milk. *The Australian Journal of Dairy Technology.* 54: 19-23.

Kurmann, J.A., Rasic, J.Lj. and Kroger, M. (1992). Encyclopedia of fermented fresh milk products. New Yok: Van Nostrand Reinhold.

Kwak, H.S., Park, S.K., and Kim, D.S. (1996). Biostabiliztion of kefir with a nonlactose-fermenting yeast. *J. Dairy Sci.* 79: 937-942.

Laffineur, E., Genetet, N., and Leonil, J. (1996). Immunomodulatory activity of beta-casein permeate medium fermented by lactic acid bacteria. *J. Dairy Sci.* 79(12): 2112-20.

La Riviere, J.W.M., Kooiman, P. and Schmidt, K. (1967). Kefiran, a novel polysaccharide produced in the kefir grain by *Lactobacillus brevis. Arch. Mikrobiol.* 59: 269-278. (English abstract)

Laursen, I., Briand, P. and Lykkesfeldt, A.E. (1990). Serum albumin as a modulator of human breast cancer cell line, MCF-7. *Anticancer Res.* 10: 343-352.

Lavrentiadou, S. N., Chan, C., Kawcak, T., Ravid, T., Tsaba, A., van Der Vliet, A., Rasooly, R. and Goldkorn, T. (2001). Ceramide-mediated apoptosis in lung epithelial cells is regulated by glutathione *Am. J. Respir. Cell Mol. Biol.* 25: 676-684.

Lee, Y.K. and Salminen, S. (1995). The coming age of probiotics. *Trends in Food Science and Technology.* 6: 241-245.

Lemonnier, L. A., Dillehay, D. L., Vespremi, M. J., Abrams, J., Brody, E. and Schmelz, E. M. (2003) Sphingomyelin in the suppression of colon tumors: prevention versus intervention. *Arch. Biochem. Biophys.* 419: 129-138.

Libudzisz, Z. and Piatkiewicz, A. (1990). Kefir production in Poland. *Dairy Ind. Int.* 55: 31-33.

Lidbeck, A., Overvik, E., Rafter, J., Nord, C.E., and Gustafsson, J.A. (1992). Effect of *Lactobacillus acidophils* supplements on mutagen excretion in feces and urine in humans. *Microb. Ecol. Health Dis.* 5: 59-67.

Link-Amster, H., Rochat, F., Saudan, K.Y., Mignot, O. and Aeschlimann J.M. (1994). Modulation of a specific humoral immune response and changes in intestinal flora mediated through fermented milk intake. *FEMS Immunol. Med. Microbiol.* 10: 55-63.

Liu, B., Andrieu-Abadie, N., Levade, T., Zhang, P. and Obeid, L.M. (1998). Glutathione regulation of neutral sphingomyelinase in tumor necrosis factor-α-induced cell death. *J. Biol. Chem.* 273(18): 11313-11320.

Liu, M., Peiling, J.C., Ju, J., Chu, E. and Brash, D.E. (1998). Antioxidant action via p53-mediated apoptosis. *Cancer Res.* 58: 1723-1729.

Liu, Y-Y, Han, T-Y, Giuliano, A.E., Ichikawa, S., Hirabayashi, Y. and Cabot, M.C. (1999). Glycosylation of ceramide potentiates cellular resistance to tumor necrosis factor-alpha-induce apoptosis. *Exp. Cell Res.* 252: 464-470.

Liu, B., and Hannun, Y. A. (1997). Inhibition of the neutral magnesium-dependent sphingomyelinase by glutathione. *J. Biol. Chem.* 272: 16281-16287.

Liu, B., Andrieu Abadie, N., Levade, T., Zhang, P., Obeid, L. M. and Hannun, Y. A. (1998). Glutathione regulation of neutral sphingomyelinase in tumor necrosis factor-a-induced cell death. *J. Biol. Chem.* 273: 11313-11320.

Lotan, R. (1995). Retinoids and apoptosis: implications for cancer chemoprevention and therapy. *J. Natl. Cancer Inst.* 87: 1655-1657.

Lovat, P.E., Di Sano, F., Corazzari, M., Fazi, B., Donnorso, R.P., Pearson, A.D., Hall, A.G., Redfern, C.P. and Piacentini, M. (2004). Gangliosides link the acidic sphingomyelinase-mediated induction of ceramide to 12-lipoxygenase-dependent apoptosis of neuroblastoma in response to fenretinide. *J. Natl. Cancer Inst.* 96(17): 1288-1299.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951). Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193: 265-323.

Luberto, C., Hassler, D. F., Signorelli, P., Okamoto, Y., Sawai, H., Boros, E., Hazen-Martin, D. J., Obeid, L. M., Hannun, Y. A. and Smith, G. K. (2002). Inhibition of tumor necrosis factor-induced cell death in MCF-7 by a novel inhibitor of neutral sphingomyelinase. *J. Biol. Chem.* 277(43): 41128 - 41139

Lucci, A., Han, T.Y., Liu, Y.Y., Giuliano, A.E. and Cabot, M.C. (1999). Modification of ceramide metabolism increases cancer sensity to cytotoxics. *Int. J. Oncol.* 15(3): 541-546.

