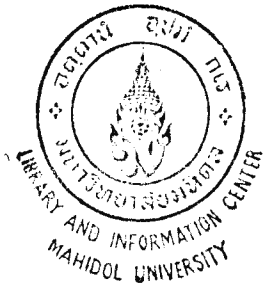

ANTIMUTAGENICITY OF YOGURT AND YOGURT CULTURES ON CYCLOPHOSPHAMIDE AND URETHANE IN THE WING SOMATIC MUTATION AND RECOMBINATION TEST USING DROSOPHILA MELANOGASTER

UMAPORN KAEWTONG

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UMAPORN KAEWTONG: ANTIMUTAGENICITY OF YOGURT AND YOGURT CULTURES ON CYCLOPHOSPHAMIDE AND URETHANE IN THE WING SOMATIC MUTATION AND RECOMBINATION TEST USING *Drosophila melanogaster*. THESIS ADVISORS: KAEW KANGSADALAMPHAI, Ph.D., ANADI NITITHAMYONG, Ph.D. 80 P. ISBN 974-663-999-4

Some epidemiological studies have shown a decreased incidence of colon cancer in people consuming fermented milk products. This study investigated yogurt and a group of lactic acid bacteria to induce strains actually used for fermenting yogurt. Expected to decrease the incidence of mutagen. The protective effects of yogurt A (fermented with a mixture of *Streptococcus thermophilus* TUA196L and *Lactobacillus acidophilus* ATCC4356), yogurt B (fermented with a mixture of *Streptococcus thermophilus* TUA196L and *Lactobacillus bulgaricus* TUA093L) or yogurt cultures (*Streptococcus thermophilus* TUA196L, *Lactobacillus acidophilus* ATCC4356 and *Lactobacillus bulgaricus* TUA093L) against the somatic mutation and recombination induced by cyclophosphamide (2.79 mg/tube) or urethane (4.45 mg/tube) in the improved high bioactivation (IHB) cross of the wing spot test in the *Drosophila melanogaster* were evaluated. Larvae trans-heterozygous (3-day-old) for the wing spot cells markers *mwh* (multiple wing hairs) and flr^3 (flare) were fed until pupation on medium containing a genotoxin or the combination of a genotoxin and UHT milk (2 ml/tube), each of yogurt (2 g/tube) or each yogurt culture (10^7, 10^8 or 10^9 cells). Subsequently, the wings of the resulting adult flies were analyzed for the frequencies and size of single and twin spots. The results showed that yogurt, milk and yogurt cultures had significant antimutagenic effect on both cyclophosphamide and urethane. The antimutagenic effect of yogurts brought the activity of the two mutagens close to the effect produced in the water control group. The results of this study suggested that 1) components of both milk and yogurt decreased the availability of the genotoxic agents to the imaginal disc cells of larva by scavenging activity, 2) binding of mutagens to cell wall of the yogurt bacteria should decrease the mutagenicity of both standard mutagens. However more research could be done in the future to substantiate these finding.

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อุมาพร แก้วทอง: ฤทธิ์ต้านการก่อกลายพันธุ์ ของ นมเปรี้ยว และ แบคทีเรียที่ใช้ผลิตนมเปรี้ยว ต่อ CYCLOPHOSPHAMIDE และ URETHANE ในการศึกษาโดยวิธี SOMATIC MUTATION AND RECOMBINATION ใน *Drosophila melanogaster* (ANTIMUTAGENICITY OF YOGURT AND YOGURT CULTURES ON CYCLOPHOSPHAMIDE AND URETHANE IN THE WING SOMATIC MUTATION AND RECOMBINATION TEST USING *Drosophila melanogaster*). คณะกรรมการควบคุมวิทยานิพนธ์: แก้ว กังสดาลอำไพ, Ph.D., อาณาดี นิติธรรมยง, Ph.D. 80 หน้า. ISBN 974-663-999-4

