AN ABSTRACT OF THE THESIS OF

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Abstract Approved: _____

Alan T. Bakalinsky

The purpose of this study was to identify antimutagens in yogurt active against the experimental colon carcinogen, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG). Our initial experiments showed that acetone extracts of yogurt, or milk fermented by various lactic acid bacteria were antimutagenic against MNNG and 3,2'-dimethyl-4-aminobiphenyl (DMAB) in the Ames test (*Salmonella typhimurium* TA 100). Further experiments carried out with milk fermented by *Lactobacillus delbrueckii* ssp. *bulgaricus* 191R showed that the putative compounds were more soluble in DMSO than in water, and that extractability of activity against MNNG and DMAB varied with pH, suggesting the presence of ionizable groups.

Subsequent experiments demonstrated the antimutagenicity of yogurt. An acetone extract of yogurt was found to be active against a range of mutagens and promutagens in the Ames test. Simulation of fermentation by addition of lactic acid, lactic acid bacteria, or both to milk did not increase antimutagenicity, suggesting that compounds responsible for the activity may be formed during fermentation. Conjugated linoleic acid (CLA), a known dairy anticarcinogen, did not inhibit MNNG or DMAB indicating that other antimutagens may be present in yogurt. Fractionation of the acetone extract by HPLC showed that anti-MNNG and anti-DMAB activities did not co-elute, indicating that different compounds were responsible for the two activities.

Using the Ames test to direct purification, isolation of an anti-MNNG active compound was accomplished using silica gel, Sephadex LH-20 and C18 reversed phase

medium pressure chromatographies. The antimutagen was identified as palmitic acid by: a) co-elution with authentic palmitic acid on GC and HPLC columns, and b) by comparison of mass and ^{13}C-NMR spectra. Minor components of milk fat such as iso methyl branched fatty acids (isopalmitic acid, isomargaric acid, isomyrsitic acid, and isostearic acid) were found to be more active than their straight chain counterparts. Isopalmitic acid also inhibited 4-nitroquinoline-*N*-oxide (4NQO) and the P450-mediated activation of 7,12-dimethylbenz[a]anthracene (DMBA). The mechanism of antimutagenesis against MNNG has not been established.

A Study of Antimutagenicity in Yogurt

by

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Chair of Department of Food Science and Technology

Dean of Graduate School

I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

N.R. Sudarshan, Author

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CONTRIBUTION OF AUTHORS

Dr. Alan T. Bakalinsky is the principal investigator of the project (Isolation of antimutagens and anticarcinogens from yogurt) and was involved in the design, analysis, and writing of each manuscript of the thesis. Drs. Steven J. Gould and John R. Carney were involved in the isolation of the antimutagen from yogurt, and writing the manuscript for Chapter 4.

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CHAPTER 1

INTRODUCTION

1.1 Yogurt

Yogurt by definition is milk fermented by a mixed starter culture of *Streptococcus salivarius* ssp. *thermophilus* (formerly known as *S. thermophilus*) and *Lactobacillus delbrueckii* ssp. *bulgaricus* (formerly known as *Lb. bulgaricus*) (1:1). Other fermented milks are produced by addition of one or more strains of other species of lactic acid bacteria. Certain strains of *Lactobacillus acidophilus*, capable of adhering to the colon, are now added to commercial yogurt in order to increase putative beneficial effects. *L. delbrueckii* ssp. *bulgaricus* is more proteolytic than *S. salivarius* ssp. *thermophilus* and produces peptides for the latter, while *S. salivarius* ssp. *thermophilus* produces formic acid, and carbon dioxide necessary for purine biosynthesis in *L. delbrueckii* ssp. *bulgaricus* (Hartley and Denariaz, 1993). Thus, the formation of yogurt is due to a symbiosis between the starter cultures (Figure 1.1).

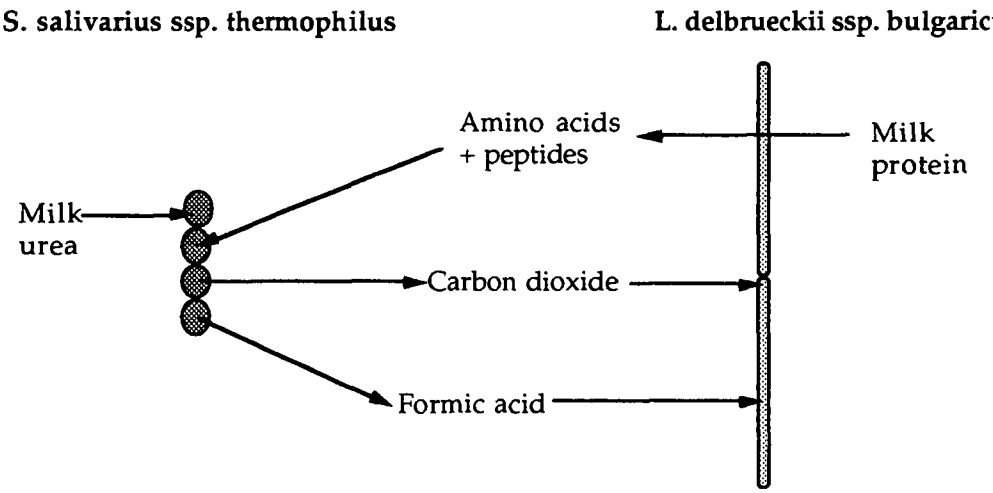

Figure 1.1. Symbiosis between *Streptococcus salivarius* ssp. *thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus*.

During fermentation, the bacterial population increases to 10^8 per ml. Generally, the ratio of *S. salivarius* ssp. *thermophilus* to *L. delbrueckii* ssp. *bulgaricus* at the end of fermentation is 4:1. β-galactosidase of the lactic acid bacteria breaks down lactose to glucose and galactose. Of the lactose present in milk, 20-30% and sometimes more is broken down by lactic acid bacteria. Glucose is converted to lactic acid while galactose remains largely unchanged. Due to the reduction in lactose content, and persisting β-galactosidase activity of lactic acid bacteria, fermented milk and yogurt may be more suitable for consumption by individuals who are lactose-intolerant.

When the pH is reduced to 4.6, the micellar calcium phosphate which holds the casein micelles together is solubilized, resulting in precipitation of the casein molecules at their isoelectric point (pH 4.6 - 4.7). Apart from lactic acid, other organic acids like succinic, fumaric and benzoic acids are formed to a lesser extent. Increase in peptides and essential amino acids occur together with production of volatile and long-chain free fatty acids. Though vitamin B_{12} in milk is used by the bacteria, they produce other vitamins including folic acid and thiamine (B_1). Flavor components produced include acetaldehyde, acetoin and diacetyl (Yukuchi et al., 1988).

Several studies have focussed on the putative health benefits of consuming yogurt and fermented milks, including: a) antimutagenicity, b) anticarcinogenicity, c) reduction of deleterious fecal enzymes, d) elevation of immune response, and e) supporting epidemiological studies. A summary of these studies follow.

1.2. Mutagens

Mutagens are compounds that alter DNA by causing base-pair changes. These compounds are usually converted to potent electrophiles which can bind macromolecules other than DNA as well. Binding to DNA causes alterations in base pairing leading to mutations. Though some mutations are innocuous, those which affect genes controlling

cell division, cell growth, and tumor suppression are believed to play key roles in the initiation of carcinogenesis.

Mutagens are of two types, direct-acting and indirect-acting. Direct-acting mutagens do not require enzymatic activation and can directly alter DNA. Indirect-acting are also called promutagens or procarcinogens. Promutagens are present in our diet and environment. Some promutagens are produced by microorganisms. As an example, *Aspergillus flavus* produces aflatoxin B1 (AFB1). Other promutagens are produced during cooking and grilling of meat (e.g. heterocyclic amines) or are produced as combustion products such as polycyclic aromatic hydrocarbons. These mutagens may be activated by the cytochrome P450 family of enzymes to the proximate or ultimate form. The liver detoxifies xenobiotics to more polar forms to facilitate their excretion. However, in the same process, promutagens or procarcinogens can become activated to more reactive forms (proximate and ultimate mutagens or carcinogens, respectively) which can then bind to macromolecules causing cellular damage including mutations.

1.3 Carcinogens

Carcinogens differ from mutagens in the fact that they act in three stages, initiation, promotion and progression. Mutagens can be classified as initiators. The subsequent process leading to the development of cancer is called promotion. Substances such as phorbol esters, and tobacco smoke are promoters. Cancer occurs more readily in dividing cells in tissues such as skin, stomach, lung, and the intestinal tract. These tissues are exposed to a variety of chemicals, and promutagens that occur in food and the environment. Mutations that occur in such rapidly dividing cells may be repaired by SOS repair enzymes, which are error-prone. Hence, there is a greater likelihood of the mutations being carried forward by the replicating cells and an increased risk for onset of cancer.

1.4 Antimutagens and anticarcinogens

Antimutagens and anticarcinogens are substances which reduce or decrease the effects of mutagens and carcinogens, respectively. Many such substances have been detected in foods (e.g. chlorophyllin, β-carotene, phenylisothiocyanate, diallyl sulfide). The process of antimutagenesis is typically studied in microbial and mammalian cell systems while the effects of anticarcinogens are ultimately based on animal studies.

Antimutagens are of two types, desmutagens and bioantimutagens. Figure 1.2 depicts different mechanisms of antimutagenesis by desmutagens and bioantimutagens. Desmutagens interact directly with the mutagens to reduce their damage on DNA. Direct interaction may involve formation of mutagen-antimutagen complexes by non-covalent or covalent binding. As an example, chlorophyllin has been shown to form a complex with planar molecules like 3-amino-1-methyl -5*H*-pyrido[4,3-*b*]indole (Trp-P-2), (Negishi et al., 1989). Desmutagens may also inhibit activation of promutagens. Brady et al., (1988) showed that diallyl sulfide (from garlic and onions) inhibited cytochrome P450 2E1-mediated xenobiotic-activation. Activated mutagens may also be detoxified by conjugation to groups such as glucuronide. Sulforophane in broccoli, induces quinone reductase and glutathione-S-transferase to aid in the excretion of activated compounds (Zhang et al., 1992). In contrast, bioantimutagens (or true antimutagens) act in vivo on the repair and replication processes of the mutagen-damaged DNA. Vanillin is an example of a true antimutagen because it enhances recA-dependent error-free recombination repair in *E. coli* (Ohta et al., 1988).

Antioxidants may be classified as desmutagens or bioantimutagens depending on their site of action. Antioxidants, include carotenoids (e.g. β-carotene), and vitamins (A, E, C), scavenge free radicals and singlet oxygen to prevent cellular damage and mutations. Synthetic compounds like butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) have been found to be antimutagenic due to their antioxidant nature (Hartman and

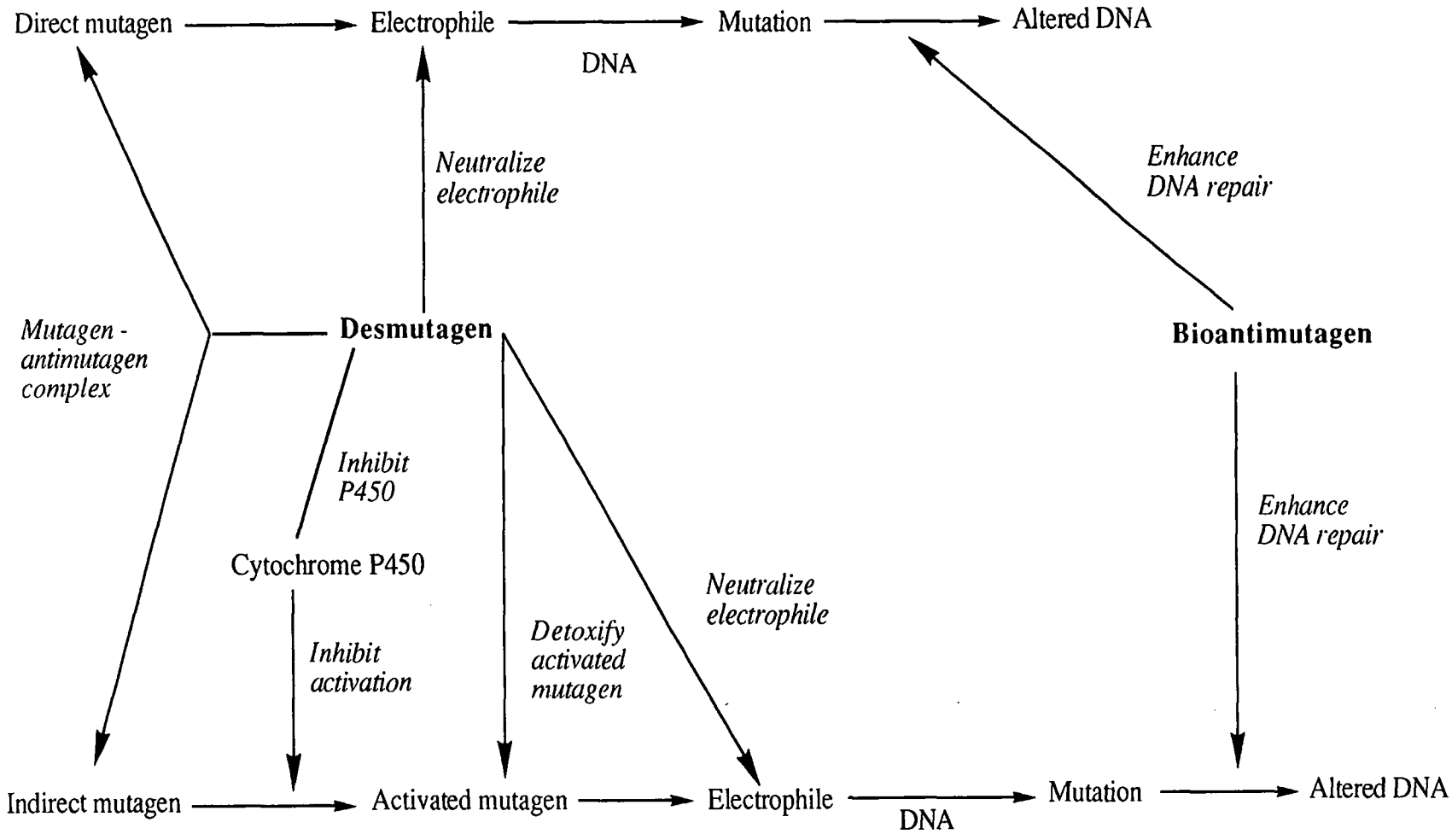

Figure 1.2. Mechanisms of inhibition of mutagenesis by desmutagens and bioantimutagens.

Shankel, 1990). β-carotene, vitamins A and E have been found to be anticarcinogenic in animal models (Ferguson, 1994).

1.5 Antimutagenicity of fermented milk and yogurt in microbial cells

1.5.1 Microbial cell systems

Many microbial cell systems are used to detect mutagenesis and antimutagenesis. The microbes used in these assays are usually auxotrophs, requiring an amino acid. Treatment with mutagens induces revertants that no longer require the nutrient the parental form was unable to synthesize. Antimutagens interfere with the action of the mutagen and decrease the frequency of reversion. The Ames test uses specially constructed *Salmonella typhimurium* strains requiring histidine. The strains have increased permeability to chemicals (due to abnormalities in the lipopolysaccharide layer), making it highly sensitive to mutagens (Maron and Ames, 1983). Other microbial cell systems include the specially constructed yeast strain *Saccharomyces cerevisiae* D7, requiring isoleucine and tryptophan (Zimmermann et al., 1975) and *Escherichia coli* B/r WP2 which requires tryptophan (Kada et al., 1960). The latter also lacks a lipopolysaccharide layer thus making it highly permeable to chemicals.

The advantages of the microbial systems are their convenience, simplicity, and relative inexpense compared to animal models. However, not all mutagens are carcinogens nor are all antimutagens anticarcinogens. The Ames test is an *in vitro* assay and thus an inhibition of mutagenesis detected in this assay cannot be assumed to predict a protective effect in humans or animal models. Nevertheless, an antimutagen detected in microbial assays may be a potential anticarcinogen. Hence, identification of specific antimutagens may help in understanding their mechanism of action and consequently their role in the process of chemoprevention. Table 1.1 summarizes a number of studies that

have been performed to demonstrate the antimutagenicity of fermented milk in microbial systems.

1.5.2 Summary of studies

Hosono et al., (1986) showed that milk fermented by *S. salivarius* ssp. *thermophilus* and *L. delbrueckii* ssp. *bulgaricus* inhibited the mutagenicity of 4-nitroquinoline-*N*-oxide (4NQO) and 2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide (AF2) in the Ames test. Incubation of fermented milk and the mutagens decreased the number of revertants. Similarly, Hosono et al., (1986) showed that milk fermented by *L. delbrueckii* ssp. *bulgaricus*, *S. faecalis*, and *Lc. lactis* had a desmutagenic effect against 4NQO and a water-extract of dog feces in *E. coli* B/r WP2 trp-hcr-. Hosoda et al., (1992a, 1992b) used the Ames test to further demonstrate the antimutagenicity of fermented milk. Incubation of fermented milk with either *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) or Trp-P-2 reduced the frequency of revertants. Inhibition varied depending on the strain and the species of lactic acid bacteria used. Antimutagenicity also increased with cell number though the effect decreased after fermentation had proceeded for more than 18 h. Reasons suggested for the antimutagenic effect were neutralization of the cationic breakdown product of MNNG or binding of both mutagens onto cell walls of the lactic acid bacteria.

Milk fermented by *S. salivarius* ssp. *thermophilus* and *L. delbrueckii* ssp. *bulgaricus*, alone or together, were shown to significantly reduce mutagenesis induced by 4NQO and 2-aminofluorene (2AF) in the Ames test (Bodana and Rao, 1990). The fermented milk was extracted with acetone or ethyl acetate to produce a DMSO-soluble extract which was used as the source of antimutagenicity rather than whole cultured milk as used in earlier work by others. The extraction of activity from fermented milk but not from unfermented milk implicated lactic acid bacteria in its formation during

Table 1.1. Positive reports of antimutagenicity of yogurt and fermented milk in microbial assays

Source of antimutagenic compounds	Mutagen	Reference
Milk fermented by *L. delbrueckii* ssp. *bulgaricus*, or *S. salivarius* ssp. *thermophilus*	AF2, 4NQO, fecal extracts	Hosono et al., 1986
Whole or digested casein; Milk fermented by *L. delbrueckii* ssp. *bulgaricus*, or *S. salivarius* ssp. *thermophilus*	Pepper extract	Hosono et al., 1988
Acetone extract of milk fermented by *L. delbrueckii* ssp. *bulgaricus, S. salivarius* ssp. *thermophilus*, yogurt	4NQO, 2AF	Bodana and Rao, 1990
Lyophilized cells of various lactic acid bacteria	NDEA , NDMA	Hosono et al., 1990
Lyophilized cells of various lactic acid bacteria	Glu-P-1, Trp-P-1, Trp-P-2 AF2	Zhang and Ohta, 1991
Milk fermented by various lactic acid bacteria	MNNG, Trp-P-2	Hosoda et al., 1992a,b
Milk fermented by various lactic acid bacteria	Nitrosated beef	Pool-Zobel et al., 1993a
Whole or digested casein	4NQO, MNU	Van Boekel et al., 1993
Acetone extract of milk fermented by various lactic acid bacteria; yogurt	MNNG, DMAB, DMBA Trp-P-2, 4NQO	Nadathur et al., 1994, 1995
Milk fermented by bifidobacteria or *Lb. helveticus*	4NQO, 2-NF, AFB1, quercetin, B[a]P	Cassand et al., 1994

The Ames test (*S. typhimurium*) was used in all studies except those of Hosono et al., (1986) who used *E. coli* B/r WP2. Nadathur et al., (1995) also used *S. cerevisiae* D7.

fermentation. A weak activity was present in the extract of unfermented milk. Studies done in our laboratory showed that in the Ames test, mutagenesis induced by MNNG and 3,2'-dimethyl-4-aminobiphenyl (DMAB) was inhibited by acetone extracts of fermented milk (Nadathur et al., 1994). Activity obtained from fermented milk was found to be 2-3 times greater than that obtained from unfermented milk. Addition of lactic acid to unfermented milk did not increase antimutagenicity indicating that normal acidification that normally occurs during fermentation was not responsible by itself for the antimutagenic activity.

Pool-Zobel et al., (1993a) showed that milk fermented by *Lactobacillus casei* was inhibitory to nitrosated beef extract-induced mutagenesis in the Ames test. Milk fermented by bifidobacteria or *Lb. helveticus* was shown to reduce mutagenicity caused by 4NQO and 2-nitrofluorene (Cassand et al., 1994). Interestingly, unfermented milk was more inhibitory than fermented milk to the indirect mutagens AFB1, benzo[a]pyrene (B[a]P), and quercetin. The authors suggested that hydrophobic interactions between the milk components and the mutagens may have decreased their availability and hence their ability to damage DNA. The higher activity of unfermented milk also suggested that fermentation may break down certain components and reduce their effectiveness.

The antimutagenic potential of casein was studied by Van Boekel et al., (1993). Casein and its digestion products were found to inhibit mutagenesis casued by B[a]P, and *N*-methylnitrosourea (MNU) in the Ames test. Mutagenesis induced by 4NQO was inhibited to a lesser extent. A reason suggested for the inhibition was the binding ability of casein and its digestion products. Studies by Zhang and Ohta (1990) showed that binding of Trp-P-2 by cells of lactic acid bacteria was pH-dependent, irrespective of whether the cells were viable or heat-killed. In a separate study (Zhang and Ohta, 1993), inactivation of 3-amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-1) and 2-amino-6-methyldipyrido[1,2-*a*:3',2'-*d*]imidazole (Glu-P-1) was studied with various bacteria. Gram-negative bacteria were found to have a greater binding capability than gram-positive

bacteria, while Trp-P-1 was bound to a greater extent than Glu-P-1. Recently, Tanabe et al., (1994) had showed that the binding of Trp-P-1 by *Lc. lactis* was pH-dependent and that binding was inhibited by sodium dodecyl sulfate (SDS). Ethylenediaminetetraacetic acid (EDTA) alone did not inhibit the binding of the mutagen indicating that there was no chelation involved. Hydrophobic interactions between cell wall components of bacteria and mutagens were suggested to be responsible for mutagen inactivation.

1.6 Antimutagenicity of fermented milk and yogurt in mammalian cells

1.6.1 Mammalian cell systems

Chinese hamster ovary (CHO) cells are used to study genotoxic effects in mammalian cells. Chromosomal aberrations arise from exposure to chemicals that induce single- and double-stranded chromosomal breaks. The extent of damage indicates the potency of the chemical. The chromosomes are observed at metaphase following the first full cell cycle after treatment, as many aberrant cells fail to survive the first mitosis. Because damage to the chromosome can cause heritable changes, a protective effect in this test is significant. The chromosomes of these cells are also used to study sister chromatid exchange (SCE) to detect and quantify genotoxic effect. Induction of SCE refers to DNA repair phenomema following DNA damage. Reduction in the frequency of SCE is inferred to be protective.

The micronuclei test detects changes in the nucleus. Micronuclei originate from chromosomal material that has lagged in anaphase and has not travelled to the appropriate pole of the spindle to be included in the main nucleus of the daughter cell. Today, the test is performed in erythrocytes. Young erythrocytes (PCE's) are distinguished from mature cells (NCE's) by differential staining. Usually the ratio of NCE:PCE is 1:1. An increase in NCE indicates a cytotoxic effect. Cytotoxicity also closely correlates with occurrence of heritable chromosomal changes.