Lupton, J.R. (1995) Butyrate and colonic cytokinetics: differences between in vitro and in vivo studies. *Eur. J. Cancer. Prev.* 4: 373-378.

Mann, G.V. (1974). Studies of a surfactant and cholesteremia in the Maasai. *Am. J. Clin. Nutr.* 27: 464-9.

Marquina, D., Santos, A, Corpas, I., Munoz, J., Zazo, J. and Peinado, J.M. (2003). Dietary influence of kefir on microbial activities in the mouse bowel. *Letters in Applied Microbiology.* 35: 136-140.

Marshall, V.M., Cole, W.M., and Brooker, B.E. (1984). Observation on the structure of kefir grains and the distribution of the microflora. *J. Appl. Bacteriol.* 57: 491-497.

Marshall, V.M. and Cole, W.M. (1985). Methods for making kefir and fermented milks based on kefir. *J. Dairy Res.* 52: 451-456.

Masuda, O., Nakamura, Y. and Takano, T. (1996). Antihypertensive peptides are present in aorta after oral administration of sour milk containing these peptides to spontaneously hypertensive rats. *J. Nutr.* 126: 3063-3068.

Matar, C., Amiot, J., Savoie, L. and Goulet, J. (1996). The effect of milk fermentation by *Lactobacillus helveticus* on the release of peptides during in vitro digestion. *J. Dairy Sci.* 79: 971-979.

Matar, C., Nadathur, S.S., Bakalinsky, A.T. and Goulet, J. (1997). Antimutagenic effects of milk fermented by *lactobacillus helveticus* L89 and a protease-deficient derivative. *J. Dairy Science.* 80(9): 1965-1970.

Mathias, S., Pena, L. A. and Kolesnick, R. N. (1998). Signal transduction of stress via ceramide. *Biochem. J.* 335: 465-480

Matsuno, T. (1991). Pathways of glutamate oxidation and its regulation in the HuH13 human hepatoma cells. *J. Cell Physiol.* 148: 290-294.

McIntosh, G.H., Regester, G.O. and Leu, L.E. (1995). Dairy proteins protect against methylhydrazine-induced intestinal cancers in rats. *J. Nutr.* 125: 809-816.

McIntosh, G.H. (1993). Colon cancer: dietary modifications required for a balanced protective diet. *Prev. Med.* 22: 767-774.

McIntosh, G.H., Regester, G.O., Le Leu, R.K. Royal, P.J. and Smithers, G.W. (1995) Dairy preoteins protect against dimethylhyrazine-induced-intestinal cancers in rats. *J. Nutr.* 125: 809-816.

Merrill, A H., Schmelz, E-M, Dillehay, D.L., Spiegel, S., Shayman, J.A., Schroder, J.J., Riley, R.T., Voss, K.A. and Wang, E. (1997). Sphingolipids- the enigmatic lipid class: biochemistry, physiology, and pathophysiology. *Toxicology and Applied Pharmacology.* 142: 208-225.

Merrill, A.H. (1991). Cell regulation by sphingosine and more complex sphingolipids. *J. Bioenerg. Biomembr.* 23: 83-104.

Merrill, A. H., Jr and Sandhoff, K. (2002). Sphingolipids: metabolism and cell signaling. Vance, D. E. Vance, J. E. eds. New Comprehensive Biochemistry: Biochemistry of Lipids, Lipoproteins, and Membranes 4th ed. 2002:373-407 Elsevier Science New York, NY.

Merrill, A.H.Jr., Schmelz, E.M., Wang, E. and Schroeder, J.J. (1995). Role of dietary sphingolipids and inhibitors of sphingolipid metabolism in cancer and other diseases. *J. Nutr.* 125(6): 1677-1680.

Mesner, P.W. Jr., Budihardjo, I.I. and Kaufmann, S.H. (1997). Chemotherapy-induced apoptosis. *Adv. Pharmacol.* 41: 461-499.

Micheli, L., Uccelletti, D., Paleschi, C. and Crescenzi, V. (1999). Isolation and characterization of a *lactobacillus* strain producing the exopolysacchsride kefiran. *Applied Microbiology and Biotechnology.* 34: 137-143.

Miyamoto, T., Morita, H., Nishioka, K., Kataoka, K., Izumimoto, M. and Kuyama, T. (1991). Constituent species of lactic acid bacteria from kefir and their desmutagenic properties. *Japan. J. Dairy Food Sci.* 40: 111-120. (English abstract)

Modrak, D.E., Cardillo, T.M., Newsome, G.A., Goldenberg, D.M. and Gold, D.V. (2004). Synergistic interaction between sphingomyelin and gemcitabine potentiates ceramide-mediated apoptosis in pancreatic cancer. *Cancer Res.* 64: 8405-8410.