ปัจจุบันมีการศึกษาพบว่านมเปรี้ยวสามารถลดการเกิดมะเร็งลำไส้ใหญ่ได้ จึงทำการทดลองผลของนมเปรี้ยวและเชื้อแบคทีเรียที่ผลิตนมเปรี้ยวต่อการต้านฤทธิ์ของสารก่อกลายพันธุ์ และคาดหวังว่านมเปรี้ยวจะสามารถต้านฤทธิ์ของสารก่อกลายพันธุ์ได้ วัตถุประสงค์ของการศึกษานี้ต้องการศึกษาว่า นมเปรี้ยวชนิด A (แบคทีเรียที่ใช้ผลิต คือ *Streptococcus thermophilus* TUA196L และ *Lactobacillus acidophilus* ATCC4356), นมเปรี้ยวชนิด B (แบคทีเรียที่ใช้ผลิตนมเปรี้ยว *Streptococcus thermophilus* TUA196L และ *Lactobacillus bulgaricus* TUA093L), นม (2 ml/tube), แบคทีเรียที่ใช้ผลิตนมเปรี้ยว (2 mg/tube) คือ *Streptococcus thermophilus* TUA196L, *Lactobacillus acidophilus* ATCC4356 หรือ *Lactobacillus bulgaricus* TUA093L (10^7, 10^8, 10^9 cells) มีผลต่อการต้านฤทธิ์ของสารก่อกลายพันธุ์ Cyclophosphamide (2.79 mg/tube) และ Urethane (4.45 mg/tube) ในแมลงหวี่สายพันธุ์พิเศษ (Improved high bioactivation cross) หรือไม่ วิธีการทดสอบโดยนำหนอนอายุ 3 วัน ของแมลงหวี่พันธุ์ทาง (trans-heterozygous) ที่มีลักษณะเซลล์ปีกแบบ *mwh* และ *flr*^{3} มาเลี้ยงในอาหารที่มีสารก่อกลายพันธุ์ หรือมีสารก่อกลายพันธุ์ (cyclophosphamide) ร่วมกับ นมเปรี้ยว หรือ นม หรือ แบคทีเรียที่ใช้ผลิตนมเปรี้ยว จากนั้น ตัดปีกแมลงหวี่ที่รอดชีวิตนำมาตรวจหาจำนวนและชนิดของเซลล์ขนที่ผิดปกติ ผลการทดลองสรุปว่า นมเปรี้ยวชนิด A, นมเปรี้ยวชนิด B, แบคทีเรียที่ผลิตนมเปรี้ยวและนม มีผลต่อการต้านฤทธิ์ของสารก่อกลายพันธุ์ทั้ง 2 ชนิด โดยนมเปรี้ยวมีผลในการลดฤทธิ์ของสารก่อกลายพันธุ์ทั้ง 2 ชนิดมากกว่า นมและแบคทีเรียที่ผลิตนมเปรี้ยว โดยนมเปรี้ยวทำให้ฤทธิ์ของสารก่อกลายพันธุ์หมดไป จากผลการทดลอง นมและนมเปรี้ยวสามารถลดความสามารถของสารก่อกลายพันธุ์ได้ก่อนที่สารก่อกลายพันธุ์จะมีฤทธิ์ต่อความผิดปกติที่ Imaginal dice cells ในตัวแมลงหวี่ ด้วยองค์ประกอบในนมและนมเปรี้ยว และ แบคทีเรียที่ผลิตนมเปรี้ยวสามารถลดความาสามารถของสารก่อกลายพันธุได้ที่องค์ประกอบของแบคทีแรีย คือที่ผนังเซลล์ของแบคทีเรีย

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LIST OF ABBREVIATION

°C	degree celsius
g	gram
h	hour
mg	milligram
min	minutes
ml	milliliter
No.	number