Another type of assay evaluates changes in the activity of phase II enzymes known to be involved in the disposition and transformation of xenobiotics. Phase II enzymes are inducible and conjugate xenobiotics activated by phase I cytochrome P450 enzymes, to make them more polar and thus facilitate their excretion. The enzymes transfer groups such as glucuronide, sulfate, glutathione and add them to liver-activated xenobiotics. Sulphoraphane, present in broccoli, was found to induce quinone reductase and glutathione transferase in vitro (Zhang et al., 1992).

1.6.2 Summary of studies

Antimutagenic effects have also been observed in mammalian cells (Table 1.2). *Lb. casei*, Ominiflora (a mixture of *Lb. gasseri*, *B. longum* and *E. coli*) and yogurt were shown to inhibit bisulfan-induced chromosome aberrations and gaps as well as micronuclei. The cell free culture broth of *Lb. casei* did not inhibit chromosome aberrations while the cell-free culture broths of the other bacteria showed weak inhibition. Lactic acid alone did not inhibit either the chromosome damage or mutagen-induced micronuclei (Renner and Münzner, 1991). Pool-Zobel et al., (1993b) observed that *L. casei* inhibited MNNG-induced DNA damage using a novel micro-gel electrophoresis test, also known as the comet assay (Singh et al., 1988). In this test, damaged DNA appears as a comet-like structure with a long tail during electrophoresis. The greater the damage, the longer the tail.

Bosselaers et al., (1994) reported that casein was antimutagenic against MNNG and 4NQO in the sister chromatid exchange test. However, soy protein, whey protein, and β-lactoglobulin did not have any protective effect. The difference in the antimutagenic activity of the different proteins was ascribed to their respective structures. Casein lacks secondary and tertiary structure and may form micelles or act as a nucleophile. Both whey proteins and β-globulin have tight secondary structures which make them less accessible to mutagens. MacDonald et al., (1994) reported that fermentation of media containing casein

Table 1.2. Positive reports of antigenotoxic effects of yogurt and fermented milk in mammalian assays

Source of antimutagenicity	Mutagen	Result	Reference
Whole cells of *Lb. casei, Lb. gasseri,* Omniflora (*B. longum, Lb. gasseri* and *E. coli*); yogurt	Bisulfan	Decrease in aberrant chromosomes and micronuclei.	Renner & Münzner, 1991
Milk fermented by *Lb. casei*	MNNG	Decrease in DNA damage to esophageal gastric, duodenal, and colonic mucosa	Pool-Zobel et al., 1993a
Tissue culture media containing casein fermented by a mixture of *S. salivarius* ssp. *thermophilus and L. delbrueckii* ssp. *bulgaricus.*	MNNG	Reduction in DNA damage to intestinal cell lines derived from colon adenocarcinoma	MacDonald et al., 1994
Whole or digested casein Soy protein, whey protein β-lactoglobuiln	4NQO MNNG	Inhibition of sister-chromatid exchange in chinese hamster cells	Bosselaers et al., 1994

by yogurt cultures reduced MNNG-induced DNA damage. Incorporation of a radiolabelled nucleotide precursor ([^{3}H]thymidine) into DNA decreased when the adenocarcinoma cell line was treated with yogurt compared to the control. Decreased incorporation of [^{3}H]thymidine suggested diminished DNA repair and hence reduction in DNA damage.

1.7 Anticancer activity of yogurt

1.7.1 Antitumor effect of fermented milk and yogurt

Ehrich ascites tumor (EAT) cells have been routinely used to quantify a decrease in tumor cell proliferation, suggesting an antitumor effect. The tumor cells are injected intraperitoneally and their proliferation is measured after an injection of different preparations of fermented milk. A decrease in cell number or in DNA content is taken to indicate an antitumor effect.

Reddy et al., (1973) reported a weak antitumor effect of dietary yogurt based on the above assay. Inhibition of tumor cells reached a maximum at the end of the 3rd day of feeding and remained the same through the 7th day. Yogurt dialyzate, but not the curd, was shown to inhibit tumor proliferation by Ayebo et al., (1981). In contrast, Friend et al., (1982) showed that antitumor activity was found in the yogurt bacterial cells and not the dialyzate. The activity decreased when the cells were sonicated suggesting that cell fragments did not have an antitumor effect. Inhibition was observed in mice when the animals were fed yogurt but not whole cells, suggesting that milk components prevented degradation of cells, before reaching the intestine. This result was substantiated when the culture cells were implanted in the intestine and were found to inhibit tumor cell proliferation. Consumption of fermented milk has been shown to inhibit the growth of certain types of tumors in mammals. Bogdanov et al., (1975) inoculated mice with sarcoma S-180 cells. After an initial increase in tumor size, the mice were given

Table 1.3. Antitumor activity and anticarcinogenicity of yogurt

Treatment	Source of anticarcinogenicity	Result	Reference
Ehrilch ascites carcinoma	Dietary yogurt	Moderate inhibition of tumor cell proliferation	Reddy et al., 1973
	Intravenous or intraperitoneal injection of yogurt dialyzate	Moderate inhibition of tumor cell proliferation	Ayebo et al., 1982
	Dietary yogurt; Intraperitoneal injection of yogurt cells	Significant inhibition of tumor cell proliferation	Friend et al., 1982
	Feeding of milk or soy milk fermented by *S. salivarius* ssp.*thermophilus* and *Lb. acidophilus*	Significant inhibition of tumor cell proliferation	Lexun et al., 1990
	Intraperitoneal injection or implantation of cell walls of *Lb. acidophilus*	Significant inhibition of tumor cell proliferation	Fernandez et al., 1991
Sarcoma-180	Cell wall glycopeptides of *L. delbrueckii* ssp. *bulgaricus* injected intravenously	Degeneration of tumor cells	Bogdanov et al., 1975
	Lyophilized cells of *L. mesenteroides*, *Lc. cremoris*, *Lb. lactis*, and *S. salivarius* ssp. *thermophilus* administered intrperitonealy	Significant regression of tumors except for *S. salivarius* ssp. *thermophilus*	Kelkar et al., 1988
	Lyophilzed ropy sour milk (långfil and ropy yogurt) injected intraperitonealy	Significant inhibition of tumors	Kitazawa et al., 1990

Table 1.3. (continued)

Treatment	Source of anticarcinogenicity	Result	Reference
Carcinogens	Milk fermented by *S. salivarius* ssp. *thermophilus* or *L. delbrueckii* ssp. *bulgaricus* fed with diet	Shift in DMH-induced tumors from ear duct to small intestine	Shackleford et al., 1983
	Feeding of milk fermented by *Lb. acidophilus*	Significant inhibition of DMH-induced tumors	Yongjun et al., 1991
	Lyophilzed cells of *B. longum*	Significant inhibition of IQ-induced colon, liver, and mammary tumors	Reddy and Rivenson, 1993
Cancer cell line	Milk fermented by *Lb. acidophilus*, or *Lb. helveticus*, or bifidobacteria; yogurt added to the culture media	Significant inhibition except for *Lb. acidophilus* (no inhibition)	Baricault et al., 1995

intravenously one dose of a cell wall lysate preparation of *L. delbrueckii* ssp. *bulgaricus*. A decrease in tumor size was reported within 1-3 days and the acquisition of immunity by the animals to replantation of the tumor was observed. These results indicated that an immune response was modulated by the injected cell wall lysate glycopeptides of *L. delbrueckii* ssp. *bulgaricus*. Similar work done by Kelkar et al., (1988), showed that intratumor administration of lyophilized cells (*Lb. mesenteroides*, *Lc. cremoris* and *Lb. lactis*.) at a dose of 20 mg/kg body weight, resulted in regression of fibrosarcoma in mice. *S. salivarius* ssp. *thermophilus* was more effective in inhibiting sarcoma-180 than the Ehrlich ascites carcinoma. The authors also reported that there was no recurrence of the tumors.

Fernandez et al., (1991) reported that *Lb. acidophilus* reduced proliferation of EAT cells and that hydrocortisone, an immunoinhibitive agent, suppressed the inhibition. This suggested an immune-based response as a plausible mechanism. Kitazawa et al., (1990) studied the mechanism of inhibition of sarcoma-180 tumor using carrageenan and delayed cutaneous hypersensitivity (DCH) to oxazolone. Carrageenan is cytotoxic to macrophages and should increase tumor growth. The DCH response to oxazolone in mice involves T-cell or macrophage-mediated events, which are also depressed by carrageenan injection. Antitumor activity of ropy sour milk was not observed when carrageenan was injected into the mice. The authors suggested from these results that the antitumor activity involved macrophage and/or T cells. An earlier experiment by Kitazawa et al., (1988) had shown that långfil, a ropy sour milk, significantly inhibited Lewis lung carcinoma in mice compared to ropy yogurt. Carrageenan inhibited the antitumor activity suggesting an immune response.

1.7.2 Anticarcinogenic effect of fermented milk and yogurt

Shackelford et al., (1983) used 1,2-dimethylhydrazine (DMH) to induce tumors in rats, and examined the effect of fermented milk on the incidence of colon tumors. Rats

fed milk fermented by *S. salivarius* ssp. *thermophilus* or *L. delbrueckii* ssp. *bulgaricus*, had a higher survival rate than rats fed unfermented milk. Also, rats fed unfermented milk had more ear-duct tumors while rats fed fermented milk had an increased incidence of small intestinal tumors. These results indicated that feeding of fermented milk altered DMH metabolism by shifting the target organ from the ear-duct to the small intestine. The authors suggested that lactic acid bacteria may produce metabolites that affect activation of procarcinogens. The effect of milk fermented by *Lb. acidophilus* on DMH-induced carcinogenesis was studied by Yongjun et al., (1991). Rats fed fermented milk had a higher survival rate than rats fed unfermented milk. The rate and number of tumors also was significantly lower in the fermented milk group. Reduction of fecal enzymes by fermented milk and consequent prevention of formation of harmful metabolites was suggested as a mechanism in decreasing the effect of DMH-induced carcinogenesis.

Lexun et al., (1990) studied the effect of dietary soya milk and soya yogurt against EAT. Soya milk was fermented by *S. salivarius* ssp. *thermophilus* and *Lb. acidophilus* to form soya yogurt. Soya yogurt significantly inhibited the growth of sarcoma-180 compared to soya milk, while there was no effect against Lewis lung carcinoma. Both soya yogurt and yogurt had a similar inhibition of sarcoma-180 leading the authors to advocate the use of soya milk to make yogurt due to its higher protein content. The inhibitory effect of *Bifidobacterium longum* on colon, mammary, and liver carcinogenesis induced by 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ) in rats was reported by Reddy and Rivenson (1993). Significant inhibition of colon and liver carcinogenesis occurred in male rats, while mammary tumors were inhibited to a lesser extent in female rats. No mechanism has been found yet, but the authors speculated that *B. longum* may have reduced procarcinogen activating fecal enzymes.

1.8 Reduction of deleterious fecal enzymes

1.8.1 Types of bacteria

An enormous number of bacteria (about 10^{14} bacteria) are present in the human large intestine. The intestinal microflora are comprised of approximately 100 species with a combined weight of 1-1.5 kg. The bacteria in the gut are classified as beneficial and harmful. Changes in the types of intestinal microflora occur with age. Bifidobacteria (present in breast milk) colonize the intestine of infants. With time, the number of bifidobacteria decreases while putrefactive bacterial species like Clostridia increase. Such changes are thought to increase production of toxins and carcinogens and cause diseases such as cancer.

1.8.1.1 Beneficial

Lactic acid bacteria include lactobacilli, lactococci and bifidobacteria. They play beneficial roles in the intestine and are also called probiotics. When fermented milks and yogurt are consumed, the lactic acid bacteria present in the dairy products affect the intestinal microflora in various ways. Lactic acid bacteria produce organic acids (lactic, acetic, formic, butyric) as well as H_2O_2 which are more or less antibacterial. The acids control many harmful bacteria including coliforms (e.g. *E. coli.*) and pathogens (e.g. *Clostridium*). Lactic acid bacteria also produce bacteriocins such as nisin, pediocin, and helveticin which are bacteriocidal towards many gram-positive bacteria.

Extracts of fermented milk have been shown to inhibit mutagenesis induced by 4NQO, and 2-AF (Bodana and Rao, 1990) and by MNNG, 4NQO, Trp-P-2, DMAB, and 7,12-dimethylbenz[a]anthracene (DMBA) (Nadathur et al., 1994, 1995). This suggested that lactic acid bacteria may also produce compounds which are antimutagenic. However, whether the bacteria synthesize these compound(s) de novo or require milk components

for their production has not yet been determined. Certain lactobacilli have been shown to degrade the procarcinogenic N-nitrosamines in vitro (Rowland and Grasso, 1975).

1.8.1.2 Harmful

Harmful bacteria include species of *Bacteroides*, *Clostridium,* and others which can convert innocuous substances to harmful metabolites capable of causing mutagenesis. Fecal bacterial enzymes play a deleterious role in the production of toxic metabolites during the excretory process. Xenobiotics are converted to polar conjugated forms by liver enzymes and are excreted. The process involves phase I (cytochrome P450 family of enzymes) and phase II conjugating enzymes. Fecal enzymes catalyze reactions that remove the conjugate and prevent excretion of the xenobiotic moiety. Instead, the now non-polar xenobiotic is re-absorbed in the intestine. As an example, removal of glucuronide by β-glucuronidase causes re-absorption of the xenobiotic in the intestine. This is also known as enterohepatic circulation. Fecal enzymes like azoreductase and nitroreductase, reduce nitrogen compounds (N=N to NH-NH and NO_2 to NH_2, respectively) and renders the nitrogen in the xenobiotic an active center for cytochrome P450-mediated activation (Damani, 1982). As a result, the amines are converted to reactive and mutagenic electrophiles.

1.8.2 Summary of studies

Table 2.4 summarizes various studies on the role of fecal enzymes in affecting cancer. Goldin and Gorbach (1980) showed that supplementation of *Lb. acidophilus* to a western-style diet of beef delayed or controlled DMH-induced colon cancer. Rats receiving beef alone had more carcinomas than rats fed beef supplemented with *Lb. acidophilus*. Rats on a grain diet or a vegetarian style diet did not have any carcinomas. The results showed that the incidence of colon cancer was either delayed or reduced by the

supplementation of *Lb. acidophilus* with the beef diet. The reduction in fecal bacterial enzymes is thought to be a plausible reason for the control of colon carcinomas.

In a subsequent paper, Goldin and Gorbach (1984a) measured levels of β-glucuronidase, azoreductase, and nitroreductase in human subjects who consumed milk alone and/or milk supplemented with *Lb. acidophilus* for a specific time period while maintaining normal dietary habits. The levels of the measured enzymes decreased considerably in subjects who consumed milk supplemented with *Lb. acidophilus* relative to subjects who consumed milk alone. Reduction in fecal enzymes may have occurred by inhibition of the bacteria or by inhibition of the enzymes by metabolites of *Lb. acidophilus*. Goldin and Gorbach (1984b) supplemented diets of rats with aromatic nitro compounds, azo dyes, and glucuronides, and measured the amounts of free amines in the feces in different groups of rats. Rats fed meat alone excreted a greater amount of free amines than rats fed a diet of meat and *Lb. acidophilus*. Administration of antibiotics caused a reduction in the excretion of free amines, suggesting that bacteria in the gut can convert nitro- and azo- compounds to free amines which may then be converted to potent electrophiles.

Johansson et al., (1990) studied the effect of a lactovegetarian diet on the activities of β-glucuronidase, β-glucosidase, and sulfatase. Though the participants consumed isocaloric meals, the lactovegetarian diet had more fibre content than the mixed diet. Analysis of the feces showed a decrease in the levels of the fecal enzymes. β-glucuronidase activity has been known to increase with an increase in glucuronide conjugates and bile flow. The lactovegetarian diet, being low in fat, caused a decrease in biliary flow thus reducing the levels and activity of β-glucuronidase. Higher fiber content also decreases contact time and consequently the likelihood of mutagenesis.

Conjugated forms of mutagens may be converted back to mutagenic forms by the bacterial enzymes during the excretory process (entero-hepatic circulation). Mutagenic

Table 1.4. Effect of changes or supplementation of diet on the activity of fecal enzymes

Subjects	Changes in diet	Mutagen	Result	Reference
Humans	Consumption of fried food	Fried pork or bacon	Increase in excretion of mutagens	Baker et al., 1982
"	Consumption of fried food	Fried pork or bacon	Increase in excretion of mutagens	Dolara et al., 1982
"	Viable cells of *Lb. acidophilus*		Reduction in β-glucuronidase, azoreductase, and nitroreductase	Goldin & Gorbach, 1984a
"	Change to a lactovegetarian diet		Reduction in β-glucuronidase, β-glucosidase, and sulfatase	Johansson et al., 1990
"	Milk fermented by *Lb. acidophilus*	Fried beef	Decrease in excretion of mutagens	Lidbeck et al., 1992
Females only	Milk fermented by *Lb. acidophilus*		Reduction in β-glucuronidase, nitroreductase, and glycholic acid hydrolase	Ling et al., 1994
Rats	Viable cells of *Lb. acidophilus*	DMH	Decrease in tumors	Goldin & Gorbach, 1980
"	Viable cells of *Lb. acidophilus*		Decrease in excretion of amines	Goldin & Gorbach, 1984b
Bacteria	Whole cells of Lactobacillus	NDMA, NDPA	Decrease in nitrosamines	Rowland & Grasso, 1975

activity was detected in the Ames test, in the urine of subjects consuming fried bacon or beef (Baker et al., 1982; Dolara et al., 1984). Felton et al., (1986) reported on the of promutagenic heterocyclic amines during grilling and charbroiling of meat. Lidbeck et al., (1992) showed that administration of *Lb. acidophilus* decreased the amount of mutagen excreted in feces and urine after ingestion of grilled beef patties. Assimilation or binding by lactobacilli was postulated to have caused the reduction in the excretion of the mutagenic metabolites. Yogurt containing viable *Lb. acidophilus* GG alone or together with fiber significantly decreased β-glucuronidase, nitroreductase, and glycholic acid hydrolase activities in feces, and the excretion of bacterial metabolites (phenol, *p*-cresol) in the urine of females (Ling et al., 1994).

Both intestinal and lactic acid bacteria were shown to bind cooked food mutagens (Orrhage et al., 1994). Binding of the mutagens may decrease the likelihood of mutagen to bioactivation by fecal enzymes. However, when subjects consumed yogurt, neither *S. salivarius* ssp. *thermophilus* nor *L. delbrueckii* ssp. *bulgaricus* were recovered when the stomach contents or feces of volunteers were examined (Pedrosa et al., 1995). Others were given a capsule containing 10^{11} organisms of a mucosal adhering strain of *Lb. gasseri* (ADH). ADH was recovered from the stomach and the feces of volunteers, suggesting the use of adherent strains.

1.9 Elevation of immune response

1.9.1 Types of immune responses

Immune responses are elicted by antigens (typically a protein or lipopolysaccharide in nature). Any substance composed of these molecules (e.g. bacteria, viruses, erythrocytes) can thus be antigenic and induce an immune respone. A number of studies suggest beneficial effects from ingestion of lactic acid bacteria either orally or through consumption of fermented milks (Table 2.5). The immunostimulatory effect of

consuming fermented milks is undoubtedly related to the presence of large numbers of lactic acid bacteria in such products.

Antigens induce three principal cell types: macrophages, and B, and T lymphocytes. B cells help synthesize the antibodies or immunoglobulins (IgA, IgE, IgD, IgG, and IgM) which are part of the humoral immune response. The humoral immune response thus, involves the formation of an antigen-antibody complex. Macrophages which are also part of the humoral response, engulf foreign particles and remove them by phagocytosis. The cell-mediated immune response involves the T cells which produce lymphokines and also contribute to delayed hypersensitivity. Macrophage activities are measured by the release of lysozomal and non-lysozomal enzymes (β-galactosidase, β-glucuronidase, lactate dehydrogenase).

1.9.2 Summary of studies

Administration of lactobacillus has been observed to increase the level of immune response in animal studies (Perdigon et al., 1986, 1987, 1988). Immune response was measured in mice fed a mixture of *Lb. acidophilus* and *Lb. casei* or *Lb. acidophilus* and *S. salivarius* ssp. *thermophilus*. In all studies, Swiss albino mice were fed cultures of lactic acid bacteria and then assayed for macrophage activities. An increase in the levels of lysozomal enzymes indicated higher macrophage activity. Initial events of the immune response in the gut occur in Peyer's patch (Owens, 1977). *Bifidobacterium breve* enhanced anti-lipopolysaccharide antibody production in Peyer's patch cells (Yasui and Ohwaki, 1991). The authors also found that the proliferation of B cells was enhanced by a soluble factor. A protective effect of milk fermented by *Lb. casei* and *Lb. acidophilus* was observed against *Shigella sonnei*-induced diarrhea by Nader de Macias et al., (1992). Higher levels of anti-shigella antibodies were detected in mice fed fermented milk than mice receiving skim milk. There was a 100% survival rate for the mice fed fermented milk. Perdigon et al., (1994) showed that yogurt enhanced IgA-secreting B cells in both

Table 1.5. Effect of fermented milk products or various bacteria on the immune response

Subjects	Source of immunomodulation	Result	Reference
Humans	Milk fermented by various lactic acid bacteria; *E. coli.*	Increase in cytokines and interferons	Solis Pereyra and Lemonnier, 1993
"	Milk fermented by *Lb. acidophilus* or Bifidobacteria	Increase in IgA response to *S. typhi*	Link-Amster et al., 1994
"	Milk fermented by *Lb. acidophilus* or bifidobacteria	Increase in phagocytosis to *E. coli*	Schiffrin et al., 1995
Mice	Milk fermented by *L. acidophilus, Lb. casei,* or *S. salivarius* ssp. *thermophilus*	Increase in phagocytic activity	Perdigon et al., 1986, 1987, 1988
"	Milk fermented by various lactic acid bacteria	Increase in IgA response to *L. delbrueckii* ssp. *bulgaricus* only.	Moineau and Goulet, 1991
"	Milk fermented by *Lb. casei, Lb. acidophilus*	Increase in levels of anti-shigella antibodies	Nader de Macias et al., 1992
"	Milk fermented by various lactic acid bacteria	Increase in IgG response to *K. pneumoniae* AD-1	Saucier et al., 1992
"	Yogurt	Increase in IgA-secreting B cells alone.	Perdigon et al., 1994
"	Cell walls of eighteen *E. coli* strains.	Increase in IgG levels; weak IgM response	Sedelmeier and Bessler, 1995
"	Milk fermented by various lactic acid bacteria	Antihypertensive effect with *Lb. helveticus* alone	Yamamoto et al., 1994
Peyer's patch	Cells of *B. breve*	Increase in B cells	Yasui and Ohwaki, 1991

small and large intestinal mucosa in mice. The increase in macrophages (detected by an increase in β-glucuronidase) did not produce an inflammatory response. No differences in the levels of IgM, IgG, or T cells were observed. Milk fermented by *Lb. helveticus* was found to have significant anti-hypertensive effects in spontaneously hypertensive rats (Yamamoto et al., 1994). Milk fermented by other lactic acid bacteria (*Lb. lactis*, *L. cremoris*, *S. salivarius* ssp. *thermophilus*, *Lb. casei* and Bifidobacteria) did not show significant anti-hypertensive effects. Cell wall-associated proteinase activity and peptide content were higher in milk fermented by *Lb. heleveticus* strains.