Morgan, S. M.; Hickey, R., Ross, R. P., Hill and Teagasc, C. (2000). Efficient method for the detection of microbially-produced antibacterial substances from food systems. *J. Appl. Microbiol.* 89(1): 56-62.

Morotomi, M.and Mutai, M. (1986). In vitro binding of potent mutagenic pyrolyzates. *J. Natl. Cancer Inst.* 77: 195-201.

Motaghi, M.M., Mazaheri, A.M., Moazami, N., Farkhondeh, A., Fooladi, A. and Goltapch, E.M. (1997). Kefir production in Iran. *World Journal of Microbiology and Biotechnology.* 13: 579-581.

Mullally, M., Meisel, H. and Fitzgerald, R. (1997). Angiotensin converting enzyme inhibitory activities of gastric and pancreatic proteinases digests of whey protein. *Int'l Dairy J.* 7: 299-303.

Murofushi, M., Shiomi, M. and Aibara, K. (1983). Effect of orally administered polysaccharide from kefir grain on delayed-type hypersensitivity and tumor growth in mice. Japan. *J. Med. Sci. Biol.* 36: 49-53.

Murofushi, M., Mizuguchi, J., Aibara, K. and Matushasi M. (1986). Immunopotentiative effects of polysaccharide from kefir grain, KGF-C, administered orally in mice. *Immunopharmacology.* 121: 29-35

Murray, W.R., Blackwood, A., Calman, K.C. and Mackay, C. (1980). Fecal bile acids and clostridia in patients with breast cancer. *Br. J. Cancer.* 42: 856-860.

Nadathure, S.R., Carney, J.R., Gould S.J. and Bakalinsky, A.T.(1996). Palmitic acid is the major fatty acid responsible for significant anti-MNNG activity in yogurt. *Mutat Res.* 359: 179-189.

Nadathure, S.R., Gould, S.J. and Bakalinsky, A.T. (1994). Antimutagenicity of fermented milk. *J. Daiy Sci.* 77: 3287-3295.

Nadathure, S.R., Zhou, L., Lowry, R.R. and Bakalinsky, A.T. (1997). Effects of hydrolysis of milk glycerides on the antimutagenicity of a hexane extract of milk. *J. Dairy. Sci.* 81: 664-671.

Nagaoka, S., Futamura, Y., Miwa, K., Awano, T., Yamauchi, K., Kanamaru, Y., Tadashi, K., and Kuwata, T. (2001). Identification of the novel hypocholesterolemic peptides derived from bovine milk beta-lactoglobulin. *Biochemical and Biophysical Research Communications.* 281: 11-17.

Nagira, T., Narisawa, J., Teruya, K., Katahura, Y., Shim, S.Y., Kusumoto, K., Tokumaru, S., Tokumaru, K., Barnes, D.W. and Shirhata, S. (2002). Supression of UVC-induced cell damage and enhancement of DNA repair by fermented milk, kefir. *Cytotechnology.* 40: 125-137.

Nakamura, Y., Yamamoto, N., Saki, K. and Takano, T. (1995). Antihypertensive effects of sour milk and peptides isolated from it that are inhibitors of angiotensin converting enzyme. *J Dairy Sci.* 78: 1253-1257.

Narva, M., Nevala, R., Poussa, T. and Korpela, R. (2004). The effect of Lactobacillus helveticus fermented milk on acute changes in calcium metabolism in postmenopausal women. *Eur. J. Nutr.* 43: 61–68

Nicodemo, A., Kubow, S. and Ismail, A.A. (1999). *FASEB*, April 17 - 21, 1999, Washington, DC

Obata, H., Yamade, K., Kahara, H. and Hamano, A. (2000). Preparation of yeast protein for therapeutic use as a substitute of pancreatic stone protein. *Jpn. Kokai Tokkyo Koho* (Patent written in Japanese, English abstract).

Obeid, L.M. and Hannun, Y.A. (1995). Ceramide: a stress signal and mediator of growth suppression and apoptosis. *J. Cell Biochem.* 58: 191–198.

Odet, G. (1995). Fermented milks. *IDF Bull.* 300: 98-100.

Okazaki, T., Kondo, T. and Tashima, M. (1998). Diversity and complexity of ceramide signaling in apoptosis. *Cell Signal.* 10: 685–692.

Olesmall, M.E., Nicheno, E.V., Mitrokhin, S.D., Nonikov, V.E. and Minaev, V.I. (1999). Effectiveness of acipole in prevention of enteric dysbacteriosis due to antibacterial therapy. *Antibiotiki I Khimioterapiia.* 44(1): 23-25. (In Russian, English abstract).

Orrhage, K., Sillerstrom, E., Gustafsson, J.A., Nord, C.E. and Rafter, J. (1994). Binding of mutagenic heterocyclic amines by intestinal and lactic acid bacteria. *Mutat. Res.* 311: 239- 248.