CHAPTER I

INTRODUCTION

The large variation of cancer from between different parts of the world and populations both in space and times shows that the vast majority of cancer is determined by environmental factors. It is likely that this environmental influence does not only depend on the exposure to carcinogens, but also to a lack of antimutagenic and anticarcinogenic agents, for instance vitamins. A systematic use of such agents to prevent cancer appears as an important parting cancer prevention. Today antimutagens and anticarcinogens have only been using to a limited extent in a systematic way. There are essentially two possible strategies to employ chemopreventive agents for this purpose, either by manipulating the composition of the diet or by applying pure compounds (1). Several epidemiological and experimental studies suggest that some dietary factors can influence and counteract the development of colonic cancer. The intake of dietary fiber is one factor that seems to be protective (2). Fiber supplementation in the form of whole wheat and oat fiber was shown to decrease the mutagenicity and secondary bile acid levels in feces of healthy volunteers (3).

Many studies clearly show that fermented milk products and lactic acid bacteria inhibit the activity of mutagenic chemical compounds. The consumption of fermented milk with the bacteria used in its production has been observed to inhibit the

acidophilus in human have been shown to result in reduction of fecal bacteria enzyme activities (β-glucuronidase, nitroreductase and azoreductase) involved in procarcinogen activation (9) and to reduce the excretion of mutagens in feces and urine (10). Zhang (11) suggested that oral administration of *Streptococcus pyogenes* A3 (lactic acid bacteria) was valued for immunotherapy of liver tumor. Its cell wall, plasma membrane, cytoplasmic fraction and some protein showed antitumor activity but the effect varied according to the tumor site and the route of administration. Mastsukara (12) reported that the freeze dried cells of lactic acid bacteria effectively bound mutagenic pyrolyzates. Heating cells at 120°C for 15 min or at 80°C for 3 h did not significantly decrease binding of mutagenic pyrolyzates by the cells. In view of the above findings and the regular intake of fermented milk or lactic acid bacteria by a sizable portion of the human population, it would be of relevance to evaluate the possible inhibitory effects of yogurt or lactic acid bacteria against the *in vivo* mutagenesis induced by standard mutagens namely, cyclophosphamide and urethane. For this purpose, the somatic mutation and recombination test in *Drosophila melanogaster* was employed in the study.

CHAPTER II

REVIEW OF LITERATURE

There are numerous chemical substances present in the environment around us and many of these can cause harmful effects to the human body. A lot of researchs were carried out into toxicity, where the appearance of the damage to the body due to chemicals was in a directly observable form. In recent years, however, there has also been active research into genetic toxicity, which causes cancer or ageing by inducing mutation in body cells so as to increase the frequency of recessive genetic expression and many insights have accumulated (13).

At one time, it was the opinion in cancer research that there was no relationship between carcinogens and mutagens, but reports that 4-nitroquinoline-*N*-oxide, a known carcinogen, also had the property of inducing spontaneous mutation in bacteria and that *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine, induce known of mutation, also induce stomach cancer, etc., led inexorably to the conclusion that there was a close relationship between the two. Today it is clear that 85% of carcinogens are mutagenic (14).

2.1 Antimutagenicity

Many substances were found in food (and its components) to contain the property of weakening the action of mutagens or carcinogens. Substances which can

groups (15). One group, desmutagens, act directly on mutagens to weaken or inactivate their mutagenicity. The other group, antimutagens act on cells to decrease substantially the induction of mutation (13).

A major focus for cancer prevention must be on managerial approaches to assuring optimal nutrition. In addition to the importance of nutrition in cancer prevention, recognition is growing that food contains specific components that function as protective agents against several diseases. Epidemiological studies revealed that reduce risk of some cancer is associated with food consumption such as yellow green vegetable, cereals, whole wheat breads and soy products. Food components that have demonstrated anticarcinogenic effect in experimental systems are given in Table 1. Prominent among phenolics, which occur in a wide array usually as glycosides, and nutrients such as minerals and vitamins. Food rich in such components are referred to as " functional food" The potential of such agents for cancer prevention deserves consideration. Moreover, the attention needs to be given to combined modification of both nutritional and functional components of the diet (16).