Fermentation was found to be necessary for the beneficial effects of an enhanced immune response against *Klebsiella pneumoniae* AD-1 (Saucier et al., 1992). Mice that received milk fermented by a mixture of eight lactic acid bacteria had elevated levels of IgG relative to the control group that received either milk or saline. When the fermented milk was pasteurized prior to administration, no elevation of IgG levels was observed. In a different study, humoral immune response was elevated significantly in mice fed milk fermented by *L. delbrueckii* ssp. *bulgaricus* (Moineau and Goulet, 1991). IgA increased significantly eight days after administration of fermented milk. However, milk fermented by other lactic acid bacteria (*B. longum*, *Lb. acidophilus*, *Lb. casei*, *Lb. helveticus*, *S. salivarius* ssp. *thermophilus*, *Lc. cremoris*, *Lb. lactis*) did not increase IgA levels.

Link-Amster et al., (1994) showed an increase in IgA levels in men against *Salmonella typhi*. Groups who received *Lb. acidophilus* and bifidobacteria showed a 4-fold increase in the levels of IgA compared to the control group. A recent study by Schiffrin et al., (1995) reported that milk fermented by *L. acidophilus* or bifidobacterium did not modify lymphocyte activity in blood cells of humans. In contrast, phagocytosis of *E. coli* was enhanced in an in vitro assay. The increase in phagocytosis coincided with colonization of the colon by *Lb. acidophilus* and bifidobacterium.

Milk fermented by *L. delbrueckii* ssp. *bulgaricus* and *S. salivarius* ssp. *thermophilus* and cell wall preparations increased cytokine production by 83% in humans

compared to milk (Solis Pereyra and Lemonnier, 1993). *Lb. casei, Lb. acidophilus*, and bifidobacteria also induced cytokines to a lesser extent. The authors noted that intestinal bacteria like *E. coli* induced some types of cytokines. Sedelmeier and Bessler (1995) showed that a bacterial extract (OM-89) containing cell wall components of eighteen *E. coli* strains increased IgG levels in mice. The authors also showed that certain components like murein, lipopeptide, and porin may contribute to the therapeutic effect of the bacterial extract.

1.10 Epidemiological studies

1.10.1 Types of studies

Changes in diet and lifestyle may influence the incidence of certain types of cancer (Doll and Peto, 1981). Though different studies suggest that consumption of fermented milk and yogurt may decrease the incidence of colon and breast cancer (Table 2.6), the data remain inconclusive. The main types of epidemiological studies are cohort, between-population and case-control studies. Cohort studies focus on populations undergoing dietary transitions. Between-population studies examine populations with large differences in lifestyles and diets. Case-control studies are done within a poulation.

1.10.2 Summary of studies

Some epidemiological studies have shown a negative correlation between risk for colon cancer and increased consumption of yogurt and fermented milk products. A large case-control study in Los Angeles county found that yogurt consumption was protective in both sexes after adjustments were made for calcium content and non-dietary risk factors. No effect was associated with consumption of fruits and vegetables, vitamins, and micronutrients. Total caloric intake pointed to an increased risk for colon cancer (Peters et al., 1992). A large prospective cohort study of adults in the US indicated a reduced risk

for colon cancer with increased intake of vegetables, and dietary fiber (Thun et al., 1992). Yogurt intake was not evaluated.

Calcium present in dairy products is thought to have a protective role against colon cancer. Dietary lipids are thought to stimulate tumor formation from cell populations exposed to initiating carcinogens by increasing cell proliferation leading to increased numbers of cells with abnormal DNA (Lipkin and Newmark, 1985). Ionic calcium from digested foods may reduce the exposure of bowel epithelium to fats and bile acids by binding lipids to form insoluble soaps (Newmark et al., 1978). Pectins in vegetables, by binding calcium in the upper gastrointestinal tract, may be beneficial by releasing ionic calcium de novo to the colon lumen and lowering colon and fecal pH. Slattery et al., (1988) found calcium to be protective against colon cancer in a case-control study in Utah. A higher protective effect was found in males than in females after adjusting for total calories. Garland et al., (1985), found a significant association between dietary vitamin D and calcium and a decreased risk for colorectal cancer in a prospective study of white males in the U.S. Sunlight was also considered as it is required for production of vitamin D. However the authors found no correlation between the association of sunlight, vitamin D, and colon cancer. A prospective cohort study of women in Iowa mentioned that calcium and vitamin D may play a role in modestly reducing colon cancer risk (Bostick et al., 1993). Vitamin D is associated with calcium metabolism and has been shown to reduce cell proliferation in human colon cell lines in vitro (Colston et al., 1981). However, a recent cohort study found that the occurrence of colorectal adenoma was neither related to calcium intake nor to milk consumption (Kampman et al., 1994), while vitamin D from supplements was found to have a slight protective effect among women. The authors suggested that the lack of association between calcium and colon cancer may be due to the long time period required for the development of tumors.

A case-control study in Utah reported that adults who consumed food high in saturated fatty acids had a slightly increased risk for prostate cancer while consumption of

Table 1.6. Factors affecting risk for certain cancers

Type of cancer	Dietary factors	Correlation	Reference
Breast	Cheese and alcohol Yogurt	Increase in risk Decrease in risk	Le et al., 1986
	Fermented milks; fiber Anti-oxidants	Decrease in risk No conclusion	Van `T Veer et al., 1991
Colon	Fiber, shorter transit time, milk Fecal bacterial steroids	Decrease in risk No conclusion	Report from IARC, 1977
	Vitamin D, calcium	Protective effect	Garland et al., 1985
	Calcium Calories, protein, fat	Protective effect Increase in risk	Slattery et al., 1988
	Yogurt Vitamins, micronutrients	Protective Not protective	Peters et al., 1992
	Vegetables, dietary fiber	Decrease in risk	Thun et al., 1992
	Calcium, fermented milk, milk Vitamin D	Not protective Slight decrease in risk	Kampman et al., 1994
Ovarian	Galactose, lactase activity	Increase in risk	Cramer et al., 1989
Pancreas	Vegetables, fermented milks, cheese, legumes	Decrease in risk	Bueno de Mesquita et al., 1991
Prostate	Saturated fatty acids (consumed at an adult age)	Increased risk	Slattery et al., 1988

fatty acids during adolescence did not increase risk (Slattery et al., 1990). Most participants in the study reported changing their dietary habits towards a healthier lifestyle as adults. Thus, changes in dietary habits including consumption of fermented milk products may have reduced the risk for prostate cancer. A population-based case-control study performed in The Netherlands suggested that intake of fiber, cruciferous vegetables, and fermented milk products significantly decreased the risk for pancreatic cancer. No single food group alone contributed to a protective effect (Bueno de Mesquita et al., 1991).

Consumption of fermented milk and yogurt have been associated with a reduced risk for breast cancer. Van`T Veer et al., (1991) showed that the combination of low fat, high fiber, and fermented milk in the diet provided protection against breast cancer. Dietary fiber controls fat accumulation by binding biliary breakdown products. Though fat intake was linked with incidence of breast cancer, recent studies indicate that the data are inconclusive. Decrease in the risk for breast cancer was observed in women, who consumed yogurt in a French study (Lê et al., 1986). The authors speculated that inhibition of fecal enzymatic activity of certain gut bacteria may have been responsible for the protective effect. Risk for breast cancer however increased with consumption of cheese, and with the cream content of milk. No association between consumption of butter and risk for breast cancer was observed. Butter contains the highest amount of retinoids and the antioxidant activity of retinoids may have counteracted the risk from fat intake.

A study done on white residents in Boston found that women with ovarian cancer consumed dairy products including yogurt and cottage cheese (Cramer et al., 1989). The study also analyzed the women for galactose-1-phosphate uridyltransferase activity which converts galactose to glucose. The risk for ovarian cancer was higher in women with a reduced transferase activity. Galactose is formed when lactose is hydrolyzed by β-galactosidase. Thus, persistence of β-galactosidase activity and the consequent increase in

levels of galactose in women with reduced transferase activity, were linked to the development of ovarian cancer. Though dairy products may also be high in fat content, no association with risk for ovarian cancer was seen.

1.11 Objective

In spite of a large number of independent observations of antimutagenicity in yogurt, no antimutagenic compounds have been identified. The primary objective of this project was to isolate and identify specific antimutagenic compounds from yogurt with a long-term view towards determining their ability to inhibit colon cancer in a rodent model.

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CHAPTER 2

ANTIMUTAGENICITY OF FERMENTED MILK

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2.1 Abstract

Reconstituted nonfat dry milk fermented by *Lactobacillus helveticus* CH65, *Lactobacillus acidophilus* BG2FO4, *Streptococcus salivarius* ssp. *thermophilus* CH3, *Lactobacillus delbrueckii* ssp. *bulgaricus* 191R, and by a mixture of the latter two organisms was freeze-dried, extracted in acetone, dissolved in dimethyl sulfoxide, and assayed for antimutagenicity in the Ames test (*Salmonella typhimurium* TA 100) against *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine and 3, 2'-dimethyl-4-aminobiphenyl. Dose-dependent activity was significant against both mutagens in all extracts. Maximal inhibitory activity against 3, 2'-dimethyl-4-aminobiphenyl and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine was 2- and 2.7-fold greater, respectively, than that exhibited by extracts of unfermented milk. Extracts of milk fermented by *L. delbrueckii* ssp. *bulgaricus* 191R were examined further. Activity against both mutagens was less soluble in aqueous solutions than in dimethyl sulfoxide. Adjustment of milk fermented by *L. delbrueckii* ssp. *bulgaricus* 191R to pH 3, 7.6, or 13 prior to freeze-drying and acetone extraction did not significantly alter the amount of 3, 2'-dimethyl-4-aminobiphenyl-specific activity recovered. In contrast, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine-specific activity was less extractable at pH 7.6. The weak antimutagenicity of unfermented milk was not increased by addition of 2% L-lactic acid.

2.2 Introduction

Lactic acid bacteria and their fermented food products are thought to confer a variety of important nutritional and therapeutic benefits to consumers, including antimutagenic and anticarcinogenic activity, or both (Fernandez et al., 1987; Gilliland, 1990; O'Sullivan et al., 1992). At least five classes of observations of such activity in fermented milk have been made. 1) Antimutagenicity has been detected against a range of mutagens and promutagens in various microbial and mammalian cell-based test systems (Bodana and Rao, 1990; Hosoda et al., 1992a; Hosoda et al., 1992b; Hosono et al.,

1986a; Hosono et al., 1986b; Hosono et al., 1990; Renner and Münzner, 1991). 2) Consumption of fermented milk inhibited growth of certain types of tumors in mice and rats [(Ayebo et al., 1981; Ayebo et al., 1982; Esser et al., 1983; Fernandez et al., 1987; Reddy et al., 1973), reviewed in (Gilliland, 1991)]. 3) Oral supplements of *Lactobacillus acidophilus* in humans reduced activities of fecal bacterial enzymes (β-glucuronidase, nitroreductase, and azoreductase) that are involved in procarcinogen activation (Goldin and Gorbach, 1984) and reduced excretion of mutagens in feces and urine (Lidbeck et al., 1992). 4) In human and animal studies, dietary lactobacilli stimulated the immune system (Gilliland, 1991; Perdigon et al., 1986; 1987; 1988; 1993; Sellars, 1991). 5) Epidemiological evidence indicates a negative correlation between the incidence of certain cancers and consumption of fermented milk products (Bueno de Mesquita et al., 1991; Peters et al., 1992; Van `T Veer et al., 1991). Of particular interest is the study by Peters et al. (1992), which found that yogurt was protective against colon cancer independent of the suggested protective effect of calcium (Sorenson et al., 1988).

In spite of these observations and the interest generated by them, no specific antimutagenic compounds produced during milk fermentation have been identified. The present report describes antimutagenic activity extracted from fermented milk relative to mutagenesis induced by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) and 3, 2'-dimethyl-4-aminobiphenyl (DMAB) and provides evidence that, in milk fermented by *Lactobacillus delbrueckii ssp. bulgaricus*, different hydrophobic compounds may be responsible.

2.3 Materials and methods

2.3.1 Chemicals

A direct-acting mutagen, MNNG, and an indirect-acting mutagen, DMAB, were purchased from Sigma Chemical Co. (St. Louis, MO). An S9 microsomal fraction from

rat liver induced by Aroclor 1254 was purchased from Molecular Toxicology (Annapolis, MD). All chemicals were reagent grade.

2.3.2 Bacterial strains

Salmonella typhimurium TA 100 was provided by Bruce N. Ames (Department of Biochemistry, University of California, Berkeley, CA). *Streptococcus salivarius* ssp. *thermophilus* CH3, *L. delbrueckii* ssp. *bulgaricus* 191R, and *Lactobacillus helveticus* CH65 were provided by W. E. Sandine (Department of Microbiology, Oregon State University, Corvallis, OR), and *Lactobacillus acidophilus* BG2FO4 was provided by T. Klaenhammer (Department of Food Science, North Carolina State University, Raleigh, NC).

2.3.3 Extraction of fermented milk

Milk was reconstituted in distilled water from nonfat dry powder (Fred Meyer, Inc., Portland, OR) as an 11% solution, hereafter referred to as milk, and was autoclaved for 12 min at 121°C, cooled immediately by transfer to 4°C, and stored at 4°C until use. Fermentations were begun with 0.5% (vol/vol) active cultures and were incubated at 35 ± 3°C for 1 to 2 d. Active cultures were prepared initially by inoculation of a small volume of autoclaved milk with cells held at -80°C and incubation at 37°C for 24 h. Cultures subsequently held at 4°C served as inocula for 1 to 2 wk.

Yogurt was prepared by inoculation of milk with a mixture of *S. salivarius* ssp. *thermophilus* CH3 and *L. delbrueckii* ssp. *bulgaricus* 191R. When used, *L. acidophilus* BG2FO4 was inoculated in milk supplemented with 0.5% yeast extract (Difco, Detroit, MI) and incubated for 4 to 5 d at 37°C. The lengthy fermentations reflect the relatively low temperature and small inocula size used. The fermented milks were freeze-dried in a lyophilizer (Labconco Inc., Kansas City, MO) and extracted with 5 volumes of acetone (Bodana and Rao, 1990). Acetone was removed by vacuum evaporation (Brinkmann

Instruments, Inc., Westbury, NY), and the residue was dissolved in dimethyl sulfoxide (DMSO) and stored at -20°C. One liter of fermented milk yielded 5 ml of extract. Extracts of unfermented milk were prepared and stored similarly.

2.3.4 Activity at different pH

To assess extractability of the antimutagenic activity as a function of pH, unfermented milk, and milk fermented by *L. delbrueckii* ssp. *bulgaricus* 191R were divided into equal portions and adjusted from pH 6.5 (unfermented) to 3, 4, and 13 or from pH 4 (fermented) to 3, 7.6, and 13 prior to freeze-drying and extraction. Adjustment of pH was by addition of 5 N NaOH or 5 N HCl. In one experiment, unfermented milk was acidified by addition of L-lactic acid to a final concentration of 2%, final pH 3.6, and incubated at 37°C for 24 h prior to extraction.

2.3.5 Solubility of antimutagenic compounds

Milk fermented by *L. delbrueckii* ssp. *bulgaricus* 191R was freeze-dried and extracted with acetone, and the acetone extracts were divided into equal portions prior to evaporation of the solvent. Residues were dissolved in DMSO (control); distilled water; 0.1 M potassium phosphate, pH 3; 0.2 M sodium phosphate, pH 7.6; or 0.2 M KCl·NaOH, pH 13; at a ratio of 1 ml of solvent/200 ml of fermented milk. The pH of the latter solution did not change after the extract was dissolved. Residues of the aqueous extracts were re-extracted with DMSO to distinguish between potential insolubility of activity and its potential instability in an aqueous environment. The volume of DMSO used in the re-extraction was also 1 ml/200 ml of fermented milk.

2.3.6 Antimutagenicity assay

The preincubation method of the Ames test (Maron and Ames, 1983) was used. Briefly, 100 µl of a 10 to 11 h culture of *S. typhimurium* TA 100 (requiring histidine)

was added to a variable amount of 0.2 M phosphate buffer, pH 7.4, followed by 1) extracts of fermented or unfermented milk and 2) freshly prepared MNNG or DMAB (both in DMSO) to final volumes of 900 (MNNG assay) or 675 µl (DMAB assay). For DMAB, 100 µl of culture was added to 500 µl of S9 mix instead of phosphate buffer. Final concentrations of MNNG and DMAB were 1 and 10 µM, respectively. DMSO (100 µl) and extract (50 µl) were added separately as controls instead of mutagen to test their effects on mutation frequency and cell viability. At the concentrations used, neither the extracts nor DMSO reduced cell viability, according to the visual test described by Maron and Ames (1983). Following 20 min of incubation at 37°C in a rotary shaker, tube contents were transferred to test tubes containing 2 ml of top agar and 0.5 mM histidine and biotin (all at 45°C). The tubes were vortexed and poured onto duplicate minimal glucose plates, and colonies were counted after 2 d at 37°C. Antimutagenic activity was expressed as a percentage of inhibition of mutagenesis:

$$\text{Percentage of Inhibition} = \{1 - [(A - E) / (B - D)]\} \times 100$$

A and B are numbers of mutagen-induced histidine non-requiring revertants observed in the presence and absence of the extracts, respectively, and E and D are numbers of spontaneous histidine non-requiring revertants observed in the extract and DMSO controls, respectively. The number of spontaneous revertants in the DMSO and extract controls typically ranged from 100 to 150.

2.3.7 Statistics

Data were analyzed by Student's t test, or by ANOVA, SAS microcomputer version 6.04, SAS Institute, Inc., Cary, NC. Significant differences detected by ANOVA were analyzed by pairwise t test protected least significant differences. Significance was at $P \leq 0.05$.

2.4 Results

Extracts of milk fermented by all organisms inhibited mutagenesis induced by MNNG and DMAB. For MNNG, all extracts exhibited a similar dose-response (Table 2.1). Inhibition at the lowest dose ranged from 30 to 60% and from 87 to 95% at the highest. In contrast, unfermented milk exhibited weaker antimutagenicity, ranging from 10 to 21% at the lowest dose and from 34 to 36% at the highest. At the maximal addition of extract, the activity of fermented milk was 2.7-fold greater than that of milk alone. Addition of 0.5% yeast extract to unfermented milk (used as a control for the similarly supplemented milk fermented by *L. acidophilus* BG2F04) did not result in significantly greater activity relative to that of unsupplemented milk.

Extracts of fermented milk exhibited a positive dose-response against DMAB-induced mutagenesis. For DMAB, unfermented milk (with and without yeast extract) also exhibited a weak dose response. At the maximal addition of extract, the activity of fermented milk against DMAB was 2-fold greater than that of milk alone.

To assess the effect of pH on extractability of activity into acetone, the pH of milk fermented by *L. delbrueckii* ssp. *bulgaricus* 191R, final pH 4.1, and *S. salivarius* ssp. *thermophilus* CH3, final pH 5.0, was adjusted to 3, 7.6, or 13 prior to freeze-drying and extraction (Figures 2.1, 2.2, and 2.3). Extraction of MNNG-specific activity from the milk fermented by *L. delbrueckii* ssp. *bulgaricus* 191R was significantly less efficient at pH 7.6 than at pH 3 or 13, based on the doses of 20 and 50 µl. At the 10-µl dose, the difference was not significant. In contrast, extractability of DMAB-specific activity from the same fermentation was insensitive to pH adjustment, suggesting hydrophobic and neutral compounds that are different from that responsible for MNNG-specific activity (Figure 2.2). Adjustment of pH had a significant effect on the extractability of DMAB-specific activity from milk fermented by *S. salivarius* ssp. *thermophilus* CH3 only at the 75-µl dose (Figure 2.3). Activity was extractable at all pH tested, but activity was

Table 2.1. Percentage of inhibition of mutagenesis by extracts of milk and fermented milk[1].

Extract (µl)	Milk	*Streptococcus salivarius* ssp. *thermophilus*[2]	*Lactobacillus delbrueckii* ssp. *bulgaricus*[3]	*Lactobacillus helveticus*[4]	Yogurt[5]	Milk[6]	*Lactobacillus acidophilus*[7]
			N-methyl-*N*'-nitro-*N*-nitrosoguanidine				
10	10.4 ± 0.9 ^{c}	49.3 ± 6.6^{ab}	29.6 ± 2.9^{bc}	35.3 ± 11.2^{b}	45.8 ± 14.4^{ab}	20.4 ± 1.3^{x}	59.8 ± 4.7^{y}
20	19.2 ± 2.5^{b}	77.4 ± 7.9^{a}	64.3 ± 5.3^{a}	73.9 ± 9.4^{a}	80.6 ± 5.4^{a}	26.5 ± 1.8^{x}	76.5 ± 4.9^{y}
50	34.2 ± 1.3^{b}	89.4 ± 5.9^{a}	93.4 ± 0.8^{a}	95.1 ± 3.1^{a}	93.6 ± 2.7^{a}	35.4 ± 1.5^{x}	95.6 ± 0.7^{y}
			3,2'-dimethyl-4-aminobiphenyl				
25	22.9 ± 2.3^{b}	42.0 ± 4.5^{a}	34.3 ± 2.3^{ab}	33.6 ± 2.7^{ab}	44.0 ± 6.9^{a}	11.1 ± 2.8^{x}	33.2 ± 1.8^{y}
50	23.1 ± 2.9^{c}	58.2 ± 3.9^{a}	43.0 ± 4.0^{b}	54.5 ± 6.4^{b}	58.7 ± 7.3^{a}	19.5 ± 2.8^{x}	49.5 ± 0.6^{y}
75	33.9 ± 2.8^{c}	61.9 ± 5.0^{ab}	56.7 ± 4.2^{b}	65.2 ± 6.1^{ab}	78.8 ± 9.9^{a}	24.6 ± 1.3^{x}	55.1 ± 0.8^{y}

[1] Means ± SEM of 3 to 4 experiments. Values in the same row that do not share at least one common superscript (eg. a, b, c, etc.) are significantly different ($P \leq .05$). Comparisons were 1) between unfermented milk supplemented with 0.5% yeast extract and supplemented milk fermented by *Lb. acidophilus* BG2FO4 (values in last 2 columns) and 2) between unfermented milk and milk fermented by each of the other organisms (values in first 5 columns).
[2] Strain CH3.
[3] Strain 191R.
[4] Strain CH65.
[5] *S. salivarius* ssp. *thermophilus* CH3 + *L. delbrueckii* ssp. *bulgaricus* 191R.
[6] Milk supplemented with 0.5% yeast extract (control for *Lb. acidophilus* BG2FO4).
[7] Strain BG2FO4.