Osada, K., Nagira, K., Teruya, K., Tachibana, H., Shirahata, S. and Murakami, H. (1993). Enhancement of interferon-b production with sphingomyelin from fermented milk. *Biotherapy.* 7(2): 115-123.

Otani, H. and Hata, L. (1995). Inhibition of proliferative responses of mouse spleen lymphocytes and Peyer's patch by bovine milk casein and their digests. *J. Dairy Res.* 62: 339-348.

Papenburg, R., Bounous, G., Fleiszer, D. and Gold, P. (1990). Dietary milk proteins inhibit the development of dimethylhydrazine-induced malignancy. *Tumour Biol.* 11: 129–136.

Parodi, .P (1998). The role of milk proteins in cancer prevention. *Aust. J. Dairy Technol.* 53(1): 37-47.

Parodi, P.W. (1996). Milk fat components: possible chemopreventative agents for cancer and other diseases. *Aust. J. Dairy Tech.* 51: 24-32.

Parodi, P.W. (1994). Conjugated linoleic acid: an anticarcinogenic fatty acid present in milk. *Aust. J. Dairy Technol.* 49: 93-97.

Pena, L.R., Hill, B.B. and McClain, C.J. (1999). Treatment with glutathione precursor decreases cytokine activity. *J. Parenter. Enteral. Nutr.* 23: 1-6.

Pence, B.C. (1993). Role of calcium in colon cancer prevention: experimental and clinical studies. *Mutat. Res.* 290: 87-95.

Pence, B.C., Dunn, D.M., Zhao, C., Patel, V., Hunter, S. and Landers, M. (1996). Protective effects of calcium from nonfat dried milk against colon carcinogenesis in rats. *Nutr. Cancer.* 25: 35-45.

Perdigon, G., Nader de Macias, M.E., Alvarez, S., Oliver, G. and Pesce de Ruiz, H. (1986). Enhancement of immune response in mice fed with *Streptococcus thermophilus* and *Lactobacillus acidophilus. J Dairy Sci.* 70: 919-926.

Pettus, B.J., Chalfant, C.E.and Hannun, Y.A. (2002). Ceramide in apoptosis: an overview and current perspectives. *Biochem. Biophys. Acta.* 1585:114-125.

Pihilanto-Leppala, A., Koskinen, P., Piilola, K., Tupasela, T. and Korhonen, H. (2000). Angiotensin I-converting enzyme inhibitory properties of whey protein digests: concentration and characterization of active peptides. *J. Dairy Res.* 67: 53-64.

Pool-Zobel, B.L., Munzner, R. and Holzapfel, W.H. (1993). Antigenotoxic properties of lactic acid bacteria in the S. typhimurium mutagenicity assay. *Nutr. Cancer.* 20: 261-70.

Pool-Zobel, B.L., Neudecker, C. and Domizlaff, I. (1996). Lactobacillus and Bifidobacterium-mediated antigenotoxicity in the colon of rats. *Nutr. Cancer.* 26: 365-80.

Raff, M.C. (1992). Social controls on cell survival and cell death. *Nature.* 356: 397-400.

Reddy, G.V., Shahani, K.M. and Banerjee, M.R. (1973). Inhibitory effects of yogurt on ehrlich ascites tumor cell proliferation. *Journal of the National Cancer Institute.* 50 (3): 815-817.

Reddy, G.V., Friend, B.A., Shahani, K.M. and Farmer, R.E. (1983). Antitumor activity of yogurt components. *J. Food Protect.* 46: 8-11.

Reddy, B.S. and Rivenson, A. (1993). Inhibitory effect of *Bifidobacterium longum* on colon, mammary and liver carcinogenesis induced by 2-amino-3-

methylimidazol [4,5-f] quinoline, a food mutagen. *Cancer Research.* 53: 3914-3918.

Regan, D.H., Lavietes, B.B., Regan, M.G., Demopoules, H.B. and Moris, H.P. (1973). Glutamate mediated respiration in tumors *J. Natl. Cancer Inst.* 51: 1013-1017.

Renner, H.W. and Munzner, R. (1991). The possible role of probiotics as dietary antimutagens. *Mutation Res.* 262: 239-245.

Renner, L.G. (1991). Cultured dairy products in human nutrition. *Bulletin of the International Dairy Federation.* 255: 4.

Richelsen, B., Krietensen, K. and Pedersen, S.B. (1996). Long-term (6 months) effect of a new fermented milk product on the level of plasma lipoproteins- a placebo-controlled and double blind study. *Eur. J. Clin. Nutr.* 50: 811-813.

Roberfroid, M.B., Bornet, F., Bouley, C. and Cummings, J.H. (1995). Colonic microflora: nutrition and health. *Nutr. Rev.* 53: 127- 130.