Table 1 Food-Borne Inhibitors of Experimental Cancer[a]

Food Component	Food	Experimental Cancer Inhibited
Bifidobacterium longum - culture - calcium	Fermented dairy product	Colon, Liver
Carotenoids - β-carotene	Green-yellow vegetables, Fruits	Colon, Stomach
Conjugated linoleic acid	Cheese, Cooking meats	Breast, Forestomach, Skin
Diallyl sulfide	Garlic, Onions	Esophagus, Forestomach, Large intestine, Liver

Table 1(cont.) Food-Borne Inhibitors of Experimental Cancer[a]

Food Component	Food	Experimental Cancer Inhibited
Fiber Cereal, Bread		Breast, Colon
Indole-3-carbinol (glucobrassicin)	Cruciferous vegetables	Breast, Endometium, Forestomach, Liver, Lung
Minerals		
- Calcium	Dairy products	Large intestine
- Selenium	Vegetables	Breast, Skin, Liver, Lung, Large intestine
Monoterpenes		
- D-carvone	Caraway seed	Forestomach, Lung
- D-limonene	Citrus fruits	Breast, Forestomach, Lung
Myo-inositol (phylate)	Fish, Poultry, Dairy products	Large intestine
Phenolics (glycosides)		
- Catechins - (-)-Epigallocatechin-3-gallate	Fruits, Vegetables, Tea, Coffee, Cereal grains	Lung, Skin, Small intestine
- Flavones	Vegetables	
- Quercetin		Breast, Colon
- Isoflavones	Soy products	
- Genistein		Breast, Large intestine
- Hydroxycinnamic acid	Fruits, Vegetables, Soy, Cereals	
- Caffeic acid		Forestomach
- Chlorogenic acid		Large intestine, Liver
- Ferulic acid		Forestomach
- Tannins	Vegetables	
- Tannic acid		Forestomach, Lung
- Ellagic acid	Fruits	Esophagus, Liver
Protease inhibitors		
- Bowman-Birk	Soy	Liver
- Edi ProA soy protein	Soy	Liver
Soy protein isolate	Soy	Breast

Table 1(cont.) Food-Borne Inhibitors of Experimental Cancer[a]

Food Component	Food	Experimental Cancer Inhibited
Thiocyanates (glucosinolates)	Broccoli, Cabbage, Watercress	Breast, Forestomach, Liver, Lung
- Benzyl isothiocyanate		Breast, Liver
- Benzyl thiocynate		Breast, Lung esophagus, Forestomach
- Phenethyl isothiocyanate		
Vitamins		
- vitamin A	Liver, Milk, Eggs	Liver, Lung
- vitamin C (ascorbic acid)	Citrus fruits, Vegetables	Kidney, Large intestine, Lung Breast,
- vitamin E (α-tocopherol)	Seeds and Nuts, Vegetable and Seed oils	Forestomach, Large intestine, Oral, Skin

[a] from Watson (16).

Epidemiological studies suggest that fermented milk products suppress the onset of carcinogenesis. The alteration in intestinal microbiota is apparently responsible for the anticarcinogenic attribute. Animal mode deployed to delineate the anticarcinogenic role fermented milk products may be broadly divided into prevention of cancer initiation and the suppression of initiated tumor (17).

2.2 Yogurt

Cultured yogurt can be characterized as a gel-like coagulated milk product, having a smooth consistency and a pleasing tart flavor. It is available in a wide range of variations, including plain yogurt and many fruit, vegetable and/or nut flavored varieties. Yogurt is also available as drink products and as frozen desserts.

The definition of yogurt in the FAO/WHO Standards is 'the coagulated milk product obtained by lactic acid fermentation through the action of *Lactobacillus bulgaricus* and *Streptococcus thermophilus* from milk and milk product's. The

standards also make provision for the use of order cultures as optional additives (18). Traditionally, the manufacture of yogurt is generally considered to be dependent on the fermentation of a 'milk mix' by a starter culture composed of *Lactobacillus bulgaricus* and *Streptococcus thermophilus*. The composition of the 'milk mix' may be varied depending on the desired formulation with regard to butterfat content, total solids, and stabilizer (19).