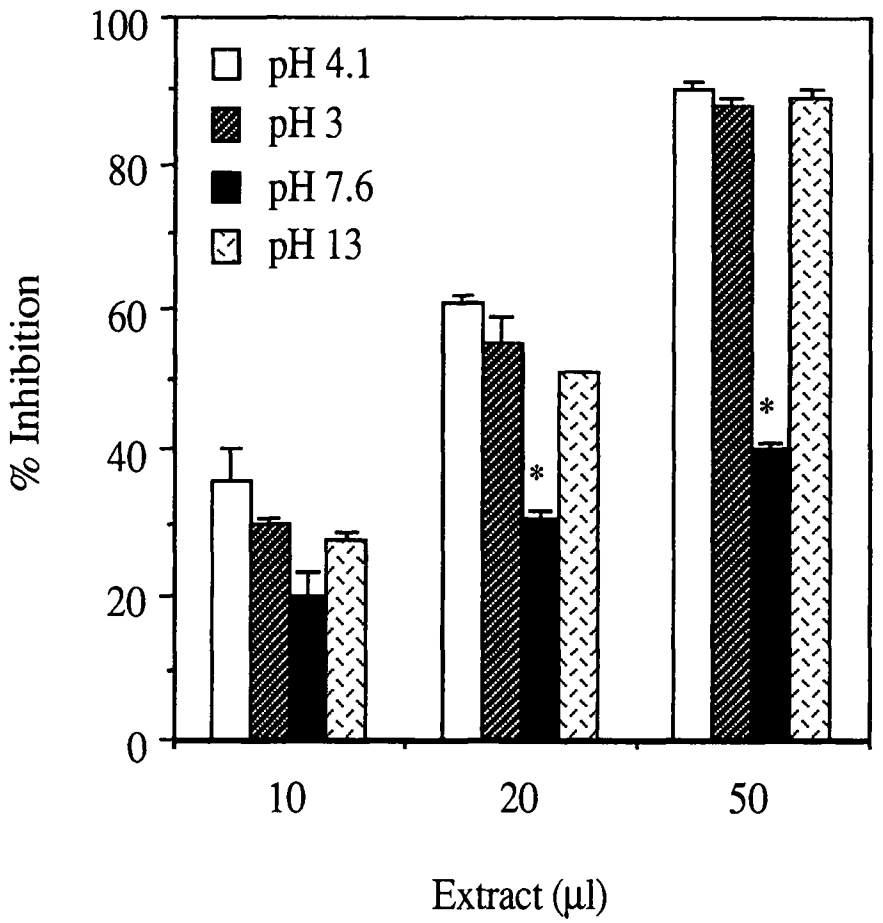

Figure 2.1. Effect of pH on extractability of compounds with activity specific for *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG). Percentage of inhibition of MNNG-induced mutagenesis by extracts of milk fermented by *Lactobacillus delbrueckii* ssp. *bulgaricus* 191R (pH 4.1) and adjusted to pH 3, 7.6, and 13 prior to freeze-drying and extraction. Values are means of two experiments. Error bars represent the standard deviation, and an asterisk over a bar indicates that significantly less activity was extracted at that pH than at the other pH treatments at the same dose (two-tailed *t* test).

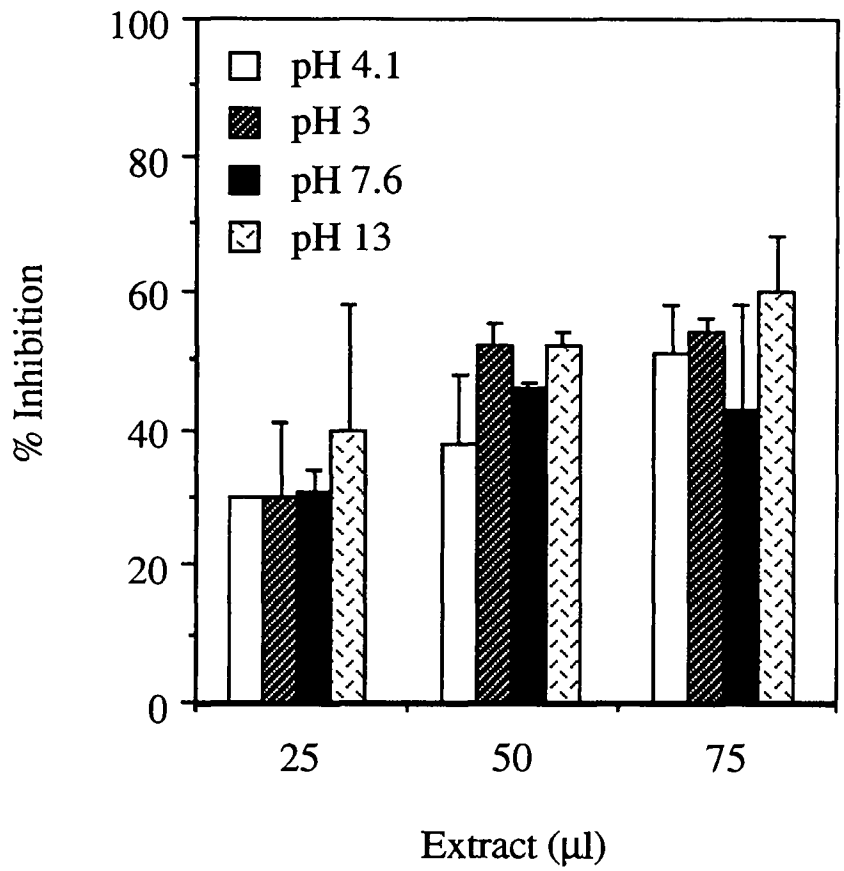

Figure 2.2. Effect of pH on extractability of compounds with activity specific for 3,2'-dimethyl-4-aminobiphenyl (DMAB). Percentage of inhibition of DMAB-induced mutagenesis by extracts of milk fermented by *Lactobacillus delbrueckii* ssp. *bulgaricus* 191R (pH 4.1) and adjusted to pH 3, 7.6, and 13 prior to freeze-drying and extraction. Values are means of two experiments. Error bars represent the standard deviation. No significant differences were found between any of the treatments (two-tailed *t* test).

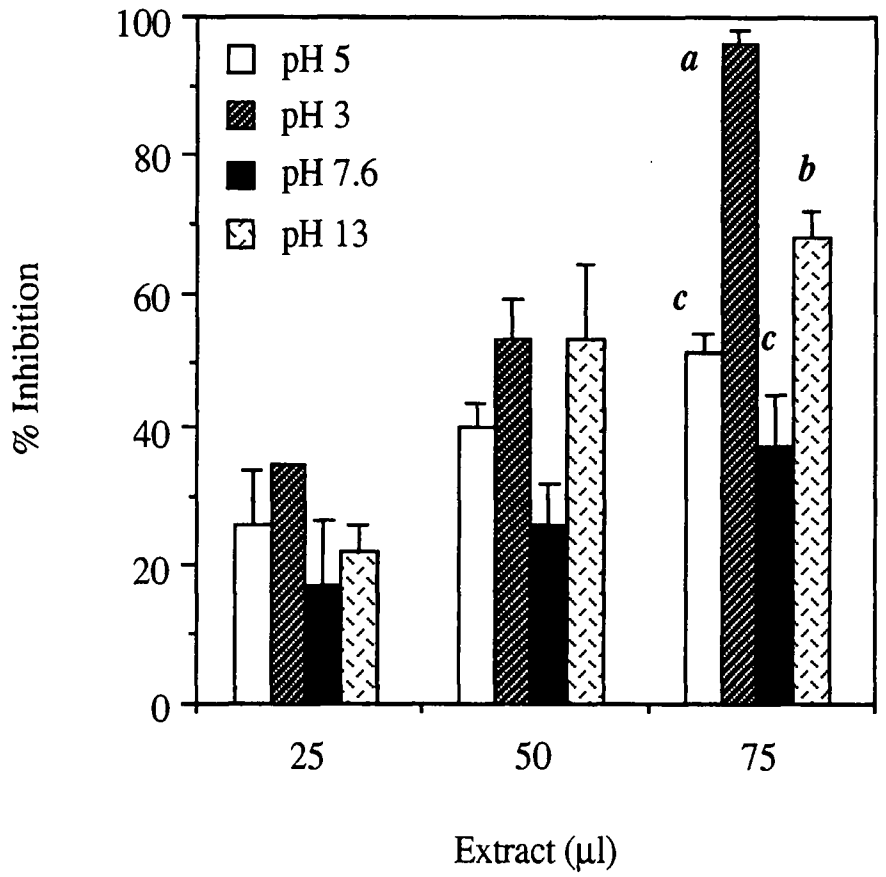

Figure 2.3. Effect of pH on extractability of compounds with activity specific for 3,2'-dimethyl-4-aminobiphenyl (DMAB). Percentage of inhibition of DMAB-induced mutagenesis by extracts of milk fermented by *Streptococcus salivarius* ssp. *thermophilus* CH3 (pH 5) and adjusted to pH 3, 7.6, and 13 prior to freeze-drying and extraction. Values are means of two experiments. Error bars represent the standard deviation. Different letters above the bars (or adjacent to the pH 3 bar) indicate significantly different activities at 75 µl (two-tailed *t* test).

greatest at pH 3, followed by pH 13; equal and lesser amounts were extracted at pH 5 and 7.6.

TABLE 2.2. Percentage of inhibition of mutagenesis by extracts of unfermented milk adjusted to different pH prior to extraction.

Extract	pH				
(µl)	6.5	3	3.6[2]	4	13
	N-methyl-N'-nitro-N-nitrosoguanidine				
10	16.5 ± 0.1	12.6 ± 0.8	15.2 ± 0.6	17.5 ± 1.4	4.3 ± 1.1
20	28.4 ± 0.3	28.2 ± 1.2	24.7 ± 1.4	32.7 ± 1.6	8.7 ± 1.3[3]
50	35.5 ± 0.6	41.1 ± 1.0	35.2 ± 0.8	41.6 ± 0.4	16.2 ± 0.6[4]
	3, 2'-dimethyl-4-aminobiphenyl				
25	13.7 ± 4.0	9.6 ± 2.8	7.1 ± 0.6	18.4 ± 0.6	9.1 ± 6.9
50	24.4 ± 0.2	15.3 ± 0.9	15.8 ± 1.0	19.7 ± 0.1	12.9 ± 3.8
75	28.3 ± 2.1	26.7 ± 0.8	25.4 ± 0.4	23.0 ± 1.6	30.9 ± 2.8

[1] Means ± SEM of 2 experiments.
[2] Milk was incubated at 37°C for 24 h after addition of 2% L-lactic acid (final concentration).
[3,4] Significantly less activity was extracted than at all other pH (two-tailed *t* test).

Adjustment of the pH of unfermented milk (by addition of concentrated HCl, NaOH, or 2% L-lactic acid) from 6.5 to 3, 3.6, 4, and 13 did not significantly change the amount of antimutagenic activity extracted relative to DMAB (Table 2.2). In contrast, extraction of MNNG-specific activity was less efficient at pH 13, suggesting that different constituents are responsible for the modest antimutagenicity in unfermented milk. Adjustment of milk pH by addition of 2% L-lactic acid did not significantly increase activity over that in untreated milk, demonstrating that acidification alone during milk fermentation is insufficient to produce antimutagenic activity.

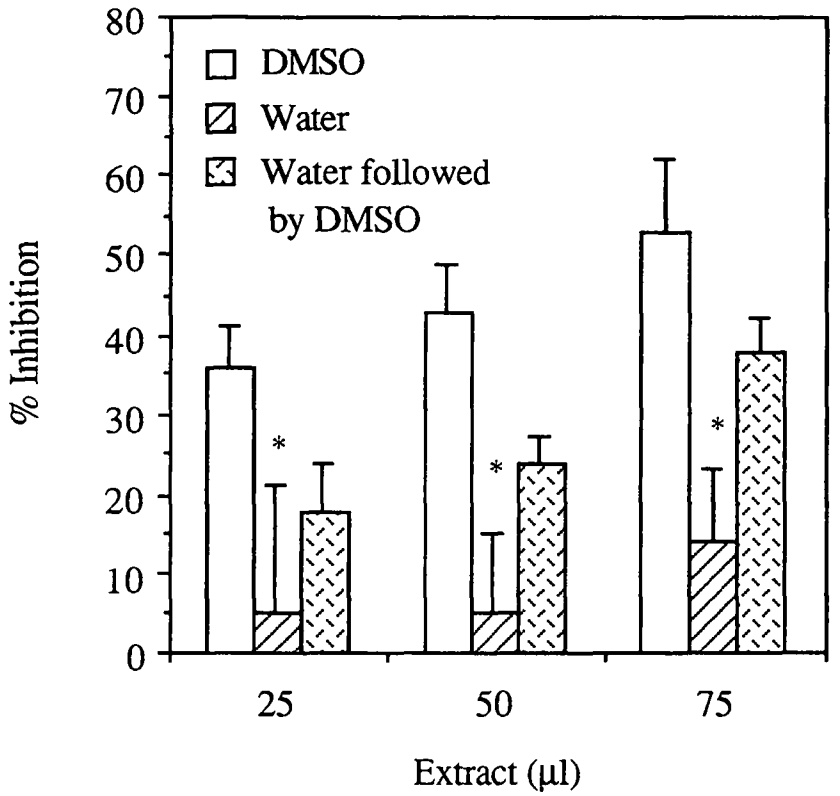

Figure 2.4. Solubility of compounds with activity specific for 3,2'-dimethyl-4-aminobiphenyl (DMAB). Percentage inhibition of DMAB-induced mutagenesis by extracts of milk fermented by *Lactobacillus delbrueckii* ssp. *bulgaricus* 191R and dissolved in dimethyl sulfoxide (DMSO), water, or water initially followed by reextraction with DMSO. Values are means of two experiments. Error bars represent the standard deviation, and an asterisk over the bar representing water indicates significantly less activity was extracted into water than DMSO at the same dose (two-tailed *t* test).

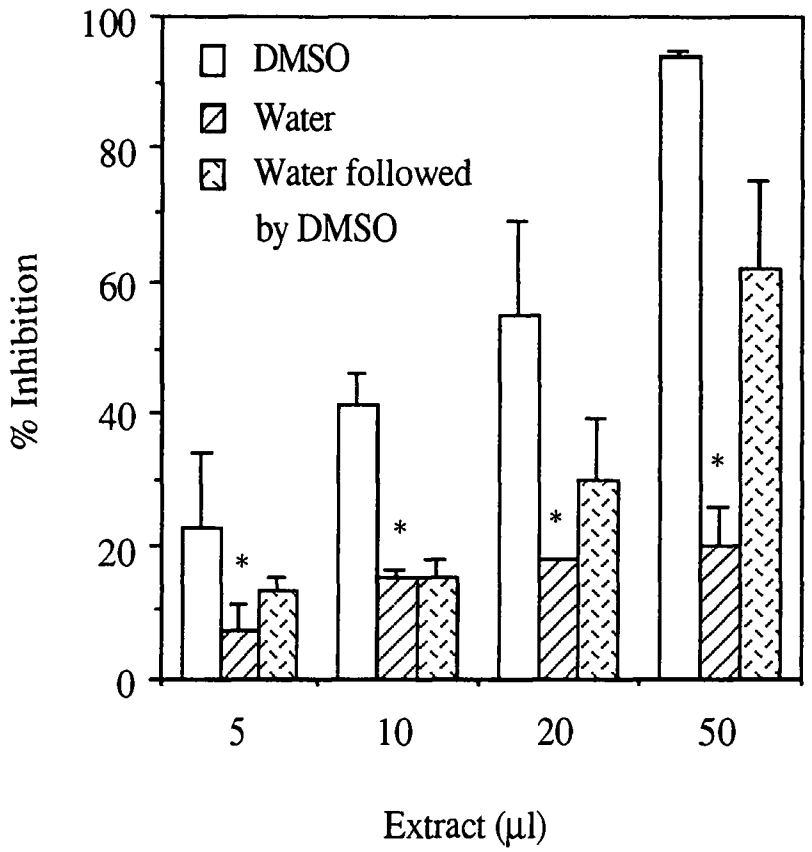

Figure 2.5. Solubility of compounds with activity specific for *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG). Percentage of inhibition of MNNG-indiced mutagenesis by extracts of milk fermented by *Lactobacillus delbrueckii* ssp. *bulgaricus* 191R and dissolved in dimethyl sulfoxide (DMSO), water, or water initially followed by re-extraction with DMSO. Values are means of twp experiments. Error bars represent the standard deviation and an asterisk over the bar represnting the water indicates significantly less activity was extracted into water than DMSO at the same dose (two-tailed *t* test).

TABLE 2.3. Differential solubility of antimutagenic activity of acetone extracts of milk fermented by *Lactobacillus delbrueckii* ssp. *bulgaricus* 191R[1].

Extract			pH			pH	
µl	DMSO	3[2]	7.6[3]	13[4]	3[2]	7.6[3]	13[4]
		Initial Extraction			Residue of initial extraction dissolved in dimethyl sulfoxide (DMSO)		
		Percentage of inhibition of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine-induced mutagenesis					
10	39.9 ± 3.8^{a}	12.0 ± 3.9^{b}	15.7 ± 2.5^{b}	12.6 ± 1.5^{b}	29.0 ± 0.3^{ab}	26.3 ± 14.3^{ab}	30.2 ± 4.3^{ab}
20	64.4 ± 3.0^{a}	20.1 ± 7.6^{cd}	27.4 ± 1.5^{cd}	19.0 ± 8.9^{d}	40.0 ± 4.6^{bc}	50.3 ± 6.6^{ab}	52.9 ± 4.6^{ab}
50	97.2 ± 0.1^{a}	37.2 ± 0.7^{c}	28.6 ± 0.2^{cd}	16.6 ± 9.5^{d}	79.8 ± 1.1^{b}	80.4 ± 1.6^{b}	88.2 ± 1.8^{ab}
		Percentage of inhibition of 3, 2'-dimethyl-4-aminobiphenyl-induced mutagenesis					
25	31.7 ± 3.0^{a}	17.2 ± 1.0^{bcd}	20.0 ± 0.8^{bc}	14.2 ± 0.9^{d}	32.3 ± 4.8^{a}	16.2 ± 0.4^{bcd}	22.3 ± 0.4^{b}
50	47.4 ± 3.7^{a}	27.9 ± 1.6^{d}	26.4 ± 1.9^{d}	26.6 ± 1.5^{d}	42.0 ± 4.2^{b}	26.7 ± 2.5^{d}	36.2 ± 1.6^{c}
75	58.4 ± 4.0^{a}	36.6 ± 1.6^{c}	36.1 ± 1.5^{c}	33.3 ± 0.6^{c}	51.0 ± 3.1^{b}	37.2 ± 0.8^{c}	56.2 ± 4.1^{ab}

[1] Mean ± S.E. of two experiments. Acetone extracts of milk fermented by *L. delbrueckii* ssp. *bulgaricus* 191R were initially extracted into DMSO or aqueous solutions. Residues of the aqueous extractions were subsequently dissolved in DMSO. Values in the same row that do not share at least one common superscript are significantly different ($P \leq .05$).
[2] Potassium phosphate, 0.1 *M*, pH 3. [3] Sodium phosphate, 0.2 *M*, pH 7.6. [4] KCl·NaOH, 0.2 *M*, pH 13.

When water was used instead of DMSO to dissolve the acetone extracts, activity against DMAB (Figure 2.4) and MNNG (Figure 2.5) was significantly less, suggesting that the compounds responsible are largely insoluble in water. To test the possibility that activity was extractable but unstable in water, the water-extracted residue was re-extracted with DMSO. More activity was recovered in the second extractions, supporting the notion that the antimutagenic compounds are relatively insoluble in water. Use of aqueous solutions instead of water in the same extraction protocol supported this possibility (Table 2.3). Activity against both mutagens was less extractable in all aqueous solutions than in DMSO. In the majority of re-extractions with DMSO, greater activity was recovered than extracted initially into the buffers or the alkaline KCl solution.

2.5 Discussion

Milk fermented by different lactic acid bacteria was extracted in acetone and exhibited dose-dependent antimutagenic activity against direct acting (MNNG) and indirect acting (DMAB) mutagens. Milk fermented by *L. delbrueckii* ssp. *bulgaricus* 191R was examined further. Extractability and solubility of the anti-MNNG and anti-DMAB activities were tested and compared with activity in unfermented milk to assess the effect of fermentation. Although alternative explanations are possible, the data support the possibility that different hydrophobic antimutagenic compounds are produced in milk fermented by *L. delbrueckii* ssp. *bulgaricus* 191R.

The findings reported herein are consistent with other observations of antimutagenicity in fermented milks. Skim milk fermented by a number of lactic acid bacteria inhibited MNNG-induced mutagenesis in the Ames test (*S. typhimurium* TA 100) (Hosoda et al., 1992a). Fermented milk rather than extracts was tested directly, and, unfortunately, water was used rather than milk as a negative control. However, periodic sampling during the course of one fermentation involving *Lb. acidophilus* LA106 (LA2) indicated that final activity was about 2.5-fold greater than that present initially in milk.

Although this activity is apparently significantly greater than that observed (given that our extract represented a relative 100-fold concentration), other differences in experimental protocols, including use of different strains of *Lb. acidophilus*, complicate a valid comparison. In a similar study, antimutagenic activity against 3-amino-1-methyl-5*H*-pyrido[4, 3-*b*]indole (Trp P-2) varied considerably among the same strains of various lactic acid bacteria compared with unfermented milk (Hosoda et al., 1992b). Hosono et al. (1986b) used streptomycin-dependent derivatives of the Ames strains to show that fermented milk, relative to unfermented milk, inhibited a different mutagen, 2-(2-furyl)-3-(5-nitro-2-furyl) acrylamide. Data suggested that activity occurred against 4-nitroquinoline-N-oxide (4NQO) and mutagenic extracts from mammalian feces, but the relative contribution of milk to the latter activities was impossible to assess because water was used as a negative control. Bodana and Rao (1990) reported that acetone and ethyl acetate extracts of fermented skim milk inhibited mutagenesis caused by 4NQO and 2-aminofluorene (2AF) in the Ames test (*S. typhimurium* TA 100 and TA 98). When the TA 100 tester was used, unfermented milk exhibited slight dose-dependent activity against 4NQO but not 2AF. Although the same extraction protocol was used (Bodana and Rao, 1991), no evidence suggests that the activity against MNNG and DMAB was due to the same compounds that inhibited mutagenesis caused by 2AF and 4NQO. Interestingly however, both 2AF and DMAB share a common activation pathway initially involving N-hydroxylation by cytochrome P450 1A2 (Aoyama et al., 1989). However, subsequent in vivo acetylation, other enzymatic modifications by the Ames strain, or both may not necessarily be the same.

MNNG and DMAB differ in their ability to cause mutagenesis; MNNG is converted nonenzymatically by glutathione to a methyldiazonium cation, which spontaneously forms CH_3^+, a potent methylating agent, which attacks the O^6 position of guanine to produce GC to AT transitions (Lawley and Thatcher, 1970). Antimutagenic

compounds in the fermented milk extracts may 1) react directly with and inactivate MNNG, 2) react with glutathione and prevent activation of MNNG, 3) neutralize the methyldiazonium ion or methyl cation, or 4) enhance repair of DNA damage. None of these possibilities is mutually exclusive, nor can additional mechanisms be ruled out.

DMAB is an indirect acting mutagen requiring metabolic activation by cytochrome P450 1A2 to form N-hydroxy DMAB which may be further transformed by acetylation (Flammang et al., 1985). Adducts formed by reaction of DNA with N-hydroxy DMAB have been identified as N-deoxyguanosin-8-yl-DMAB and 5-deoxyguanosin-N^2-yl-DMAB (Westra et al., 1985). These adducts presumably interfere with normal base pairing and cause GC-AT transitions. Mechanisms of protection exhibited by the extracts against DMAB may involve one or more of the following activities: 1) direct chemical reaction with DMAB; 2) inhibition of cytochrome P450 1A2; 3) inhibition of subsequent activating enzymes such as acetylase; 4) reaction with N-hydroxy DMAB or other activated forms of DMAB; or 5) enhancement of DNA repair. On theoretical grounds, a single compound is not likely to inhibit MNNG and DMAB.

The role of milk in the formation of MNNG- and DMAB-specific antimutagenicity is unresolved, but at least two possibilities are worth consideration: 1) inactive milk constituents (precursors) may be converted to antimutagens by the fermenting organisms or 2) milk may lack such precursors but serve as a neutral medium for organisms that ordinarily produce antimutagens during fermentative growth. A combination of those possibilities cannot be ruled out. One explanation for the antimutagenic activity of unfermented milk has been provided by Yoshida and Xiuyun (1992), who showed pH-dependent binding of mutagenic heterocyclic amines, including Trp P-2, by bovine milk caseins in vitro. (At the acidic pH of fermented milk, little binding occurred.) The relevance of this activity to that observed in the present study in unfermented milk against different mutagens remains to be established.