Roberts, J.C. and Francetic, D.J. (1991). Time course for elevation of glutathione in numerous organs of L210-bearing CDF1 mice given L-cysteine prodrug Ribcystine. *Toxicol. Lett.* 59: 245-251.

Rogers, M.L., Goddard, C., Regester G.O., Ballard F.J. and Belford D.A. (1996). Transforming growth factor beta in bovine milk: concentration, stability and molecular mass forms. *J. Endocrinology.* 151: 77-86.

Rokka, T., Syvaojia, E.L., Tuominen, J. and Korhonen, H. (1997). Release of bioactive peptides by enzymatice proteolysis of Lactobacillus GG fermented UHT milk. *Milchwissenschaft.* 52(12): 675-677.

Ronco, A.L., Stefani, E. and Dattoli, R. (2002). Dairy foods and risk of breast cancer: a case-control study in Montevido, Uruguay. *European Journal of Cancer Prevention.* 11: 457-463.

Rose, D.M., Hochwald, S.N., Harrison, L.E. and Burt, M. (1995). Selective glutathione repletion with oral oxothiazolidine carboxylate (OTZ) in the radiated tumor-bearing rat. *Journal of Surgical Research.* 62: 224-228.

Rosi, J. (1978a). The kefir microorganisms: the yeasts. *Sci. Tecni. Lattiero-Casearia.* 29: 59-67.

Rosi, J. (1978b). The kefir microorganisms: the acetic acid bacteria. *Sci. Tecni. Lattiero-Casearia.* 29: 221-227.

Rosi, J. (1978). The kefir microorganisms: the lacteric acid bacteria. *Sci. Tecni. Lattiero-Casearia.* 29: 291-305.

Rotesein, J.B. and Slaga, T.J. (1988). Effect of exogenous glutathione on tumor progression in the murine skin multistage carcinogenesis model. *Carcinogenesis.* 9: 1547-1551.

Rudnick, S.E., Hilser, V.J. and Worosila, G.D. (1994). Comparison of the utility of capillary zone electrophoresis and high-performance liquid chromatography in peptide mapping and separation. *Journal of Chromatography.* 672: 219-229.

Russo, A., DeGraff, W., Friedman, N. and Mitchell, J.B. (1986). Selective modulation of glutathione levels in human normal versus tumor cells and subsequent differential response to chemotherapy drugs. *Cancer Res.* 46: 2845-2848.

Ryan, M.P., Rea, M.C., Hill, C. and Ross, R.P. (1996). An application in Cheddar cheese manufacture for a strain of Lactococcus lactis producing a novel broad-spectrum bacteriocin, lacticin 3147. *Appl. Environ. Microbiol.* 62: 612-619.

Safrit, J.T. and Bonavida, B. (1992). Sensitivity of resistant human tumor cell lines to tumor necrosis factor and adriemycin used in combination: correlation between down-regulation of tumor necrosis factor-messenger RNA induction and overcoming resistance. *Cancer Research.* 52: 6630-6637.

Sanders, M.E. (2000). Considerations for use of probiotic bacteria to modulate human health. *J. Nutr.* 130: 384S-390S.

Satterlee, L.D., Kendrick, J.G., Marshall, H.F., Jewell, D.K., Ali, R.A., Keckman, M.M., Steinke, H.F., Larson, P., Phillips, R.D., Sarwar, G. and Slump, P. (1982). In vitro assay for predicting protein efficiency ratio as measured by rat bioassay: collaborative study. *J. Assoc. Off. Anal. Chem.* 65(4): 798-809.

Schaafsma, G. (1996). State of the art concerning probiotic strains in milk products. *Newsletter of the International Dairy Federation.* 145: 23-24.

Schanbacer, F.L., Talhouk, R.S., Murray, F.A., Gherman, L.I. and Willett, L.B. (1998). Milk-bone bioactive peptides. *Int. Dairy Journal.* 8:393-403.

Schillinger, V. (1990). Bacteriocins of lactic acid bacteria, p. 55-79 In D.D. Bills and S. Kung ed.: Biotechnology and food safety. Butterworth-Heinemann, Boston, Mass.

Schmelz, E.-M., Dillehay, D. L., Webb, S. K., Reiter, A., Adams, J. and Merrill, A. H., Jr. (1996). Sphingomyelin consumption suppresses aberrant colonic crypt foci and increases the proportion of adenomas versus adenocarcinomas in CF1

mice treated with1,2-dimethylhydrazine: implications for dietary sphingolipids and colon carcinogenesis. *Cancer Res.* 56: 4936–4941.

Schonberg, S. and Kordan H.E. (1995). The inhibitory effects of conjugated linoleic acid derivative (CLA) of linoleic acid on the growth of the human tumor cell lines is in part due to increased lipid peroxidation. *Anticancer research.* 15: 1241-1246.

Schröder, C.P., Godwin, A.K., O'Dwyer, P.J., Tew, K.D., Hamilton, T.C. and Ozols, R.F. (1996). Glutathione and drug resistance. *Cancer Invest.* 14: 158-68.