The cultures used to manufacture yogurt are characterized as thermophilic lactic starter cultures (20). The organisms primarily included are *Lactobacillus bulgaricus* and *Streptococcus thermophilus*. Both of these organisms contribute to the product during the fermentation process. The primary function of both these cultures is to produce the lactic acid required for the formation of the coagulum. Additionally they produce certain volatile compounds, such as acetaldehyde and acetone, which contribute to the flavor of the product. The latter are produced primarily by *Lactobacillus bulgaricus* (19). The combination of the lactic acid and volatile components is responsible for the typical flavor and texture of cultured yogurt.

While *Lactobacillus bulgaricus* and *Streptococcus thermophilus* are the primary organisms involved in the manufacture of cultured yogurt, other starter cultures also have been utilized for producing yogurt or similar products. One of these is *Lactobacillus acidophilus*, which may be used to produce a yogurt like product. For some products the cell of *Lactobacillus acidophilus* or bifidobacteria may be added to which has been fermented by *Lactobacillus bulgaricus* and *Streptococcus thermophilus*. Caution should be exercised in adding cells of

Lactobacillus acidophilus to cultured yogurt because of the possibility of the cells of *Lactobacillus acidophilus* not surviving during storage of the product (19).

The growth and action of a starter culture composed of *Lactobacillus bulgaricus* and *Streptococcus thermophilus* during the manufacture of yogurt can create a number of changes in the milk. The most obvious change milk resulting from the fermentation by the yogurt starter culture is the formation of the gel-like coagulum resulting from the precipitation of casein. The yogurt culture produces a mixture of D- and L-lactic acid during the fermentation (21, 22). The production of lactic acid is from the fermentation of lactose in milk, thus the lactose content is decreased during the fermentation. As much as 20-30% of the lactose present in the original milk is utilized during the fermentation (19). Only that amount which is requires for growth and acid production by using them during the fermentation. The yogurt starter cultures preferentially utilize the glucose moiety of lactose, which often in the accumulation of free galactose in yogurt (21, 22).

Changes in milk resulting from fermentation during manufacture of yogurt

1. Production of acid (primarily D-, L-acid)
2. Partial utilization of lactose
3. Improvement of digestibility of milk
4. Increased amounts of free amino acids
5. Alteration of vitamin content

2.2.1 Prevention of Cancer Initiation

Consumption of fermented milk products (FMP) containing viable lactic acid bacteria (LAB) may reduce the possible initiation of colon cancer. The favorable change in intestinal microbiota can directly and indirectly reduce the conversion of procarcinogens to carcinogen.

2.2.1.1 Direct Reduction of Procarcinogens

Nitrites used in food processing can be convert into nitrosamines in the gastrointestinal tract. The conversion could possibly be reduced if LAB deplete nitrite through cellular uptake in the gastrointestinal tract, which has been show *in vitro* (23, 24). This phenomenon has yet to be demontrated in the gastrointestinal tract and the end products identified (25). Secondly, bile salts and their derivations may initiate colon carcinogenesis (26, 27). Clostridium Bacteriods and Eubacterium are some of the genera that transform bile salt from the primary to secondary. *Lactobacillus acidophilus* decreased the rate of conversion of the primary bile acid, chenodeoxy cholic acid to its secondary derivative *in vitro* (28). Based on this observation it was extrapolated that high numbers of viable *Lactobacillus acidophilus* in the gastrointiatinal tract may reduce the potential for cancer initiation.

2.2.1.2 Indirect Reduction of Procarcinogens

Bacterial procarcinogenic enzymes in feces (such as azoreductase, β-glucuronidase and nitroreductase) are used to monitor mucosal carcinogenesis as they convert the procarcinogens to carcinogen (17, 29). The potential for initiation of

FMP reduces the level of the enzymes in feces (9). The level of procarcinogenesis enzymes may be high in undesign fecal flora and low or absent in LAB (17). Accordingly, there is a need to examine the presence of procarcinogenic enzymes among intestinal bacteria (25).