Although speculatation about compounds responsible for the MNNG-specific activity in milk fermented by *L. delbrueckii* ssp. *bulgaricus* would be premature with only a crude acetone extract available, extractability in acetone at pH 3 and 13, but not at pH 7.6, suggests the following possibility. The compounds responsible for activity may be neutral and extractable at all three pH, but an inhibitor may be uncharged and extractable only at pH 7.6. A compound possessing single amino and carboxyl groups would have the property described for the hypothetical inhibitor.

Evidence that fermented milk products are antimutagenic in various assay systems against different mutagens and carcinogens is significant because of the importance of such foods in the human diet, because multiple mechanisms of action are suggested, and because ingestion of natural anticarcinogens plays a role in counteracting the unavoidable intake of carcinogenic substances present in food (Sugimura, 1990). Although antimutagencity has been observed in the Ames test against mutagens used to produce chemically-induced colon cancer in mammalian models, such activity can not yet be labeled anticarcinogenic. Not all mutagens are carcinogens, and not all antimutagens exhibit anticarcinogenicity. Our on-going efforts to purify and to identify active constitutents of acetone extracts of milk fermented by *L. delbrueckii* ssp. *bulgaricus* 191R will aid experimental efforts to determine whether the compounds are anticarcinogenic in an appropriate animal model, and ultimately, to determine their contribution, if any, to anticarcinogenesis in humans.

2.6 Acknowledgments

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CHAPTER 3

ANTIMUTAGENICITY OF AN ACETONE EXTRACT OF YOGURT

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3.1 Abstract

Reconstituted non-fat dry milk powder fermented by a mixture of *Streptococcus salivarius* ssp. *thermophilus* CH3 and *Lactobacillus delbrueckii* ssp. *bulgaricus* 191R to produce yogurt, was freeze-dried and extracted in acetone. After evaporation of the acetone, the extract was dissolved in dimethyl sulfoxide (DMSO) and tested for antimutagenicity. In the Ames test, significant dose-dependent activity was observed against *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG), 4-nitroquinoline-*N*-oxide (4NQO), 3,2'-dimethyl-4-aminobiphenyl (DMAB), 7,12-dimethylbenz[*a*]anthracene (DMBA), and 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole acetate (Trp-P2). Weak activity was observed against 1,2,7,8-diepoxyoctane (DEO), and no activity was observed against methylmethanesulfonate (MMS), ethylmethanesulfonate (EMS), or aflatoxin B1 (AFB1). In a related assay (*Saccharomyces cerevisiae* D7), significant antimutagenic activity was detected against MNNG and 4NQO.

Activity against the experimental colon carcinogens MNNG and DMAB was examined further, as assayed in the Ames test (*Salmonella typhimurium* TA 100). Compounds responsible for both activities were less soluble in aqueous solutions than in DMSO. Adjustment of yogurt pH to 3, 7.6, or 13 prior to freeze-drying and acetone extraction did not significantly alter the amount of anti-MNNG activity recovered. In contrast, extractability of anti-DMAB activity was significantly greater at acidic pH. Conjugated linoleic acid, a known dairy anticarcinogen, failed to inhibit mutagenesis caused by either mutagen, suggesting that other yogurt-derived compound(s) are responsible. Unfermented milk was treated with lactic acid, yogurt bacteria without subsequent growth, or both, to determine if formation of antimutagenic activity required bacterial growth. Extracts of the milk treatments exhibited the same weak antimutagenicity observed in unfermented milk, approximately 2.5-fold less than in the yogurt extracts, suggesting that antimutagenic activity is associated with bacterial growth.

3.2 Introduction

Lactic acid bacteria and their fermented food products are believed to confer a variety of important nutritional and therapeutic benefits including antimutagenic and/or anticarcinogenic activity (Rao et al., 1986; Fernandez et al., 1987; Gilliland, 1990). At least five classes of observations have been made with respect to such activity.

1) Antimutagenicity has been detected against a range of mutagens and promutagens in various microbial and mammalian cell-based test systems (Bodana and Rao, 1990; Hosoda et al., 1992a, 1992b; Hosono et al., 1986a, 1986b; Hosono et al., 1990; Renner and Münzer, 1991; Nadathur et al., 1994; Pool-Zobel et al., 1993a, 1993b). 2) Consumption of fermented milk or the bacteria used in their production has been observed to inhibit growth of certain types of tumors in mice and rats (Reddy and Rivenson, 1993, Shackelford et al., 1983; Ayebo et al., 1981, 1982; Esser et al., 1983; Fernandez et al., 1991; Reddy et al., 1973). 3) Oral supplements of *Lactobacillus acidophilus* in humans have been shown to result in reduction of fecal bacterial enzyme activities (β-glucuronidase, nitroreductase, and azoreductase) involved in procarcinogen activation (Goldin and Gorbach, 1984) and to reduce excretion of mutagens in feces and urine (Lidbeck et al., 1992). 4) In human and animal studies, dietary lactobacilli have been observed to stimulate the immune system (Solis Pereyra and Lemonnier, 1993; Perdigón et al., 1986, 1987, 1988, 1993). 5) Epidemiological evidence indicates a negative correlation between the incidence of certain cancers and consumption of fermented milk products (Bueno de Mesquita et al., 1991; Peters et al., 1992; Van 'T Veer et al., 1991). Of particular interest is the study by Peters et al. (1992), which found that yogurt was protective against colon cancer independent of the suggested protective effect of calcium (Sorenson et al., 1988).

In spite of these observations and the interest they have generated, no specific antimutagenic compounds produced during milk fermentation have been identified. The present report describes antimutagenic activity extracted from yogurt, with emphasis on

activity specific for the experimental colon carcinogens, MNNG and DMAB (Jones et al., 1989; Cleveland et al., 1967).

3.3 Materials and methods

3.3.1 Chemicals

N-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG), 4-nitroquinoline-*N*-oxide (4NQO), methylmethanesulfonate (MMS), ethylmethanesulfonate (EMS) , 7,12-dimethyl-benz[*a*]anthracene (DMBA), aflatoxin B1 (AFB1), 1,2-dimethylhydrazine hydrochloride (DMH) and 3,2'-dimethyl-4-aminobiphenyl (DMAB) were from Sigma Chemical Co. (St. Louis, MO); 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole acetate (Trp-P2) and 1,2,7,8-diepoxyoctane (DEO) were purchased from Aldrich Chemical Co. (Milwaukee, WI); diethylnitrosamine (DEN) was from Fluka Chemical Corp. (Ronkonkoma, NY), and nitrosonornicotine (NNN) was from Chemsyn Science Laboratories (Lenexa, KS). Aroclor 1254-induced rat liver S9 microsomal fraction was from Molecular Toxicology (Annapolis, MD). Conjugated linoleic acid, CLA, is a mixture of positional and geometric isomers of linoleic acid (*c*9, *c*12-octadecadienoic acid) and was provided under nitrogen by Dr. M.W. Pariza (Department of Food Microbiology and Toxicology, University of Wisconsin, Madison, WI). CLA was stored at -20 °C and handled under O_2-purged nitrogen gas. All solvents and other chemicals were reagent grade.

3.3.2 Microbial strains and media

Salmonella typhimurium strains TA 100, 98, and 97 were provided by Dr. Bruce N. Ames (Department of Biochemistry, University of California, Berkeley, CA). *Streptococcus salivarius* ssp. *thermophilus* CH3 and *Lactobacillus delbrueckii* ssp. *bulgaricus* 191R were provided by Dr. W. E. Sandine (Department of Microbiology, Oregon State University, Corvallis, OR). *Saccharomyces cerevisiae* D7, *MATa/MATα ade2-40/ade2-119 trp5-12/trp5-27 ilv1-92/ilv1-92*, has been described (Zimmermann et

al., 1975). Yeast minimal medium (M) is 0.67% Bacto yeast nitrogen base without amino acids (Difco Laboratories, Detroit, MI) containing 2% glucose and supplemented where indicated with adenine sulfate (ade), 10 mg/ L; isoleucine (ile), 30 mg/ L; and tryptophan (trp), 20 mg/L. Elliker broth plates (Difco Laboratories, Detroit, MI), prepared as a 4.85% solution supplemented with 0.77% nonfat milk powder were used for differential enumeration of *L. delbrueckii* ssp. *bulgaricus* and *S. salivarius* ssp. *thermophilus*. On this medium, *L. delbrueckii* ssp. *bulgaricus* produced large, raised, white colonies, whereas *S. salivarius* ssp. *thermophilus* produced small, flat colonies with a slight yellow hue.

3.3.3 Preparation and extraction of yogurt

Milk was reconstituted from non-fat dry powder (Carnation Co., Los Angeles, CA) as an 11% solution, hereafter referred to as milk, and was autoclaved for 12 minutes at 121° C, cooled immediately by transfer to 4° C, and stored at 4° C until use. Fermentations were begun with 0.5% (v/v) active cultures and were incubated at 37° C for 24 hours. Active cultures were prepared initially by inoculating a small volume of autoclaved milk with cells held at -80 °C and incubating at 37°C for 24 hours. Cultures subsequently held at 4 °C served as inocula for 1-2 weeks.

Yogurt was prepared by inoculating milk with a mixture of *S. salivarius* ssp. *thermophilus* CH3 and *L. delbrueckii* ssp. *bulgaricus* 191R. The fermented milks were freeze-dried in a Labconco lyophilizer (Kansas City, MO) and portions of the lyophilized powder were extracted continuously in a sintered glass funnel with approximately 2 volumes of acetone (Bodano and Rao, 1990). Acetone was removed by vacuum evaporation (Brinkmann Instruments, Inc., Westbury, NY) and the residue was dissolved in dimethyl sulfoxide (DMSO) and stored at -20 °C. One liter of fermented milk yielded 5 ml of extract. Extracts of unfermented milk were prepared and stored similarly.

3.3.4 Antimutagenicity assays

3.3.4.1 Ames test

The pre-incubation method of the Ames test was used (Maron and Ames, 1983). Briefly, 100 µl of a 10-11 hour culture of *S. typhimurium* TA 100 (histidine-requiring) were added to 700 µl of 0.2 M sodium phosphate, pH 7.4, followed by 1) extracts of yogurt, unfermented milk, or unfermented milk subjected to various treatments; and 2) freshly prepared mutagens dissolved in DMSO. (AFB1 was dissolved in 95% ethanol.) In the case of DMAB, DMBA, AFB1, Trp-P2, DEN, NNN, and DMH, 100 µl of culture were added to 500 µl of S9 mix instead of phosphate buffer. Final concentrations of the mutagens were 1 µM (MNNG and AFB1); 2 mM (DEO); 2 µM (4NQO); 7 mM (MMS); 4 mM (EMS); 10 µM (DMAB); 30 µM (DMBA); 10 µM (Trp-P-2). Concentrations were chosen that induced a significant frequency of his+ revertants without causing detectable toxicity according to the visual test described by Maron and Ames (1983). The chosen concentrations were at least two-fold less than those found toxic in preliminary assays. DMSO (100 µl), 95% ethanol (10 µl), and extract (75 µl) were added separately as controls instead of mutagen to test their effects on mutation frequency and cell viability. At the maximum concentrations used, neither the extracts nor DMSO were found to be mutagenic or toxic. Following 20 minute incubation at 37 °C in a rotary shaker, tube contents were transferred to test tubes containing 2 ml of top agar and 0.5 mM histidine and biotin (all at 45 °C). In the case of 4NQO, EMS, MMS and DEO, the tubes were vortexed and poured onto duplicate minimal glucose plates without incubation. Colonies (representing his+ revertants) were counted after 2 days at 37 °C. Antimutagenic activity was expressed as % inhibition of mutagenesis:

$$\% \text{ Inhibition} = [1 - (A - E) / (B - D)] \times 100.$$

A and B are numbers of mutagen-induced his+ revertants observed in the presence and absence of the extracts, respectively; and E and D are numbers of spontaneous his+ revertants observed in the extract and DMSO controls, respectively.

3.3.4.2 Saccharomyces cerevisiae D7

Antimutagenesis was measured as a reduction in the frequencies of ile+ revertants and trp+ gene convertants in *S. cerevisiae* D7 (Zimmermann et al., 1975). Briefly, 500 µl of stationary phase cells of D7 (containing approximately 6 x 10^7 viable cells) were added to 1400 µl of 0.1M potassium phosphate pH 7.0, followed by 1) extracts of yogurt or unfermented milk; and 2) freshly prepared mutagen or promutagen in DMSO to final volumes of 2000 µl. When DMAB, DMH or AFB1 were tested at concentrations ranging from 10 nM to 1 mM, 500 µl of culture were added to 1000 µl of S9 mix instead of phosphate buffer. The treated cells were held in an incubator-shaker at 37 °C and 260 rpm for 2 hours prior to addition of an equal volume of ice cold phosphate buffer or 10 µM freshly prepared thiosulfate (2 µM 4NQO treatment only). Cells were immediately pelleted and washed twice by centrifugation (900 x g at 4 °C for 5 minutes) in phosphate buffer, resuspended in 2 ml buffer, serially diluted, and plated in duplicate on diagnostic media: M + ade + ile + trp for survival; M + ade + ile for trp+ gene convertants; and M + ade + trp for ile+ revertants. No more than 300 colonies were counted on a single plate. Percent inhibition of reversion or gene conversion was calculated by the above formula where his+ revertants were replaced by the number of mutagenic events expressed per 10^6 (trp+ gene convertants) or 10^7 (ile+ revertants) survivors. DMSO (60 µl) and extract (50 µl) added separately as controls had no measurable effect on mutation frequency or cell viability at the maximum concentrations used. The spontaneous (control) frequencies of trp+ gene convertants and ile+ revertants ranged from 5-15/10^6 survivors and 0.3-2/10^7 survivors, respectively.

3.3.5 Activity at different pH values

To assess extractability of the antimutagenic activity as a function of pH, yogurt was divided into equal portions and adjusted by addition of 5 N NaOH or 5 N HCl from pH 4 (unadjusted) to 3, 7.6, and 13 prior to freeze-drying and extraction.

3.3.6 Solubility of antimutagenic compounds

Yogurt was freeze-dried, extracted with acetone, and the acetone extracts were divided into equal portions prior to evaporation of the solvent. Residues were dissolved in DMSO (control); distilled water; 0.1 M potassium phosphate, pH 3; 0.2 M sodium phosphate, pH 7.6; or 0.2 M KCl-NaOH, pH 13; at a ratio of 1 ml of solvent per 200 ml of yogurt. The pH of the latter solution did not change after dissolving the extract. Residues of the aqueous extracts were re-extracted with DMSO to distinguish between potential insolubility of activity and its potential instability in an aqueous environment. The volume of DMSO used in the re-extraction was also 1 ml per 200 ml of yogurt.

3.3.7 Simulation of fermentation

Experiments were undertaken to determine whether antimutagenicity could be produced in unfermented milk by adding yogurt bacteria, L-lactic acid, or both to typical final concentrations in the absence of subsequent bacterial growth.

3.3.7.1 Acidification

L-lactic acid was added aseptically to sterilized milk to a final concentration of 2% (vol/vol). The acidified milk was incubated at 37° for 24 hours, freeze-dried, and extracted with acetone as described above.

3.3.7.2 Addition of cells

Cells were harvested from freshly prepared yogurt by use of Reagent A (Promega, Madison, WI) according to the manufacturer's instructions. Reagent A contains a

separating agent, detergent, and cell carrier, and facilitates separation of bacterial cells from milk by centrifugation. To 200 ml of yogurt, 100 ml of Reagent A was added, mixed, and centrifuged at 16,000 x g for 10 min at room temperature. The supernatant was discarded and the pelleted cells were washed by centrifugation in 0.1 M potassium phosphate, pH 7, and resuspended in 10 ml buffer. Approximately 6.5 x 10^8 viable cells/ml were recovered from yogurt in a ratio of 5:1 (*S. salivarius* ssp. *thermophilus*: *L. delbrueckii* ssp. *bulgaricus*), typical of a mixed yogurt culture. The washed cells were added to 200 ml of sterilized milk which was immediately freeze-dried and extracted with acetone as described above. In a separate experiment, cells were added to milk acidified with L-lactic acid (final concentration 2% vol/vol) and freeze-dried immediately and extracted with acetone.

3.3.8 Statistics

Data were subjected to analysis of variance (ANOVA), SAS microcomputer version 6.04, SAS Institute, Inc., Cary, NC. When significant differences were detected by ANOVA, they were analyzed by pairwise t-test protected least significant differences. Significance is indicated at the $p \leq 0.05$ level.

3.4 Results

Acetone extracts of yogurt exhibited dose-dependent inhibition of mutagenesis induced by direct-acting MNNG and indirect-acting DMAB in the Ames test (Table 3.1). At the 20 and 50 µl doses, the responses seen in *S. typhimurium* TA 97 and TA 100 against MNNG were the same. However, at the 10 µl dose, the inhibition observed in TA 97 was 37% less than in TA 100. Against DMAB, the inhibition was approximately 1.6-fold greater in TA 100 than in TA 98 at all doses. Relative to yogurt, acetone extracts of unfermented milk exhibited significantly less activity at all doses tested, approximately 33

and 44% inhibition against MNNG and DMAB, respectively, as assayed in TA 100 (compare Table 3.1 with column 1 of Table 3.2).

TABLE 3.1. Inhibition of MNNG- and DMAB-induced mutagenesis in *Salmonella typhimurium* by an acetone extract of yogurt.

Extract (µl)	TA 100	TA 97	Extract (µl)	TA 100	TA 98
	MNNG % Inhibition			DMAB % Inhibition	
0	0	0	0	0	0
	(3695 ± 872)	(816 ± 25)		(1172 ± 38)	(282 ± 35)
10	42.8 ± 7.4	26.9 ± 3.5	25	38.7 ± 1.2	20.2 ± 2.7
	(2153)	(593)		(718)	(225)
20	65.2 ± 5.7	60.2 ± 1.1	50	61.7 ± 2.8	39.1 ± 4.6
	(1321)	(325)		(448)	(171)
50	90.8 ± 1.9	89.3 ± 2.5	75	80.2 ± 4.7	65.7 ± 1.2
	(333)	(88)		(232)	(97)

Values are means ± S.D. of 2 (TA 97 and TA 98) or 3 (TA 100) experiments. MNNG was not mutagenic against TA 98 (15-20 net revertants) and DMAB was not assayed against TA 97. Mean corrected colony counts (total minus spontaneous his$^+$ revertants) are in parentheses. Mean colony counts of the spontaneous his$^+$ revertants in the DMSO and yogurt extract controls were 150 (TA 100), 134 (TA 97), 34 (TA 98); and 161 (TA 100), 162 (TA 97), 40 (TA 98), respectively. Percentages of inhibition of MNNG- and DMAB-induced mutagenesis were significantly greater at all doses than the corresponding acetone extractions of unfermented milk ("untreated milk", Table 2), $p \leq 0.05$.

To determine whether two major consequences of milk fermentation could account for the antimutagenic activity, yogurt bacteria, L-lactic acid, or both were added to unfermented milk to final concentrations typical of yogurt, but without permitting subsequent bacterial growth. The amount of anti-MNNG activity recovered from these milk treatments was the same as recovered from unfermented and untreated milk

TABLE 3.2. Inhibition of MNNG- and DMAB-induced mutagenesis in *Salmonella typhimurium* TA 100 by acetone extracts of different milk treatments.

Extract (µl)	Milk (untreated)	Milk (acidified)	Milk (+ cells)	Milk (acidified + cells)
	% inhibition of MNNG-induced mutagenesis			
0	0 (4404 ± 60)	0 (1900 ± 40)	0 (4332 ± 48)	0 (2030 ± 73)
10	11.2 ± 2.1 (3919)	6.7 ± 1.6 (1773)	15.5 ± 3.5 (3662)	17.1 ± 5.3 (1682)
20	22.3 ± 3.4 (3430)	15.6 ± 4.4 (1604)	21.7 ± 5.9 (3395)	25.9 ± 6.7 (1502)
50	33.9 ± 2.6 (2905)	28.8 ± 3.4 (1351)	33.0 ± 5.8 (2902)	35.3 ± 1.7 (1313)
	% inhibition of DMAB-induced mutagenesis			
0	0 (954 ± 34)	0 (1007 ± 59)	0 (1146 ± 192)	0 (1105 ± 71)
25	17.0 ± 3.7 (792)	11.9 ± 4.4 (892)	7.1 ± 6.0 (1065)	20.5 ± 1.6 (878)
50	25.1 ± 1.7 (716)	19.8* ± 0 (808)	21.7 ± 2.9 (898)	27.0 ± 1.5 (808)
75	36.7 ± 2.8 (601)	29.8 ± 0.8 (707)	34.6 ± 6.1 (750)	36.8 ± 2.5 (705)

Values are means ± S.D. of 2 experiments. Mean corrected colony counts (total minus spontaneous his$^+$ revertants) are in parentheses. Mean colony counts of the spontaneous his$^+$ revertants in the controls were 117 (DMSO), 112 (untreated milk), 95 (acidified milk), 152 (milk + cells), and 138 (acidified milk + cells).
*Significantly less antimutagenic than the acetone extract of untreated milk at $p \leq 0.05$.

(Table 3.2). The differences in anti-DMAB activity extracted among the treatments were minor. At the 50 µl dose, a small but statistically significant difference in anti-DMAB

activity was observed between the untreated and acidified milks. However, this difference was not observed at the 25 or 75 µl doses.

To assess the specificity of yogurt-derived antimutagenic activity, a number of other mutagens (Table 3.3) and a eukaryotic test system (Table 3.4) were examined. No activity was seen against MMS or EMS (both alkylating agents) or against the fungal procarcinogen AFB1. Weak inhibitory activity was observed against DEO, and significant dose-dependent activity was observed against 4NQO, confirming an earlier report by Bodana and Rao (1990) that significant anti-4NQO activity was found in acetone and ethyl acetate extracts of skim milk fermented by different strains of *S. salivarius* ssp. *thermophilus* and *L. delbrueckii* ssp. *bulgaricus*. Significant dose-dependent activity was also observed against DMBA, a skin and mammary carcinogen in rodents, and against Trp-P2, a dietary heterocyclic amine. Inhibition of DMH, DEN, and NNN could not be determined because none was mutagenic against strain TA 100 at the maximum concentrations tested: 10 mM for DMH and NNN; and 1 mM for DEN.

In *S. cerevisiae* D7, acetone extracts of yogurt exhibited approximately 2-fold greater anti-4NQO activity than acetone extracts of unfermented milk. Anti-MNNG activity observed in yogurt was at least 25-fold greater than that found in unfermented milk. In both cases, inhibition of trp^+ gene convertants and ile^+ revertants was approximately the same. Inhibition of the promutagens DMAB, DMH, and AFB1 could not be determined because none of the agents was mutagenic whether activated endogenously by use of log phase cells grown in 20% glucose as recommended by Kelly and Parry (1983), or by addition of S9. The variance observed among replicates of the D7 assays was generally greater than that seen in the Ames test (all strains).

To examine the effect of pH on extractability of antimutagenic activity into acetone, the pH of yogurt (final pH 4.1) was adjusted to 3, 7.6 or 13 prior to freeze-drying and extraction (Table 3.5). Although extractability of MNNG-specific activity was highest at pH 3, the amount of activity extracted was largely insensitive to the pH

TABLE 3.3. Inhibition of mutagenesis by an acetone extract of yogurt in *Salmonella typhimurium* TA 100.