Schutze, S., Machleidt, T., and Kronke, M. (1994). The role of diacylglycerol and ceramide in tumor necrosis factor and interleukin-1 signal transduction. *J. Leukoc. Biol.* 56: 533–541.

Schwandner, R., Wiegmann, K., Bernardo, K., Kreder, D. and Kronke, M. (1998). TNF receptor death domain-associated proteins TRADD and FADD signal activation of acid sphingomyelinase *J. Biol. Chem.* 273: 5916-5922.

Sekine, K., Ushida, Y., Kuhara, T., Ligo, M., Baba-Toriyama, H., Moore, M.A., Murakoshi, M., Satomi, Y., Nishino, H., Kakizoe, T. and Tsuda, H. (1997a). Inhibition of initiation and early stage development of aberrant crypt foci and enhanced natural killer activity in male rats administered bovine lactoferrin concomitantly with azoxymethane. *Cancer Lett.* 121: 211-216.

Sekine, K, Watanabe E., N akamura, J., Takasuka, N., Kim, D.J., Asamoto, M., Krutovskikh, V., Baba-Toriyama, H., Ota, T., Moore, M.A., Masuda, M., Sugimoto, H., Nishino, H., Kakizoe, T. and Tsuda, H. (1997b). Inhibition of azoxymethane-initiated colon tumor by bovine lactoferrin administration in F344 rats. *Jpn. J. Cancer Res.* 88: 523-526.

Selzner, M., Bielawska, A., Morse, M.A., Rudiger, H.A., Sindram, D., Hannun, Y.A. and Clavien, P.A. (2001). Induction of apoptotic cell death and prevention of tumor growth by ceramide analogues in metastatic human colon cancer. *Cancer Res.* 61: 1233–1240.

Shau, H., Kim, A. and Golub, S.H. (1992). Modulation of natural killer and lymphokine-activated killer cell cytotoxicity by lactoferrin. *J. Leukoc. Biol.* 51: 343 – 349.

Shimizu, S., Eguchi, Y., Kosaka, H., Kamiike, W., Matusuda, H. and Tsujimoto, Y. (1995). Prevention of hypoxia-induced cell death by Bcl-2 and Bcl-xL. *Nature.* 374: 814-816

Shiomi, M., Sasaki, K., Murofushi, M. and Aibara, K. (1982). Antitumor activity in mice orally administrated polysaccharide from kefir grains. *Jap. J. Med. Sci. Bio. 35*: 75-80.

Simstein, R., Burow, M., Parker, A., Weldon, C., and Beckman, B. (2003). Apoptosis, chemoresistance, and breast cancer: insights from the MCF-7 cell model system. *Exp. Biol. Med.* 228: 995-1003.

Singh, I., Pahan, K., Khan, M. and Singh, A. K. (1998). Cytokine-mediated induction of ceramide production is redox-sensitive. *J. Biol. Chem.* 273: 20354-20362.

Smith, C.A., Farrah, T. and Goodwin, R.G. (1994). The TNF receptor superfamily of cellular and viral proteins: activation, constimulation, and death. *Cell.* 76: 959-964.

Sreekumar, O. and Hosono, A. (1998). The antimutagenic properties of a polysaccharide produced by Bifidobacterium longum and its cultured milk against some heterocyclic amines. *Canadian Journal of Microbiology.* 44(11): 1029-1036.

St-Onge, M.P., Farnworth, E.R., Savard, T., Chabot, D., Mafu, A. and Jones, P.H. (2002). Kefir consumption does not alter plasma lipid levels or cholesterol fractional synthesis rates relative to milk in hyperlipidemic men: a randomized controlled trial. *BMC Complementary and Alternative Medicine*. 2: 1-7.

Struckhoff, A.P., Bittman, R., Burow, M.E., Clejan, S., Elliott, S., Hammond, T., Tang, Y. and Beckman, B.S. (2004). Novel ceramide analogs as potential chemotherapeutic agents for breast cancer. *The Journal of Pharmacology and Experimental Therapeutics.* 309(2): 523-532.

Sudarshan, R., Nadathure, T., Lin, Z., Lowry, R. and Bakalinsky, S. (1997). Effects of hydrolysis of milk glycerides on antitmutagenicity of hexane extract of milk. *J. Dairy Sci.* 81: 664-671.

Svensson, M., Sabharwal, H., Mossberg, A.K., Lipniunas, P., Leffler, H., Svanborg, C. and Linse, S. (1999). Molecular characterization of alpha-lactalbumin folding variants that induce apoptosis in tumor cells. *J. Biol. Chem.* 274(10): 6388-6396.

Takizawa, S., Kojima, S., Tamura, S., Fujinaga, S., Benno, Y., and Nakase, T. (1998). The composition of the lactobacillus flora in kefir grains. *System. Appl. Microbiol.* 21: 121-127.