Extract (µl)	DEO	MMS	EMS % Inhibition	4NQO	DMBA	Trp-P2	AFB1
0	0	0	0	0	0	0	0
	(566 ± 68)	(4166 ± 398)	(4281 ± 1126)	(1044 ± 161)	(285 ± 23)	(79 ± 14)	(567 ± 247)
10	7.5 ± 0.9	0.9 ± 0.6	1.4 ± 1.1	-	28.3 ± 5.2	18.3 ± 1.2	3.2 ± 0.8
	(524)	(4126)	(4214)	-	(204)	(65)	(549)
20	15.9 ± 3.1	1.6 ± 0.5	2.6 ± 0.8	-	43.0 ± 5.9	46.7 ± 5.7	5.2 ± 1.3
	(477)	(4101)	(4164)	-	(162)	(43)	(536)
25	-	-	-	26.4 ± 3.7	-	-	-
	-	-	-	(771)	-	-	-
50	29.1 ± 7.0	3.9 ± 2.1	3.5 ± 1.1	44.6 ± 1.7	65.4 ± 7.1	60.1 ± 0.9	8.2 ± 3.3
	(407)	(4001)	(4126)	(580)	(118)	(32)	(525)
75	-	-	-	70.6 ± 1.5	-	-	-
	-	-	-	(309)	-	-	-

Values are means ± S.D. of 2 experiments. Mean corrected colony counts (total minus spontaneous his+ revertants) are in parentheses. Mean colony counts of the spontaneous his+ revertants in the controls were 110 (DMSO) and 124 (yogurt extract).
The indirect-acting mutagens DMBA, Trp-P2, and AFB1 required S9-mediated activation.
Statistical comparisons were not made between the treatments.

TABLE 3.4. Inhibition of MNNG- and 4NQO-induced trp+ gene convertants and ile+ gene revertants in *S. cerevisiae* D7 by an acetone extract of yogurt.

Treatment	TRP+ % Inhibition	ILE+ % Inhibition
30 μM MNNG /60 μl milk	0 ± 3 (562)	-0.5 ± 6 (503)
30 μM MNNG /60 μl yogurt	29* ± 3 (593)	23* ± 4 (625)
2 μM 4NQO/30 μl milk	18 ± 6 (537)	12 ± 8 (232)
2 μM 4NQO/30 μl yogurt	33* ± 10 (507)	25* ± 4 (380)
2 μM 4NQO/60 μl milk	26 ± 12 (493)	28 ± 5 (223)
2 μM 4NQO/60 μl yogurt	44 ± 21 (435)	54* ± 11 (267)

Values are means ± S.D. For the MNNG experiments, n = 3; for 4NQO, n = 4. The mean numbers of gene convertant and revertant colonies are in parentheses.
* Significantly more antimutagenic than the corresponding acetone extractions of milk, $p \leq 0.05$.

adjustments. In contrast, the amount of DMAB-specific activity extracted was maximal at pH 3, and minimal at neutral and alkaline values, suggesting that different compounds may be responsible for the two activities. Direct evidence in support of this possibility includes our observation that the activities did not co-elute from a reversed phase HPLC C18 column in a 10 to 100% acetonitrile gradient (data not shown).

When water was used instead of DMSO to dissolve the acetone extracts, little inhibitory activity was observed against MNNG or DMAB, suggesting that the compound(s) responsible are largely insoluble in water (Table 3.6). To test the possibility that activity was extractable but unstable in water, the water-extracted residue was re-extracted with DMSO. More activity was recovered in the second extraction, supporting the notion that the antimutagenic compounds are relatively insoluble in water. Use of aqueous solutions instead of pure water in the same extraction protocol supported

this possibility. Activity against both mutagens was less extractable in all aqueous solutions than in DMSO. In all the re-extractions with DMSO (except one), greater

TABLE 3.5. Inhibition of MNNG- and DMAB-induced mutagenesis by yogurt extracted into acetone at different pH values as assayed in *Salmonella typhimurium* TA 100.

Extract (µl)	pH 4 (unadjusted)	pH 3	pH 7.6	pH 13
	% inhibition of MNNG-induced mutagenesis			
10	43.4 ± 4.1^{ab} (2525)	48.6 ± 1.9^{a} (2295)	45.4 ± 4.6^{ab} (2212)	37.2 ± 0.7^{b} (2801)
20	62.8 ± 4.4^{a} (1663)	65.2 ± 1.8^{a} (1555)	63.6 ± 4.0^{a} (1627)	55.7 ± 1.4^{a} (1977)
50	87.9 ± 2.7^{ab} (540)	92.1 ± 0.8^{a} (354)	82.9 ± 1.7^{bc} (761)	81.9 ± 2.0^{c} (806)
	% inhibition of DMAB-induced mutagenesis			
25	24.2 ± 3.1^{a} (788)	28.3 ± 1.2^{a} (741)	9.0 ± 2.9^{b} (940)	10.1 ± 2.1^{b} (928)
50	41.7 ± 0.5^{a} (603)	45.3 ± 4.6^{a} (565)	19.6 ± 0.7^{b} (830)	25.2 ± 0.2^{b} (778)
75	71.1 ± 2.8^{b} (299)	92.3 ± 1.3^{a} (80)	32.7 ± 6.9^{c} (695)	29.9 ± 2.3^{c} (724)

Values are means ± S.D. for 2 experiments. Mean corrected colony counts (total minus spontaneous his^{+} revertants) are in parentheses. Mean colony counts of MNNG- and DMAB-induced his^{+} revertants in the absence of extracts were 4460 ± 50 and 1032 ± 116, respectively. Mean colony counts of the spontaneous his^{+} revertants in the DMSO and extract controls were 153 ± 16. Percentages of inhibition of mutagenesis at the same dose of extract are significantly different from one another if they do not share at least one common superscript, $p \leq 0.05$.

activity was recovered than extracted initially into the buffers or the alkaline KCl solution. However, the amount of anti-DMAB and anti-MNNG activity extracted at pH 7.6 was

Table 3.6. Differential solubility of antimutagenic activity of acetone extracts of yogurt assayed in *Salmonella typhimurium* TA 100.

Extract	DMSO	Water	pH 3	pH 7.6	pH 13	Water	pH 3	pH 7.6	pH 13
		Initial extraction				Residue of initial extraction dissolved in DMSO			
		% inhibition of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine-induced mutagenesis							
10 µl	43.1 ± 5.6[a]	9.0 ± 6.7[d]	8.2 ± 3.4[d]	20.1 ± 1.6[cd]	22.1 ± 3.5[cd]	21.7 ± 9.6[c]	38.5 ± 1.3[ab]	27.2 ± 2.0[bc]	21.4 ± 2.9[c]
	(2329)	(3330)	(4068)	(3540)	(3480)	(2934)	(2726)	(3225)	(3485)
20	64.2 ± 4.8[a]	26.3 ± 6.5[d]	23.9 ± 2.0[d]	41.1 ± 2.2[c]	34.5 ± 4.1[d]	48.9 ± 3.8[bc]	58.9 ± 2.1[a]	46.8 ± 1.1[bc]	54.3 ± 1.0[ab]
	(1497)	(2687)	(3373)	(2613)	(2903)	(1869)	(1824)	(2359)	(2026)
50	89.7 ± 2.4[a]	38.2 ± 3.4[d]	37.1 ± 2.3[d]	56.5 ± 1.6[c]	42.7 ± 1.6[d]	73.5 ± 4.5[b]	77.7 ± 2.1[b]	63.0 ± 0.4[c]	80.1 ± 2.3[b]
	(393)	(2270)	(2793)	(1929)	(2541)	(966)	(991)	(1641)	(884)

Table 3.6 (con'd).

	% inhibition of 3,2'-dimethyl-4-aminobiphenyl-induced mutagenesis								
25	35.5 ± 5.0^{a}	15.2 ± 6.5^{e}	16.1 ± 2.4^{e}	19.1 ± 3.8^{cde}	18.7 ± 4.2^{de}	21.8 ± 9.5^{bcd}	26.9 ± 5.3^{bc}	25.7 ± 0.3^{bcd}	32.4 ± 0.4^{ab}
	(750)	(995)	(967)	(932)	(939)	(918)	(844)	(856)	(779)
50	58.4 ± 5.7^{a}	25.1 ± 6.0^{d}	32.5 ± 3.7^{cd}	32.0 ± 3.3^{cd}	29.5 ± 2.7^{cd}	46.2 ± 2.6^{b}	50.7 ± 2.1^{ab}	37.3 ± 0.6^{c}	50.3 ± 3.4^{ab}
	(484)	(879)	(778)	(785)	(812)	(630)	(568)	(723)	(575)
75	76.9 ± 5.5^{a}	34.6 ± 6.1^{e}	48.0 ± 4.4^{c}	43.6 ± 2.1^{cd}	38.4 ± 4.6^{de}	58.6 ± 1.6^{b}	64.1 ± 1.8^{b}	49.5 ± 0.9^{c}	63.8 ± 3.4^{b}
	(268)	(769)	(605)	(650)	(710)	(485)	(414)	(583)	(417)

Acetone extracts of yogurt were initially extracted into DMSO or aqueous solutions, and residues of the aqueous extractions were subsequently dissolved in DMSO. Values are means ± SD of 2 experiments (n), except for the initial extractions in DMSO (n = 5) and water (n =3).
pH 3 = 0.1 M potassium phosphate, pH 3; pH 7.6 = 0.2 M sodium phosphate, pH 7.6; pH 13 = 0.2 M KCl-NaOH, pH 13.
Mean corrected colony counts (total minus spontaneous his^{+} revertants) are in parentheses. Mean colony counts of MNNG- and DMAB-induced his^{+} revertants in the absence of extracts were 4039 ± 370, and 1163 ± 9, respectively. Mean colony counts of the spontaneous his^{+} revertants in the DMSO, water, and aqueous solution controls were 134 ± 9.
Percentages of inhibition of mutagenesis at the same dose of extract are significantly different from one another if they do not share at least one common superscript, $p \leq 0.05$.

statistically equivalent to what was recovered in the re-extractions with DMSO.

Because CLA is a recognized anticarcinogen (Ha et al., 1989) and a natural constituent of dairy foods, it was assayed in the Ames test as a putative antimutagen to determine its contribution, if any, to the antimutagenicity of yogurt. At concentrations as high as 1 μg/ml (3.6 μM), CLA failed to inhibit mutagenesis induced by MNNG or DMAB. CLA also failed to inhibit DMBA, the procarcinogen it has been reported to inhibit *in vivo* (Ip et al., 1991; Ha et al., 1987). CLA concentrations higher than 1 μg/ml were found to be toxic to *S. typhimurium* TA 100 as indicated by the absence of a normal background lawn on minimal glucose plates (Maron and Ames, 1983). In contrast to our findings, Pariza and Hargraves (1985) reported that partially purified CLA preparations containing an undetermined amount of the anticarcinogen exhibited no apparent toxicity to *S. typhimurium* TA98, according to the visual test, at doses that significantly inhibited rat liver S-9 mediated DMBA mutagenesis. Unfortunately, a direct comparison of these *in vitro* results is confounded by use of different *S. typhimurium* strains, use of an impure CLA preparation in the 1985 study, and a qualitative test of toxicity.

3.5 Discussion

Acetone extracts of yogurt (milk fermented by *L. delbrueckii* ssp. *bulgaricus* and *S. salivarius* ssp. *thermophilus*) were shown to exhibit dose-dependent antimutagenic activity against a number of direct-acting and indirect-acting mutagens. Differential extractability of anti-MNNG and anti-DMAB activities supported the possibility that more than one antimutagenic compound may be present. Treatments of unfermented milk not involving bacterial growth failed to produce antimutagenic activity. CLA did not inhibit MNNG- or DMAB-induced mutagenesis suggesting that the trace amounts of CLA that might be present in yogurt made from non-fat milk do not account for the observed antimutagenicity.

The findings reported here are consistent with other observations of antimutagenicity in fermented milks. In a related study of fermented non-fat milk, Nadathur (1994) found that anti-MNNG and anti-DMAB activites were produced in separate fermentations with either *L. delbrueckii* ssp. *bulgaricus* 191R or *S. salivarius* ssp. *thermophilus* CH3. Skim milk fermented by a number of lactic acid bacteria was reported to inhibit MNNG-induced mutagenesis in the Ames test (*S. typhimurium* TA 100) (Hosoda et al., 1992a). Fermented milk rather than extracts was tested directly, and unfortunately, water rather than milk was used as a negative control. However, periodic sampling during the course of one fermentation involving *L. acidophilus* LA106 (LA2) indicated that final activity, as in the present study, was also about 2.5-fold greater than that present initially in milk. While the actual degree of inhibition would appear to represent significantly greater activity than what we observed (taking into account that our extract represented a relative 100-fold concentration), other differences in experimental protocols, including use of a different bacterial species complicate a rational comparison. In a similar study, antimutagenic activity against Trp-P2 was shown to vary considerably among the same strains of various lactic acid bacteria in comparison to unfermented milk (Hosoda et al., 1992b). Hosono et al., (1986b) used streptomycin-dependent derivatives of the Ames strains to show that fermented milk, relative to unfermented milk, inhibited a different mutagen, 2-(2-furyl)-3-(5-nitro-2-furyl) acrylamide. Data were presented suggesting activity against 4-nitroquinoline-*N*-oxide (4NQO) and mutagenic extracts from mammalian feces as well, but the relative contribution of milk to the latter activities was impossible to assess because water was used as a negative control. Bodana and Rao (1990) reported that acetone and ethyl acetate extracts of skim milk fermented at 45 °C by different strains of *L. delbrueckii* ssp. *bulgaricus* and *S. salivarius* ssp. *thermophilus* inhibited mutagenesis caused by 4NQO and 2-aminofluorene (2AF) in the Ames test (*S. typhimurium* TA 100 and TA 98). When the TA 100 tester was used, unfermented milk exhibited slight dose-dependent activity against 4NQO but not 2AF. Although we used

the same extraction protocol (Bodana and Rao, 1990), we have no evidence that the activity we observed is due to the same compound(s) that inhibited mutagenesis caused by 2AF and 4NQO. It is of interest however, that 2AF, DMAB, and Trp-P2 share a common activation pathway initially involving N-hydroxylation by cytochrome P450 1A2 (Aoyama et al., 1989).

Our observation that CLA failed to inhibit DMBA-induced mutagenesis is an illustration of one of the unavoidable limitations of *in vitro* assays for putative anticarcinogens: not all anticarcinogens are antimutagenic and detectable in such assays, nor are all antimutagens anticarcinogenic. *In vivo*, CLA was reported to inhibit DMBA-induced skin carcinogenesis in mice (Ha et al., 1987) and mammary carcinogenesis in rats (Ip et al., 1991; 1994). The present *in vitro* results provide no evidence that CLA acts as an antimutagen. Ip et al. (1991, 1994) have suggested that CLA-mediated inhibition of mammary carcinogenesis cannot be accounted for its antioxidant activity nor by affects on Phase I or Phase II enzymes.

One explanation for the activity seen against mutagens and promutagens that do not necessarily share a common mode of action or activation pathway (MNNG, 4NQO, DEO, DMBA, DMAB, and Trp-P2) is that multiple antimutagenic compounds may be present in the complex yogurt extract. While we have evidence to support the possibility that different compounds inhibit MNNG and DMAB, it is not clear if these same compounds or others inhibit 4NQO, DEO, DMBA, and Trp-P2.

The role of milk in the formation of antimutagenicity is unresolved. Inactive milk constituents (precursors) may be converted to antimutagens by the fermenting organisms; or milk may lack such precursors but serve as a neutral medium for organisms that ordinarily produce antimutagens during fermentative growth. A combination of the above possibilities cannot be ruled out. Our finding that the production of antimutagenic activity is associated with bacterial growth is consistent with both possibilites. One explanation for the antimutagenic activity of unfermented milk has been provided by

Yoshida and Xiuyun (1992) who showed pH-dependent binding of mutagenic heterocyclic amines, including Trp-P2, by bovine milk caseins *in vitro*. (At the acidic pH of fermented milk, little binding occurred.) Van Boekel et al. (1993) reported that casein was antimutagenic towards a number of mutagens including 4NQO, and that antimutagenicity increased as a function of pepsin-mediated hydrolysis. While casein hydrolysis by bacterial proteases occurs naturally during milk fermentation, the relevance of this activity to the production of antimutagenicity remains to be established directly.

Reports providing evidence that fermented milk products are antimutagenic in various assay systems against different mutagens/carcinogens are significant because of the importance of such foods in the human diet, because multiple mechanisms of action are suggested, and because ingestion of natural anticarcinogens is recognized to play a role in counteracting the unavoidable intake of carcinogenic substances present in food (Sugimura, 1990). While we have observed antimutagencity in yogurt against a number of mutagens, including experimental colon carcinogens, it is premature to conclude that such activity will be anticarcinogenic. Our on-going efforts to purify and identify active constitutents of acetone extracts of yogurt will aid experimental efforts to determine if they are anticarcinogenic in an appropriate animal model, and ultimately, to determine their contribution to anticarcinogenesis in humans.

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CHAPTER 4

PALMITIC ACID IS THE MAJOR FATTY ACID RESPONSIBLE FOR SIGNIFICANT ANTI-MNNG ACTIVITY IN YOGURT

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Mutation Research
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4.1 Abstract

We describe here the isolation and identification of palmitic acid as being responsible for significant anti-*N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) activity in yogurt. The Ames test (*Salmonella typhimurium* TA 100) was used to direct fractionation of activity. Yogurt was freeze-dried and extracted with acetone to yield a crude extract. The crude extract was purified by normal phase silica gel, Sephadex LH-20, and reversed phase medium pressure liquid chromatographies. The major compound in the active medium pressure liquid chromatographic fractions was determined to be palmitic acid on the basis of gas chromatography-mass spectrometry (GC/MS), MS/MS, by co-elution with authentic palmitic acid on GC and high pressure liquid chromatography (HPLC) systems, and by nuclear magnetic resonance (NMR) analysis. Other saturated straight chain and methyl branched fatty acids were detected by GC/MS and were later shown to possess anti-MNNG activity. Of the straight chain fatty acids, palmitic acid had the highest anti-MNNG activity. All ω-1 methyl branched fatty acids tested were more active than their straight chain counterparts. A trace amount of isopalmitic acid (14-methyl pentadecanoic acid), a minor milk lipid, was detected in one of the active fractions, and was later shown to be five times more active than palmitic acid. Isopalmitic acid also inhibited mutagenesis induced by 4-nitroquinoline-*N*-oxide (4NQO), and 7,12-dimethyl benz[*a*]anthracene (DMBA), and was found to inhibit the metabolic activation of DMBA.

4.2 Introduction

Antimutagens are compounds that decrease the deleterious effects of mutagens. Such compounds have been classified mechanistically on the basis of whether they interact directly with mutagens or their precursors ("desmutagens"), or whether they affect the repair or replication of the mutagenized DNA ("bioantimutagens"). Some of the known mechanisms of antimutagenesis include: a) formation of a mutagen-antimutagen complex (Negishi et al., 1989; Dashwood et al., 1991), b) prevention of activation of promutagens

by cytochrome P450 (Brady et al., 1988), c) detoxification of activated promutagens (Zhang et al., 1992), and d) induction of DNA repair (Ohta et al., 1988). A number of antimutagens have been isolated from natural dietary sources including fruits and vegetables (Amonkar et al., 1986; Loub et al., 1975; Kada et al., 1985; Wood et al., 1982; Kakinuma et al., 1984; Sayer et al., 1982). While few of these compounds have been tested *in vivo*, anticarcinogenic activity has been demonstrated experimentally for some: chlorophyllin (Breinholt et al., 1995), diallyl sulfide (Wargovich, 1987), and (-)-epigallocatechin gallate (Fujiki et al., 1990).

A number of studies have examined the antimutagenic, anticarcinogenic, and anti-cancer activities of yogurt, other fermented milks, and the dairy lactic acid bacteria used in their production (Adachi, 1992; Fernandez et al., 1987; Gilliland, 1990; Rao et al., 1986; Sanders, 1993) Yogurt and extracts thereof have been shown to be antimutagenic against a range of mutagens and promutagens, in microbial and mammalian cell systems (Bodana and Rao, 1990; Renner and Munzner, 1991; Nadathur et al., 1994, 1995). Although certain epidemiological studies have suggested that consumption of yogurt and fermented milk may reduce the incidence of colon or breast cancer (Peters et al., 1992; Lê et al., 1986), others have been equivocal (Young and Wolf, 1988; Kampman et al., 1994).

This report describes the first identification of an antimutagen from yogurt. We previously reported that an acetone extract from yogurt was antimutagenic against MNNG in the Ames test (Nadathur et al., 1994, 1995). We describe here the Ames test-directed isolation and identification of palmitic acid as the major fatty acid responsible for significant anti-MNNG activity in this extract. We also report on the anti-MNNG activity of other straight-chain and branched fatty acids, some of which were detected as minor components of the extract.

4.3 Materials and methods

4.3.1 Chemicals

N-Methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) (CAS No. 70-25-7), was purchased from Aldrich (Milwaukee, WI). 3,2'-dimethyl-4-aminobiphenyl (DMAB) (CAS No. 1162-65-8), 7,12-dimethylbenz[*a*]anthracene (DMBA) (CAS No. 57-97-6), 4-nitroquinoline-*N*-oxide (4NQO) (CAS No. 56-57-5), and all fatty acids were purchased from Sigma Chemical Co. (St. Louis, MO). Aroclor 1254-induced rat liver S9 microsomal fraction (S9) was purchased from Molecular Toxicology (Annapolis, MD). All chemicals were reagent grade.

4.3.2 Bacterial strains

Salmonella typhimurium TA 100 was provided by Dr. Bruce N. Ames (Department of Biochemistry, University of California, Berkeley, CA). *Streptococcus salivarius* ssp. *thermophilus* CH3, and *Lactobacillus delbrueckii* ssp. *bulgaricus* 191R were provided by Dr. W. E. Sandine (Department of Microbiology, Oregon State University, Corvallis, OR).

4.3.3 Preparation and extraction of yogurt

Milk was reconstituted from non-fat dry powder (Carnation Inc., Los Angeles, CA) as an 11% solution, hereafter referred to as milk, and was autoclaved for 12 minutes at 121° C, cooled immediately by transfer to 4° C, and stored at 4° C until use. Fermentations were begun with 1% (v/v) active cultures and were incubated at 37° C for 18 h. Active cultures were prepared initially by inoculating a small volume of autoclaved milk with cells held at -80° C and incubating at 37° C for 24 hours. Cultures subsequently held at 4° C served as inocula for 1-2 weeks.

Yogurt was prepared by inoculating milk with a mixture of *S. salivarius* ssp. *thermophilus* CH3 and *L. delbrueckii* ssp. *bulgaricus* 191R. The fermented milks were

freeze-dried in an industrial unit (model #651M, Hull Corporation, Hatboro, PA), soaked in acetone overnight, and filtered through a sintered glass funnel with approximately 2 volumes of acetone, relative to the volume of powder (Bodana and Rao, 1990). Acetone was removed by a rotary evaporator (< 40° C) (Brinkmann Instruments, Inc., Westbury, NY) and the residue was stored at -20° C. One liter of yogurt yielded approximately 200-300 mg of extract.