Tetsuya, K., Masaaki, L., Takehito, I., Yoshihiko, U., Kasunori, S., Nobuyuki, T., Haruki, O. and Hiroyuki, T. (2000). Orally administered lactoferrin exerts an

antimetastatic effect and enhances production of IL-18 in the intestinal epithelium. *Nutr. Cancer.* 38(2): 198-200.

Thompson, C.B. (1995). Apoptosis in the pathogenesis and treatment of disease. *Science*. 267: 1456-1462.

Tokumaru, S. (1999). Healthy diet. *Jpn. Kokai Tokkyo Koho.* (Patent written in Japanese, English abstract).

Trickler, D., Shklar, G. and Schwartz, J. (1993). Inhibition of oral carcinogenesis by glutathione. *Nutr. Cancer.* 20: 139-144.

Tsai, W.Y., Chang, W.H., Chen, C.H. and Lu, F.J. (2000). Enhancing effect of patented whey protein isolate (Immunocal) on cytotoxicity of an anticancer drug. *Nutr. Cancer.* 38: 200-208.

Tsuda, H., Sekine, K., Takasuka, N., Toriyama-Baba, H. and Iigo, M. (2000). Prevention of colon carcinogenesis and carcinoma metastasis by orally administered bovine lactoferrin in animals. *Biofactors.* 12(1-4): 83-88.

Tsuda, H., Sekine, K., Ushida, Y., Kuhara, T., Takasuka, N., Iigo, M., Han, B.S. and Moore, M.A. (1999). Milk and dairy products in cancer prevention: focus on bovine lactoferrin. *Mutation Research.* 462: 227-233.

Ushida, Y., Sekine, K., Kuhara, T., Takasuka, N., Iiog, M., Maeda, M. and Tsuda, H. (1999). Possible chemopreventive effects of bovine lactoferrin on esophagus and lung carcinogenesis in the rat. *Jpn. J. Cancer Res.* 90: 262-267.

van't Veer, P., Dekker, M.J., Lamer, W.J., Kok, F.J., Shouten, E.G., Brants, A.H., Sturmans, F. and Hermus, R.J. (1989). Consumption of fermented milk products and breast cancer: a case control study in the Netherland. *Cancer Res.* 49: 4020-4023.

Verheij, M., Bose, R., Lin, X. H., Yao, B., Jarvis, W. D., Grant, S., Birrer, M. J., Szabo, E., Zon, L. I., Kyriakis, J. M., Haimovitz, F. A., Fuks, Z. and Kolesnick, R. N. (1996). Requirement for ceramide-initiated SAPK/JNK signaling in stress-induced apoptosis. *Nature.* 380: 75–79.

Wade, M.G., Desaulniers, D., Leingartner, K. and Foster, W.G. (1997). Interactions between endosulfan and dieldrin on estrogen-mediated processes in vitro and in vivo. *Reprod. Toxicol.* 11: 791-798.

Wang, J. H., Redmond, H. P., Watson, R. W. and Bouchier Hayes, D. (1997). Induction of human endothelial cell apoptosis requires both heat shock and oxidative stress responses. *Am. J. Physiol. Cell physiology.* 272: C1543-C1551.

Ware, C.F., VanArsdale, S. and VanArsdale, T.L. (1996). Apoptosis mediated by the TNF-related cytokine and receptor families. *J. Cell Biochem.* 60: 47-52.

Webb, K. E. (1986). Amino acid and peptide absorption from the gastrointestinal tract. *Fed. Proc.* 45: 2268–2271.

Weissmahr, J.A. (2000). Dietary supplement derived from fermented milks for the prevention of osteoporosis. *PCT Int. Appl.* (Patent written in English)

Westwick, J. K., Bielawska, A. E., Dbaibo, G., Hannun, Y. A. and Brenner, D. A. (1995). Ceramide activates the stress-activated protein kinases. *J. Biol. Chem.* 270: 22689–22692.

Whitefield, J.F., Bird, R.P., Chakravarthy, B.R., Isaacs, R.J. and Morley, P. (1995). Calcium-cell cycle regulator and maybe, tumor promotor. *J. Cell Biochem.* Suppl. 22: 74-91.

Wieder, T., Orfanos, C. E. and Geilen, C. C. (1998). Induction of ceramide-mediated apoptosis by the anticancer phospholipid analog, Hexadecylphosphocholine. *J. Bio Chem.* 273(18): 11025-11031.

Williams, G.T. (1991). Programmed cell death: apoptosis and oncogenesis. *Cell.* 65: 1097-1098.

Winters, R.A., Zukowski, J., Ercal, N., Mattews, H.R. and Spitz, D.R. (1995). Analysis of glutathione, glutathione disulfide, cysteine, homocysteine and other biological thiols by high-performance liquid chromatography following derivatization by N-(1-pyrenyl)maleimide. *Analytical biochemistry.* 227: 14-21.

Wollowski, I., Rechkemmer, G. and Pool-Zobel, B.L. (2001). Protective role of probiotics and prebiotics in colon cancer. *Am. J. Clin. Nutr.* 73: 451S-455S.

Wollowski, I. (1998). Studies on the protective effects of bacterial metabolites on molecular processes of colon carcinogenesis. Giessen, Germany: Fachverlag Kohler. (in German, English abstract).

Wright, S.C., Zhang, J. and Larrick, J.W. (1994). Inhibition of apoptosis as a mechanism of tumor promotion. *FASEB J.* 8: 654-660.

Wszolek, M., Tamine, A.Y., Muir, D.D. and Barclay M.N.I. (2001). Properties of kefir made in Scotland and Poland using bovine, caprine and ovine milk with different starter culture. *Lcbcnsm.-Wiss. U.-Technol.* 34: 251-261.

Wyllie, A.H., Kerr, J.F.R., and Currie, A.R. (1992). Cell death: the significance of apoptosis. *Int. Rev. Cytol.* 68: 251-260.

Yamamoto, N., Akino, A. and Takano, T. (1994). Anti hypertensive effects of different kinds of fermented milks in spontaneously hypertensive rats. *Biosci. Biotech. Biochem.* 58: 776-778.

Yoo, Y.C., Watanabe, R. and Koike, Y. (1997). Apoptosis in human leukemic cells induced by lactoferricin, a bovine milk protein-derived peptide: involvement of reactive oxygen species. *Biochem. Biophys. Res. Commun.* 237: 624-628.

Yoo, Y.C., Watanabe, S., Watanabe, R., Hata, K. Shimazaki, K., and Azuma, I. (1997b). Bovine lactoferrin and lactoferricin, a peptide derived from bovine lactoferrin, inhibit tumor metastasis in mice. *Jpn. J. Cancer Res.* 88: 184-190.

Yoon, Y.H., Cho, J.K., Baek, Y.J. and Huh, C.S. (1999). Antimutagenic activity of lactobacillus spp. isolated from kefir and yogurt and non-starter strains. *Korean Journal of Animal Science.* 41(1): 39-44. (English abstract).

Yoshimura, S., Sakai, H., Ohguchi, K., Nakashima, S., Banno, Y., Nishimura, Y., Sakai, N. and Nozawa, Y. (1997). Changes in the activity and mRNA levels of phospholipase D during ceramide-induced apoptosis in rat C6 glial cells. *J. Neurochem.* 69: 713-720.

Young, G.P. and Gibson, R.P. (1994). Butyrate and the colorectal cancer cell. In: Short chain fatty acids (Binder, H.J., Cummings J and Soergel K., eds), London. U.K.

Zacconi, C., Parisi, M.G., Sarra, P.G., Dallavalle, P. and Bottazzi, V. (1995). Competitive exclusion of Salmonella kedougou in kefir fed chicks. *Microbiol. Alim. Nutr.* 12: 387-390.

Zeisel, S.H., Char, D. and Shear, N.F. (1986). Choline, phosphatidylcholine and sphingomyelin in human and bovine milk and infant formulas. *J. Nutr.* 116: 50-58.

Zhang, X.B. and Ohta, Y. (1993). Antimutagenicity of cell fractions of microorganisms on potent mutagenic pyrolysates. *Mutat. Res.* 298: 247 – 253.

Zhang, X.B. and Ohta, Y. (1991). In vitro binding of mutagenic pyrolyzates to lactic acid bacterial cells in human gastric juice. *J. Dairy Sci.* 74: 752-757.

Zhang, Y. and Kolesnick, R. (1995). Signaling through the sphingomyelin pathway. *Endrocrinology.* 136: 4157-4160.

Zommara, M., Tachibana, N., Sakono, M., Suzuki, Y., Oda, T., Hashiba, H. and Imaizumi, K. (1996). Whey preotein from cultured skim milk decreases serum cholesterol and increases antioxidant enzymes in liver and red blood cells in rats. *Nutrition Research.* 16(2): 293-302.

Zommara, M., Takagi, H., Sakono, M., Suzuki, Y. and Imaizumi, K. (1994). Effect of milk whey and its fermentation products by lactic acid bacteria on mitochondria lipid peroxide and hepatic injury in bile duct-ligated rats. *Biosci. Biotech. Biochem.* 58: 1213-1217.

Zommara, M., Toubo, H., Sakono, M. and Imatzumi, K. (1998). Prevention of peroxidative stress in rats fed on a low vitamin E-containing diets by supplementing with a fermented bovine milk whey preparation: effects of lactic acid and β-lactoglobulin on the antiperoxidative action. *Biosci. Biotech. Biochem.* 62 (4): 710-717.

Zourari, A. and Anifantakis, E.M. (1988). Le kéfir: Caractères physico-chimiques, microbiologiques et nutritionnels. Technologie de production. Une revue. *Lait.* 68: 373-392. (English abstract).

APPENDICES