4.3.4 Antimutagenicity assay

The pre-incubation method of the Ames test was used (Maron and Ames, 1983). Briefly, 100 µl of a 10-11 hour culture of *S. typhimurium* TA 100 (histidine-requiring) was added to 700 µl of 0.2 M sodium phosphate buffer, pH 7.4, followed by: 1) extracts of yogurt or various fractions from the isolation, or different fatty acids; and 2) freshly prepared MNNG dissolved in DMSO. When DMBA was used, it was added to 500 µl of S9 mix instead of phosphate buffer. In the case of DMBA and 4NQO, TA 100 was added after the incubation of mutagen and fatty acid. Final concentrations of the mutagens were: 2 µM (MNNG and 4NQO) and 30 µM (DMBA). Concentrations were chosen that induced a significant frequency of his^+ revertants without causing detectable toxicity according to the visual test described by Maron and Ames (1983). DMSO (75 µl) and extract (75 µl) were added separately as controls instead of mutagen to test their effects on mutation frequency and cell viability. At the concentrations used, neither the extracts nor DMSO were found to be mutagenic or toxic. Following a 10 minute incubation at 37° C in a rotary shaker, tube contents were transferred to test tubes containing 2 ml of top agar and 0.5 mM histidine and biotin (all at 45° C). During the testing of authentic fatty acids, the incubation time was reduced to 5 minutes. In the case of 4NQO, there was no incubation. The tubes were vortexed, poured onto duplicate minimal glucose plates, and colonies were counted after 2 days at 37° C. Antimutagenic activity was expressed as % inhibition of mutagenesis:

% Inhibition = {1 - [(A - E) / (B - D)]} x 100.

A and B are numbers of mutagen-induced his+ revertants observed in the presence and absence of the extracts, respectively; and E and D are numbers of spontaneous his+ revertants observed in the extract and DMSO controls, respectively. The number of spontaneous revertants in the DMSO and extract controls typically ranged from 100 to 150. One unit of activity was defined as 1% inhibition of mutagenesis. Specific activity was defined as one unit per mg of antimutagen or antimutagenic extract. Percentages of inhibition against MNNG-induced mutagenesis were plotted against mass (μg range) for each fatty acid to yield slopes equivalent to specific anti-MNNG activities.

4.3.5 Isolation of anti-MNNG activity

The Ames test was used to direct fractionation of anti-MNNG activity in yogurt. Yogurt (3.14 kg) was freeze-dried and extracted with 10 liters of acetone. The solvent was removed *in vacuo* to give a yellowish oily suspension of 11 g (crude extract). The crude extract was loaded on a silica gel column (40-63 μm, 3.5 x 25 cm) and fractions were eluted using a solvent gradient of 5% ethyl acetate in hexane to 100% ethyl acetate. Fractions of 200 ml were collected and the solvent was evaporated. Fractions 8 and 9 were highly active and were combined (3.9 g) and chromatographed on 100 g of Sephadex LH 20 (Pharmacia, Piscataway, NJ) packed in dichloromethane-methanol (1:1). Elution was carried out with the same solvent mixture. Sixteen fractions of 10 ml each and a final fraction of 200 ml were collected. Following evaporation of the solvent, active fractions 7, 8, 9, and 10 were pooled to yield 133 mg. These combined fractions were then separated by medium pressure liquid chromatography (MPLC) (20-30 psi, 6 ml/min) on a C18-reversed phase Lichroprep column (40-63 μm, 25 x 310 mm) (EM Science, Gibbstown, NJ), eluting with 5% acetonitrile in methanol. An initial fraction of 100 ml was followed by 16 fractions of 10 ml each. A final 100 ml fraction was collected by eluting with 100% acetonitrile. Activity typically eluted between 150 and 220 ml.

4.3.6 Gas chromatography and mass spectrometry

Fatty acids (in dichloromethane), were converted to their methyl esters by the addition of trimethylsilyldiazomethane (in hexane) until a yellow color persisted. Reaction vials were sealed and left for 20 min. The solvent was evaporated under a stream of nitrogen and subjected to gas chromatography-mass spectrometry (GC-MS) on three different systems arbitrarily designated "system 1", "system 2", and "system 3". "System 1": Varian 3400 GC (San Fernando, CA) coupled to a Finnigan (San Jose, CA) quadrupole automated mass spectrometer. An SE 54 capillary column (10 m x 0.25 mm, Alltech Associates, Onc., Deerfield, IL) was used with an injection temperature of 280° C and a program of 50° to 280° C at a rate of 20° C per minute. "System 2": Hewlett-Packard (HP) (Wilmington, DE) 5890 series II GC coupled to a HP 5971 series II MS. An HP-1 cross-linked methylene silicone column (12 m x 0.2 mm) with a film thickness of 0.33 μm was used. Injection temperature was 250° C with a program of 70° to 240° C at a rate of 10° C per minute and an initial hold of 0.5 min at 70° C. "System 3": same as "system 2" except the column was 50 m, and had a film thickness of 0.1 mm; the injection temperature was 270° C, with a program of 100° to 150° C at a rate of 10° C per minute, a 1 minute hold at 150° followed by a ramp of 3° per minute to 282°, with a final 5 minute hold; the HP 5971 MS was the original model. In all systems, helium was the carrier gas, the ion source energy was 70 eV, and spectra were obtained in electron impact mode.

4.3.7 Tandem mass spectrometry (MS-MS)

Fast atom bombardment (FAB) mass spectra of the free acids were obtained using a Kratos MS-50TC triple analyzer tandem mass spectrometer (Manchester, UK). Each sample was dissolved in a diethanolamine matrix. The samples were bombarded with 8 KeV Xe atoms, and the ions produced were accelerated through 8 KeV. All spectra were acquired in the negative ion mode. The scan rate was 10 seconds/decay with a resolution of 1400.

4.3.8 High performance liquid chromatography (HPLC)

The high performance liquid chromatography (HPLC) system comprised a Waters 6000A chromatography pump (Waters Associates, Inc., Milford, MA), a C18 column (5 μm, 4.6 x 250 mm) (Rainin, Emoryville, CA), and a Waters R401 differential refractometer. The eluant was methanol-water (6:1) at a flow rate of 1.2 ml per minute at 2500 psi.

4.3.9 Nuclear magnetic resonance spectroscopy (NMR)

^{1}H- and ^{13}C-NMR spectra were obtained on a Bruker AM 400 spectrometer (Bruker Analytische, Silberstreifen, Germany). Samples were prepared in $CDCl_3$. The ^{13}C NMR spectrum of HPLC-purified palmitic acid from MPLC fraction "6-10" was obtained using a Nalorac microprobe under the following conditions: pulse angle 30 degrees, acquisition time 1.311 sec, 64,000 data points, 25,000 Hz sweep width, broadband 1H decoupling, 37,504 scans, line broadening 3 Hz. The spectrum of authentic palmitic acid was obtained under identical conditions using a standard 5 mm broadband probe, except that the number of scans required was 34.

4.3.10 Antimutagenicity of C_{16} fatty Acids against DMBA and 4NQO

The inhibitory effect of palmitic, isopalmitic, and anteisopalmitic acids was examined against DMBA-, and 4NQO-induced mutagenesis. Fatty acids were incubated with DMBA during the activation of the latter by S9 microsomal enzymes. Isopalmitic acid was added during or after the activation of DMBA to distinguish between an effect on activation and an interaction with the activated mutagen. Potential formation of a mutagen-fatty acid complex was tested by incubating different concentrations of the C_{16} fatty acids with DMBA and 4NQO in DMSO and examining changes in absorption in a UV/Vis 160A recording spectrophotometer (Shimadzu Corporation, Kyoto, Japan).

4.3.11 Statistics

Percentages of inhibition of MNNG-induced mutagenesis were plotted as a function of dose of fatty acids (antimutagens) and were subjected to regression analysis (SAS microcomputer version 6.04, SAS Institute, Inc., Cary, NC). Slopes from such plots yielded values of specific inhibition as units per mg fatty acid.

4.4 Results

A crude acetone extract of yogurt was purified and yielded palmitic acid as the major fatty acid having significant anti-MNNG activity. A representative purification starting with 28 liters of yogurt which yielded 3.14 kg of freeze-dried powder is shown in Table 4.1. Specific activity increased 26-fold following the silica gel and Sephadex LH-20 separations. The material obtained from the latter separation was divided in half and each was subjected to separate MPLC fractionations, runs "7" and "9". The most pure fractions from the two MPLC separations were "7-9" which contained a specific activity of 2,150 units per mg, representing an increase of about 4-fold relative to the LH-20 separation. A separate lot of 24 liters of yogurt was subjected to an identical fractionation scheme and yielded MPLC runs "5" and "6".

Proton and carbon nuclear magnetic resonance spectroscopic (^{1}H-NMR and ^{13}C-NMR) analyses of several active MPLC fractions from runs "5" and "6" dissolved in deuterated acetone (CD_3COCD_3) indicated the presence of free fatty acids, and thus, GC analysis was undertaken. GC-MS analysis of derivatized (methyl esterified) fraction "7-9" is compared to an analysis of derivatized authentic palmitic acid in Figures 4.1 and 4.2. The mass spectrum shown is that of the methyl palmitate peak (8:00 minutes). The peaks at 5:00, 8:10, and 8:40 minutes common to the authentic and experimental samples are an artifact, residual free palmitic acid, and the trimethylsilyl derivative of palmitic acid, respectively; the latter resulting from the derivitization reaction. Peaks seen at longer

retention times in the experimental fraction (not shown) are derived from the solvent and plasticware stabilizer.

Table 4.1. Purification of anti-MNNG activity from yogurt.

Extract	Mass[1] (mg)	Specific activity[2] (U/mg)	Total activity (Units)	Fold purification	Mass remaining (%)
24 liters yogurt	3,135,000	-	-	-	-
Acetone extract	11,000	22	242,000	1	100
Silica gel	3,866	32	123,712	1.5	35.1
Sephadex LH-20	133	580	77,140	25	1.20
Medium pressure C18 reversed phase[3]					
Fraction 7-6	5	1,000	5,000	45	0.05
Fraction 7-7	5	1,100	5,500	50	0.05
Fraction 7-8	4	2,000	8,000	91	0.04
Fraction 7-9	5	2,150	10,750	97	0.05
Fraction 7-10	4	2,125	8,500	97	0.04
Fraction 7-11	3	1,000	3,000	45	0.03
Fraction 9-6	4	1,313	5,252	60	0.04
Fraction 9-7	3	1,167	3,501	53	0.03
Fraction 9-8	6	1,417	8,502	64	0.05
Fraction 9-9	4	1,688	6,752	77	0.04
Fraction 9-10	5	1,550	7,750	70	0.05
Fraction 9-11	4	1,667	5,001	76	0.04

[1] Determinations of mass for the MPLC fractions were subject to an error of about ± 25-50%.
[2] Specific activities are based on unreplicated assays of antimutagenesis.
[3] The prefixes used in designating MPLC fractions ('5' and '6') refer to independent separations, each using half of the material from the LH-20 column.

Several derivatized MPLC fractions were analyzed by low-resolution mass spectrometry, and one--"6-10", by high resolution MS. For the major compound in each

fraction, the former indicated indicated a nominal molecular weight of 270, and the latter indicated a molecular formula of $C_{17}H_{34}O_2$ (found 270.2559, calculated 270.2559). The major compound was determined to be palmitic acid by: a) comparison with the mass spectrum and the MS-MS fragmentation pattern of authentic palmitic acid, b) co-elution with authentic palmitic acid on different gas chromatography columns ("system 1" and "system 2"), and c) co-elution with authentic palmitic acid on a C18 reversed phase HPLC column. The tandem mass spectrometric analysis of fractions "7-9", "7-10", and the most active fractions from runs "5" and "6" clearly excluded the presence of a methyl branch (Jensen and Gross, 1986). An independent GC/MS analysis ("system 3") of MPLC fraction "6-10" detected palmitic acid, trace amounts of isomargaric, anteisomargaric acid, margaric acid, oleic acids, and larger amounts of other non-fatty acids whose mass spectra suggested contaminants (David C. White, University of Tennessee, unpublished data, 1995). The major component of MPLC fraction "6-10" was purified by HPLC and subjected to NMR analysis. The ^{13}C NMR spectrum of the HPLC-purified component matched that of authentic palmitic acid (data not shown). By GC analysis, palmitic acid constituted 99% of the total fatty acids in the purest fractions from runs "5" and "6", and at least 99% in fraction "7-9".

A variety of saturated straight chain fatty acids were examined for their anti-MNNG activity in the Ames test. Palmitic acid (C_{16}) had the highest activity, with over 1300 units per mg (Table 4.2). The others ranged between 0 and 689 units per mg. Margaric (C_{17}) and stearic (C_{18}) acids were detected as minor components in active MPLC fractions from run "6". In fraction "7-10", a trace amount of margaric acid was detected. Authentic margaric and stearic acids exhibited about 10 and 20% of the activity of palmitic acid, respectively. Oleic acids were also detected in these fractions, but Hayatsu et al. (1981) have previously shown them to be inactive against MNNG using *E. coli* WP2 *uvrA* /pKM101.

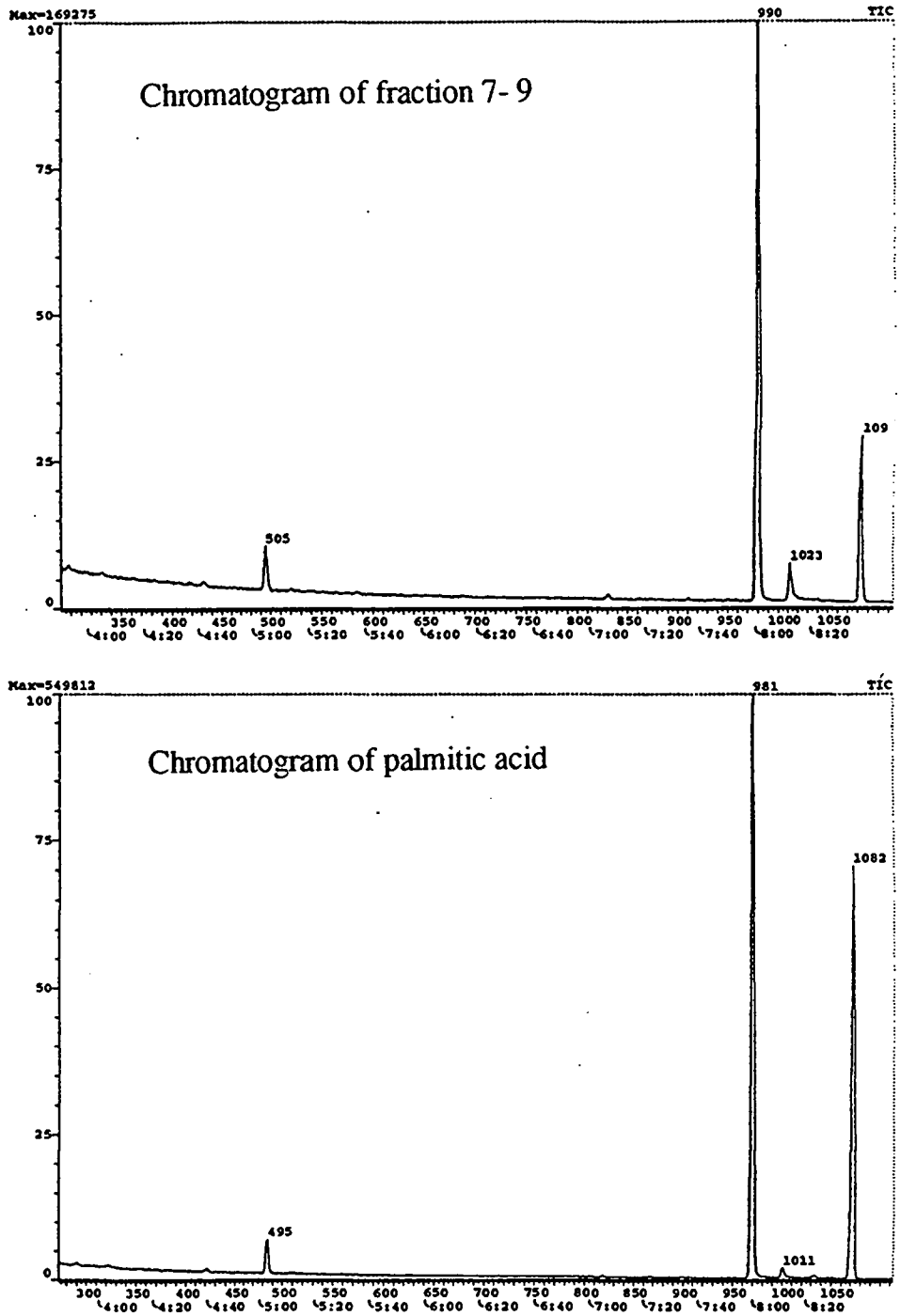

Figure 4.1. Identification of fatty acid methyl esters by GC-MS. Top, GC trace of fraction "7-9"; bottom, GC trace of authentic palmitic acid. The peaks at 5:00, 8:10, and 8:40 minutes common to the authentic and experimental samples are an artifact, residual free palmitic acid, and the trimethylsilyl ester of palmitic acid respectively; the latter resulting from the derivatization reaction. Peaks seen at longer retention times in the experimental fraction (not shown) are derived from solvent and plasticware stabilizer. The x-axis is labelled in minute:seconds, and number of scans.

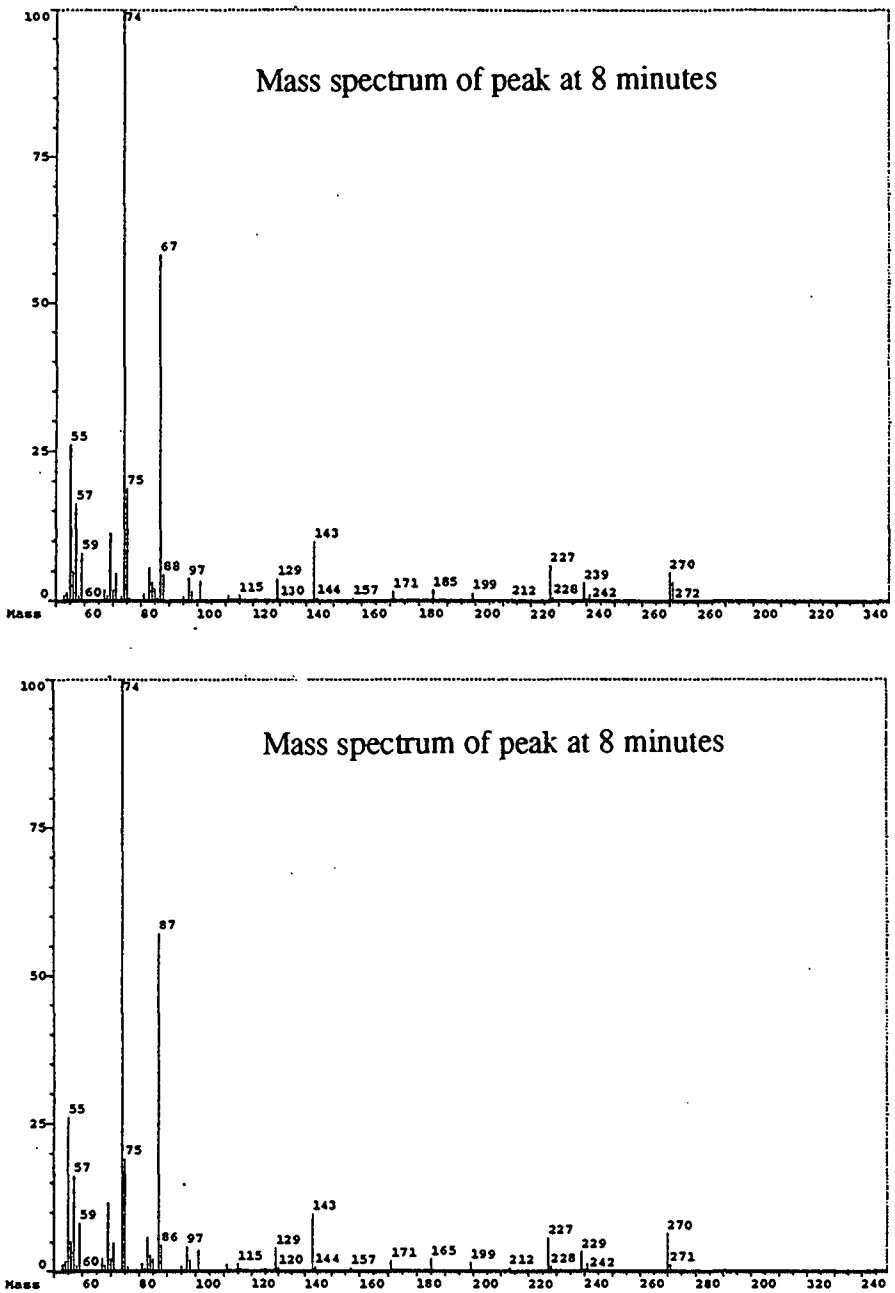

Figure 4.2. Comparison of mass spectra. Top, mass spectrum of fraction "7-9"; bottom, mass spectrum of authentic palmitic acid.

Table 4.2. Specific inhibition[1] of MNNG-induced his+ revertants by saturated fatty acids.

Fatty acid	Specific inhibition (U/mg) ± standard error of the slope
Straight chain	
Caproic (C_6)	236 ± 118
Caprylic (C_8)	212 ± 46
Capric (C_{10})	292 ± 76
Lauric (C_{12})	689 ± 74
Tridecanoic (C_{13})	0
Myristic (C_{14})	0
Pentadecanoic (C_{15})	toxic
Palmitic (C_{16})	1,322 ± 137
Margaric (C_{17})	176 ± 59
Stearic (C_{18})	244 ± 173
Arachidic (C_{20})	660 ± 101
Behenic (C_{22})	388 ± 80
Lignoceric (C_{24})	588 ± 89
Branched chain	
12-methyl tridecanoic (i[2] C_{14})	3,993 ± 682
14-methyl pentadecanoic (i C_{16})	6,553 ± 493
13-methyl pentadecanoic (a C_{16})	0
15-methyl hexadecanoic (i C_{17})	4,625 ± 841
16-methyl heptadecanoic (i C_{18})	2,007 ± 601

[1] Values of specific inhibition were calculated as described in Materials and Methods. For the straight chain fatty acids, experiments at 3 to 4 doses (in the μg/plate range) were performed at least twice; for the branched fatty acids, experiments at 4 to 6 doses (in the same range) were performed at least twice. Spontaneous his+ revertants in the DMSO controls typically ranged from 100 to 150 colonies. MNNG-induced his+ revertants numbered 3,853 ± 644.

[2] Prefixes i (iso), and a (anteiso) denote the position of the methyl branch on the fatty acid.

The short chain fatty acids (C_6 to C_{10}), had about 20% of the activity of palmitic acid, while the medium chain fatty acids (C_{17} to C_{18}) had about 14%. Lauric (C_{12}), and arachidic acids (C_{20}) were found to be about half as active as palmitic acid, while tridecanoic (C_{13}) and myristic acids (C_{14}) were found not to inhibit MNNG-induced mutagenesis in the Ames test. Pentadecanoic acid (C_{15}) was toxic at all doses tested ranging from 3 to 12.5 μg/plate.

The branched fatty acid isomargaric acid (15-methyl hexadecanoic acid) was detected by GC-MS in active MPLC fractions from run "6" and in fraction "7-10", and was assayed with other branched fatty acids for anti-MNNG activity. All iso-methyl branched chain fatty acids were more active than the straight chain fatty acids tested (Table 4.2). 14-Methyl pentadecanoic acid (isopalmitic acid), a natural but minor constitutent of bovine milk, had the highest activity of all fatty acids tested--6,360 units per mg--(five times more active than palmitic acid), and was detected in trace amount in active MPLC fraction "6-12". Interestingly, 13-methyl pentadecanoic acid (anteiso palmitic acid) was not effective in inhibiting MNNG-induced mutagenesis and was found to be toxic at concentrations greater than 4 μg/plate.

Table 4.3. Percentage of inhibition[1] of DMBA-induced mutagenesis by C16 fatty acids

Fatty acid (μg/plate)	Palmitic	Isopalmitic	Anteisopalmitic
5		35.0 ± 19.2 (108)	15.3 ± 11.2 (131)
10		38.2 ± 7.9 (101)	25.6 ± 9.5 (115)
12.5	19.1 ± 7.2 (198)		
15		55.4 ± 6.3 (82)	36.3 ± 5.5 (98)
25	26.3 ± 9.6 (165)		
37.5	29.6 ± 12.1 (155)		

Values are means ± S.D. for 3 experiments. Mean corrected colony counts (total minus spontaneous his+ revertants) are in parentheses below the corresponding percentages of inhibition. The mean colony count of the spontaneous his+ revertants in the DMSO and extract controls was 108 ± 10. The mean corrected colony count of DMBA-induced his+ revertants in the absence of extract was 185 ± 48. Based on the tabulated values, the specific activities of palmitic, isopalmitic, and anteiso palmitic acids against DMBA-induced mutagenesis are 768 ± 180, 3,120 ± 578, and 2,387 ± 370 U/mg ± standard error, respectively.

Iso-, anteiso- and straight chain palmitic acids were tested as potential inhibitors of DMBA-induced mutagenesis (Table 4.3). A dose-dependent inhibition of mutagenesis was observed, with isopalmitic being more effective in inhibiting DMBA than anteisopalmitic acid. At the maximum dose tested (15 μg/plate), the inhibition by isopalmitic acid was about 1.5 times greater than that exhibited by anteisopalmitic acid. Palmitic acid was found to be less effective, yielding about half the inhibition of isopalmitic acid at 2.5 times greater dose (29.6 % inhibition at 37.5 μg/plate vs. 55.4 % inhibition at 15 μg/plate).

Table 4.4. Percentage of inhibition of DMBA-induced mutagenesis by isopalmitic acid as a function of order of addition of reagents in the Ames test (*S. typhimurium* TA 100).

Treatment	% Inhibition[1]
1. Control: DMBA + S9 + DMSO; Incubate; Add TA 100.	0 (135)
2. DMBA + S9 + 10 μg Isopalmitic acid; Incubate; Add TA 100.	35.0 ± 11.3 (88)
3. DMBA + S9; Incubate; Add 10 μg Isopalmitic acid; Add TA 100.	0 (150)
4. DMBA + S9 + TA 100; Incubate; Add 10 μg Isopalmitic acid.	0 (148)

[1] Values are means ± S.D. for 2 experiments. Mean corrected colony counts (total minus spontaneous his$^+$ revertants) are in parentheses below the corresponding percentages of inhibition. The mean colony count of the spontaneous his$^+$ revertants in the DMSO and extract controls was 125 ± 10. The mean corrected colony count of DMBA-induced his$^+$ revertants in the absence of extract was 135 ± 4.9.

To determine a mechanism of action, isopalmitic acid was added during or after the activation of DMBA by S9 liver microsomes (treatments 2 and 3, respectively, Table 4.4). Addition of isopalmitic acid during the activation of DMBA decreased the number of revertants, while its addition after activation of DMBA did not, suggesting that isopalmitic

acid may interfere with S9-mediated activation (Table 4.4). Addition of isopalmitic acid after mutagen treatment did not result in an inhibition of mutagenesis, indicating that the fatty acid was unable to reverse the damage (treatment 4, Table 4.4). No changes in absorption were detected when the mixture of fatty acid and DMBA (unactivated) was scanned in a spectrophotometer, suggesting the absence of a complex.

Table 4.5. Percentage of inhibition[1] of 4NQO-induced mutagenesis by C16 fatty acids

Fatty acid (μg/plate)	Palmitic	Isopalmitic	Anteiso palmitic
2		7.5 ± 4.9 (574)	0 (360)
4	0 (471)	18.0 ± 1.4 (498)	0 (351)
6		26.5 ± 2.1 (478)	0 (384)
8	0 (472)	33.0 ± 5.7 (382)	0 (384)
10		41.5 ± 3.5 (330)	0.5 ± 0.7 (350)
12	0 (443)	51.5 ± 6.4 (253)	3.0 ± 0 (345)

Values are means ± S.D. for 2 experiments. Mean corrected colony counts (total minus spontaneous his+ revertants) are in parentheses below the corresponding percentages of inhibition. The mean colony count of the spontaneous his+ revertants in the DMSO and extract controls was 113 ± 16. The mean colony count of 4NQO-induced his+ revertants in the absence of extract was 557 ± 131. Based on the tabulated values, the specific activities of palmitic, isopalmitic, and anteiso palmitic acids against 4NQO-induced mutagenesis are 0, 4,241 ± 217, and 236 ± 71 U/mg ± standard error, respectively.

Table 4.5 summarizes the antimutagenic activities of palmitic acid and its monomethyl branched isomers against 4NQO. A dose-dependent inhibition of mutagenesis by isopalmitic acid was observed with an inhibition of 52% at the maximum dose tested (12 μg/plate). At the same dose, a very weak inhibition by anteisopalmitic acid (3%) was detected. At lower doses of anteiso palmitic acid, and at all doses of palmitic acid, no

inhibition of 4NQO-induced mutagenesis was observed. No changes in absorption were detected spectrophotometrically when the fatty acids were incubated with 4NQO.

4.5 Discussion

An activity-based (Ames test) purification of yogurt was accomplished using a sequence of silica gel, Sephadex LH-20, and reversed phase C18 medium pressure chromatographies. Palmitic acid was identified for the first time as having significant anti-MNNG activity in yogurt. Branched iso fatty acids, some of which were detected in active fractions, were tested later and found to be significantly more active than palmitic acid. Isopalmitic acid was found to have five times the anti-MNNG activity of palmitic acid. Palmitic acid did not inhibit DMAB-, DMBA- or 4NQO-induced mutagenesis, while isopalmitic acid was found to be antimutagenic towards DMBA and 4NQO.

The mechanism of anti-MNNG activity is unclear. One possibility is entraspment of MNNG by micelles formed by the fatty acids. Hayatsu et al., (1981) showed that oleic acid inhibited the mutagenic activity of 3-amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-1) and 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-2) by entrappment of the mutagens. Such a mechanism may explain the differential activity of fatty acids as chain length influences the ability to form a micelle (Bailey, 1951). The presence and position of methyl branching may also play a role in orientation (Abrahamsson et al., 1963) and affect interactions with the mutagen. This may account for the greater activity of iso-branched fatty acids and the inability of anteisopalmitic acid to inhibit MNNG-mutagenesis. Fatty acids may also interfere with the permeability of the bacterial membrane. Yamaguchi (1989) found that the removal of substances by ion-exchange treatments of a methanol-water extract of various cheeses caused the treated extract to become mutagenic in the Ames test. The antimutagenic substances removed were identified as fatty acids. Butyric, myristic, palmitic, stearic, and oleic acids were found in blue cheese, one of the cheeses tested. The author suggested that the fatty acids may

interfere with uptake of the mutagens. Iwado et al., (1991) found that the mutagenicity of air-borne particulates in the Ames test decreased due to the presence of palmitic, stearic, oleic, and linoleic acids. A direct interaction of the fatty acids with the mutagens was proposed to be responsible for the antimutagenicity.

We previously showed that an acetone extract of yogurt inhibited various mutagens and promutagens, that the compounds responsible for activity were more soluble in DMSO than in aqueous solutions, and that anti-MNNG and anti-DMAB activities were not due to the same compound(s) (Nadathur et al., 1995). The present study is consistent with those findings since the anti-MNNG-active compound, palmitic acid, is sparingly soluble in water, and exhibited only weak inhibition towards DMAB, (specific activity, 312 ± 53 units/mg). Work is underway to isolate compounds with anti-DMAB activity.

Isopalmitic acid was found to be antimutagenic against 4NQO and DMBA. The decrease in revertants when isopalmitic acid was added during the activation of DMBA was not observed when it was added after activation. This suggests that isopalmitic acid may interfere with the metabolic activation of DMBA, perhaps by inhibiting the activating enzyme, cytochrome P450 1A1, or by sequestering the substrate, the enzyme, or both, in a micelle. No interaction was detected spectrophotometrically between unactivated DMBA and isopalmitic acid. Negishi and Hayatsu (1984) found that short chain saturated fatty acids (C_5-C_{12}) inhibited the mutagenicity of *N*-nitrosodimethylamine (NDMA) in *E. coli* WP2 *uvrA*/pKM101. Data were presented supporting prevention of uptake of the mutagen or its activated metabolites by caproate (C_6). The authors showed that laurate (C_{12}) suppressed enzymatic demethylation of NDMA, and proposed that other fatty acids ($\geq C_8$) inhibited the metabolic activation of NDMA.

Approximately 400 fatty acids (C_2 to C_{28}) have been identified in the lipid fraction of milk, essentially as glycerides (Jensen, 1992). Branched fatty acids are present as

minor constituents (about 2.5% of total fatty acids). The smaller branched chain fatty acids ($\leq C_{10}$) are known to impart characteristic flavors in cheeses (Ha and Lindsay, 1990). Our study demonstrates a potential novel use for the isobranched C_{14}, C_{16}, C_{17}, C_{18} fatty acids. It is interesting to note that our study was accomplished using non-fat milk containing less than 0.5% fat. Fermentation of milk by lactic acid bacteria has been shown generally, but not always, to cause a modest increase in levels of free saturated fatty acids, presumably due to bacterial lipase activity. Rao and Reddy (1984) reported a 6.8% increase in free palmitic acid in milk fermented by *Lactobacillus delbrueckii* ssp. *bulgaricus*. Rasic et al., (1973) reported an increase of 5.6% in yogurt (milk fermented by *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus salivarius* ssp. *thermophilus*). The data of Blanc (1973), (as reviewed in Rasic and Kurmann, 1978), indicated a 2.5-fold increase in total free fatty acids, though changes in individual acids were not noted. The free fatty acid content of milk is relatively low because most are present as components of triacylglycerols, which presumably exhibit little anti-MNNG activity. Thus, lipase activity of lactic acid bacteria may play a role in causing the increase in antimutagenicity observed in fermented milk and yogurt (Bodana and Rao, 1990; Nadathur et al. 1994, 1995). Alternatively and less likely, the bacteria may synthesize and excrete the fatty acids *de novo* during fermentation. The implications of these findings for human health await demonstration that palmitic acid possesses anticarcinogenic activity.

4.6 Acknowledgements

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CHAPTER 5

CONCLUSIONS

5.1 Summary

The purpose of this study was to identify antimutagens in yogurt active against the experimental colon carcinogen, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG). Our initial experiments showed that acetone extracts of yogurt, or milk fermented by various lactic acid bacteria were antimutagenic against MNNG and 3,2'-dimethyl-4-aminobiphenyl (DMAB) in the Ames test (*Salmonella typhimurium* TA 100) (Nadathur et al., 1994). Further experiments carried out with milk fermented by *Lactobacillus delbrueckii* ssp. *bulgaricus* 191R showed that the putative compounds were more soluble in DMSO than in water, and that extractability of activity against MNNG and DMAB varied with pH, suggesting the presence of ionizable groups.

Subsequent experiments demonstrated the antimutagenicity of yogurt. An acetone extract of yogurt was found to be active against a range of mutagens and promutagens in the Ames test (Nadathur et al., 1995a). Simulation of fermentation by addition of lactic acid, lactic acid bacteria, or both to milk did not increase antimutagenicity, suggesting that compounds responsible for the activity may be formed during fermentation. Conjugated linoleic acid (CLA), a known dairy anticarcinogen, did not inhibit MNNG or DMAB indicating that other antimutagens may be present in yogurt. Fractionation of the acetone extract by HPLC showed that anti-MNNG and anti-DMAB activities did not co-elute, indicating that different compounds were responsible for the two activities.

Using the Ames test to direct purification, isolation of an anti-MNNG active compound was accomplished using silica gel, Sephadex LH-20 and C18 reversed phase medium pressure chromatographies. The antimutagen was identified as palmitic acid by: a) co-elution with authentic palmitic acid on GC and HPLC columns, and b) by comparison of mass and ^{13}C-NMR spectra. Minor components of milk fat such as iso-

methyl branched fatty acids (isopalmitic acid, isomargaric acid, isomyrsitic acid, and isostearic acid) were found to be more active than their straight chain counterparts. Isopalmitic acid also inhibited 4-nitroquinoline-N-oxide (4NQO) and the P450-mediated activation of 7,12-dimethylbenzanthracene (DMBA). The mechanism of antimutagenesis against MNNG has not been established.

Known mechanisms of antimutagenesis by saturated and unsaturated fatty acids include: a) entrapment of the mutagen, b) prevention of cytochrome P450-mediated activation of promutagens, c) inhibition of demethylase activity, and d) prevention of uptake of the mutagen or its metabolites (Hayatsu et al., 1981; Negishi et al., 1984; Iwado et al., 1991; Yamaguchi, 1989). A combination of some of these mechanisms may occur as well. Isopalmitic acid inhibited the direct-acting mutagen, 4NQO, and the cytochrome P450-mediated activation of DMBA. Hydrophobic interactions between the mutagen and fatty acid or steric interference of P450-mediated activation may be possible mechanisms.

5.2 Health aspects of palmitic acid and dietary fats

Palmitic acid is a saturated fatty acid and over consumption of saturated fatty acids has been implicated as being harmful to health. A summary of dietary fat and its role in cancer and cardiovascular disease follows.

Epidemiological studies have shown that the risk for colon cancer as a function of dietary fat is equivocal (Giovannucci and Willett, 1994; Kolonkel, 1987). But some studies found that total fat intake and in particular, animal fat to be strongly associated with colon carcinogenesis (McKeown-Eyssen and Bright-See, 1984). The consumption of meat and the risk for colon cancer was found to be equivocal. Haenszel et al., (1973) reported a positive association with intake of meat while a study by Graham et al., (1985) found no association between intake of meat and risk for colon cancer. A recent prospective cohort study by Goldbohm et al. (1994) also found no correlation between consumption of red meat, dietary fat and the risk for colon cancer. Manousos et al.,

(1983) found that risk for colon cancer increased in people with a high meat and low vegetable intake. Most studies found that total caloric intake was a risk factor for cancer. Jain et al., (1980) found a positive association between total caloric intake and risk for colon cancer. A lone study by Garland et al., (1985) found no such association.

Animal studies have shown that dietary fat is associated with incidence of cancer, but it has been shown to depend not only on the amount of fat, but also the type of fat. Typical fatty acid compositions of a few common edible oils are shown in Table 5.1. Because animals don't develop colon tumors spontaneously, a variety of carcinogens have been used to induce tumors in mice and rats. The authors then observed the effect of fatty acids on the enhancement or reduction of tumors. Reddy et al., (1976a) found that rats fed a 20% corn oil or lard diet and treated with DMH had a higher incidence of colon cancer than did rats fed a 5% corn oil or lard diet. Reddy et al., (1985) found a lack of tumor promoting effect of high dietary olive oil, coconut oil, and trans fat in contrast to that of high dietary corn oil, safflower oil, beef fat, and lard. This suggested that the fatty acid composition of the fat was one of the determining factors in colon carcinogenesis. Though in most cases, a high fat diet appeared to enhance tumorigenesis, it has been suggested that lower intake of essential nutrients was the actual cause and not fat intake. Feeding of unsaturated but not saturated fat, during the promotional phase enhanced colon carcinogenesis induced by multiple injections of azoxymethane (AOM) (Sakaguchi et al., 1984). Dietary fat increases the concentration of metabolites with carcinogenic or promoting activity in the fecal stream. Secondary bile acids such as deoxycholic acid and lithocholic acid were shown to have promoting activity in the colon (Reddy et al., 1976b). The authors found that in germ-free rats, bile acids had a promoting effect in colon carcinogenesis induced by MNNG.

High fat diets enhanced the promotion of skin carcinogenesis. When fatty acids were tested, oleic and lauric acids promoted skin carcinogenesis initiated by DMBA whereas palmitic and stearic acids did not (Holsti, 1959). Dietary fats are also known to

Table 5.1. Partial fatty acid compositions of common edible oils[1]

Oil/Fatty acid	Lauric	Myristic	Palmitic	Stearic	Oleic	Linoleic
Butterfat	3.1	11.7	26.2	12.5	28.2	2.9
Coconut oil	48.5	17.6	8.4	2.5	6.5	1.5
Corn oil	-	-	12.2	2.2	27.5	57.0
Lard	0.1	1.5	24.8	12.3	45.1	9.9
Olive oil	-	-	13.7	2.5	71.1	10.0
Palm oil	0.3	1.1	45.1	4.7	38.8	9.4
Safflower oil	-	0.1	6.5	2.4	13.1	77.7
Sesame oil	-	-	9.9	5.2	41.2	43.3
Soybean oil	-	0.1	11.0	5.2	23.4	53.2
Tallow (beef)	0.1	3.3	25.5	21.6	38.7	2.2

[1] Values are expressed as percent total fatty acids
Adapted from Fatty Acids in Foods and Their Health Implications. C.K. Chow (Ed). Marcel Dekker, New York, NY. pp 238-239.

enhance liver cancer. Newberne et al., (1979) found that increasing dietary corn oil (but not beef fat) during and after the administration of aflatoxin B1 (AFB1) increased the incidence of hepatic tumors, but not when the diets were fed after AFB1 administration. Thus, the effect of fat on enhancement of cancer was found mainly during the initiation stage. But, increasing the safflower or palm oil content of diet did not promote diethylnitrosamine (DEN)-induced γ-glutamyl-transpeptidase (GGT)-positive foci (Glauert and Pitot, 1986) in rats. The appearance of foci has been correlated with the later development of malignant neoplasms of the liver (Emmelot and Scherer, 1980). But when fats enhanced hepatocarcinogenesis, polyunsaturated fats had a greater effect. Rats fed diets high in unsaturated fatty acids (but not saturated fatty acids) before receiving DEN, developed more GGT-positive foci than rats fed low fat diets (Glauert et al., 1991).

Dietary fats also enhanced pancreatic cancer in animal studies. Roebuck (1986) found that high fat diets enhanced pancreatic carcinogenesis in rats. Polyunsaturated fat but not saturated fat was found to enhance tumor development. In a separate study, beef tallow (which is high in saturated fat) was found to enhance pancreatic carcinogenesis (Birt et al., 1990). The mechanism is still unknown though it appears that the fat and not the caloric intake enhances pancreatic cancer.

While most studies have reported on the initiation and enhancement of carcinogenesis, some studies have shown that fats and fatty acids may have a protective effect. Palm oil was shown to inhibit B[a]P-induced stomach cancer in rats (Azuine et al., 1992). Conjugated linoleic acid (CLA) inhibited DMBA-induced mammary carcinogenesis in rats (Ip et al., 1994). Though a high intake of fats is harmful, some fats may have protective effects against carcinogenesis. Other factors such as intake of fiber, fruits, and vegetables would decrease the level of fat intake and offset the risk for cancer.

Cardiovascular disease has been associated with intake of dietary fat and cholesterol. However, since the incidence of cardiovascular disease is associated with many factors such as diet, alcohol, smoking, and exercise, the linkage of diet and serum

cholesterol is still poorly understood. However, lowering of serum cholesterol has been found to be beneficial in reducing the risk for cardiovascular disease. Recent findings that palmitic, stearic and oleic acids may lower serum cholesterol have been reported (Bruckner, 1992). Palm oil (which contains 45% palmitic acid) has been criticized due to its high content of saturated fatty acids. Palmitic acid was implicated as the major saturated fatty acid responsible for the elevation of serum cholesterol (Grundy and Denke, 1990). However, Hegsted et al., (1965) showed that palmitic acid had the least effect, showing only a weak correlation while myristic acid was shown to account for 70% of the variance in serum cholesterol with variance in serum cholesterol associated with coconut oil. Coconut oil which has been shown to be atherogenic contains 45% lauric, 17% myristic, and 8% palmitic and the fatty acid data are consistent with the effect of the coconut oil on serum cholesterol (Wood, 1992).

5.3 Can palmitic acid explain yogurt's putative anticarcinogenicity?

Palmitic acid (in the triglyceride form) is present in higher amounts in nuts, oils, and meats than in yogurt. Consumption of such foods has not been associated with a reduced incidence of colon cancer. Salerno and Smith (1991) showed that sesame oil, palmitic acid, and other free fatty acids inhibited the HT-29 colon cancer cell line in vitro. Inhibition of the HT-29 colon cancer cell line by conjugated linoleic acid in vitro was also shown by Shultz et al., (1992). Because triglycerides are hydrolyzed in the small intestine, the site of action of the liberated free fatty acids is less likely to be the colon making it difficult to rationalize their result. Also, yogurt ought to provide no more palmitic acid than the milk from which it is made, and yet milk consumption has not been associated with a reduced incidence of cancer. How can the free fatty acids explain yogurt's putative anticarcinogenicity? The small intestine is a site for cytochrome P450 enzyme activation of promutagens. In vitro assays including ours have shown that fatty acids inhibit P450-mediated activation of promutagens and procarcinogens (Nadathur et

al., 1995b, Negishi et al., 1984). Thus, a probable mechanism is that fatty acids may act as desmutagens in vivo and inhibit the activation of promutagens in the small intestine.

We isolated palmitic acid as being antimutagenic against MNNG. Acetone extracts of yogurt and milk fermented by *Lactobacillus delbrueckii* ssp. *bulgaricus* were found to inhibit DNA damage caused by MNNG in the comet assay (Pool-Zobel, personal communication). Butyric acid was also found to be antimutagenic in the same assay (Pool-Zobel, 1995). However, if MNNG were present in our diet, its reactivity would preclude the possibility of reaching the colon intact. Hence, it is perplexing to extrapolate our finding to an in vivo situation. Therefore, a suitable animal model using a different experimental procarcinogen such as heterocyclic amines may aid in the explanation of palmitic acid's and yogurt's anticarcinogenicity.

Our study does not rule out the presence of other compounds in yogurt which may contribute to yogurt's antimutagenicity and anticarcinogenicity. This is supported by the fact that anti-MNNG and anti-DMAB activities did not co-elute when fractionated on HPLC. Hence, screening for activity against other mutagens and promutagens may aid in the identification of other antimutagens in yogurt.

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APPENDIX

List of Mutagens

Mutagen	Abbreviation
1,2-dimethylhydrazine	DMH
2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide	AF2
2-amino-3-methylimidazo[4,5-*f*]quinoline	IQ
2-amino-6-methyldipyrido[1,2-*a*:3',2'-*d*]imidazole	Glu-P-1
2-aminofluorene	2AF
2-nitrofluorene	2NF
3-amino-1-methyl-5*H*- pyrido[4,3-*b*]indole	Trp-P-2
3-amino-1,4-dimethyl-5*H*- pyrido[4,3-*b*]indole	Trp-P-1
3,2'-dimethyl-4-aminobiphenyl	DMAB
4-nitroquinoline-*N*-oxide	4NQO
7,12-dimethylbenz[a]anthracene	DMBA
Aflatoxin B1	AFB1
Benzo[a]pyrene	B[a]P
Diphenylnitrosamine	DPN
N-methyl-*N'*-nitro-*N*-nitrosoguanidine	MNNG
N-methylnitrosourea	MNU
N-nitrosodiethylamine	NDEA
N-nitrosodimethylamine	NDMA
N-nitrosodiphenylamine	NDPA