2.2.2 Suppression of Initiated Cancer

Feeding and/or injection of yogurt and LAB suppress the growth of implanted tumors (25). Tumor growth was suppressed significantly in mice in a short-term study of 1-2 weeks (30) but survived rate for rodents was not significantly increased in a long-term study (3, 31). Lee (28) induced colonic tumors chemically in rats and fed them a diet supplememted with *Lactobacillus acidophilus* to assess the effect on progression of the tumors. Rats on an *Lactobacillus acidophilus* supplemented diet activity initially (26th week) in comparison with the control group. However, towards the end (40th week) there was no difference in tumors sites and the level of ornithine decarboylaxe activity between the two groups of rats, indicating that *Lactobacillus acidophilus* may be efficacious as an antitumors agent during tumor initiation (28).

2.3 Milk

Milk is the first food ingested by young mammals including human infants. It continues to be the sole constituent of the diet for a considerable period of time. Milk is a complex biological fluid. The composition and physical characteristics of which vary from species to species, reflecting the dietary needs of the young mammal. The major constituent of milk is water, but according to species, milk contains varying

quantities of lipids, proteins and carbohydrates which are synthesized within the mammary gland. Smaller quantities of minerals and other fat soluble and water soluble components derived directly from blood plasma, specific blood proteins and intermediates of mammary synthesis are also present (Table 2).

Table 2 Average Composition of Cow's Milk[a]

Component	Percentage	Percentage of solids
Lactose	4.8	37.5
Fat	3.7	28.9
Protein	3.4	26.6
Non-protein nitrogen	0.19	1.5
Ash	0.7	5.5

[a] from Gilliland (21).

2.3.1 Milk and Cancer Prevention

Cow's milk suppresses spontaneous mutagenicity in *Salmonella typhimurium* TA1535 due to *N*-methyl-*N*-nitrosourea. Cow's milk thus works to decrease the activity of alkylatings agents. Therefore, cow's milk can be expected to stop the development of stomach cancer due to alkylating agents. Vegetable juices are similar to milk in preventing. The action of alkylating agents ascorbic acid contained in vegetable juices reacts with nitrites to form dehydro ascorbic acid and nitrate, this hindering nitroso reaction. Stomach cancer is also related to chronic gastritis. Cow's milk has the effect of regulating stomach acidity and can be used when the stomach is in bad condition since it protects the cells from direct contact with carcinogenic substances by its preserving action on the gastric mucous (32).

Cow's milk is an outstanding source of vitamin A; 100 g cow's milk contains 27 μg retinal and 11 mg carotene showing 110 IU of vitamin A activity. Epidemiological investigations indicate that foods containing a large amount of vitamin A are associated with a decreased risk of cancer and animal experiments also indicate that vitamin A works to suppress chemically induced tumors. Vitamin A has an adjuvant effect such as it is shown by immune activators (the effect of producing specific antibodies and increasing cellular immunity when administered mixed with an antigen) raising both humor and cellular immunity.

Gridley, *et al.* (33) reported that when they gave cow's milk to rats with transplanted epitheliomas (epithelial tumors: mainly malignant tumors produced from the epithelium of epidermis or mucosa), the multiplication of the tumors was suppressed. Gridley, *et al.*(33) also reported that in mice reared on low-fat feed containing cow's milk, the rate of spontaneous tumors was low and conjectured that cow's milk protein might have a suppressing effect on the development of tumor (33).

2.4. Lactic Acid Bacteria

Lactic acid bacteria (LAB) is a group of gram-positive bacteria united by a constellation of morphological, metabolic, and physiological characteristics. The general description of the bacteria included in the group is Gram-positive, nonsporing, nonrespiring cocci or rods, which produce lactic acid as the major end product during the fermentation of carbohydrates. The boundaries of the group have been subject to some controversy, but there has been general agreement that the genera *Lactobacillus, Leuconostoc, Pediococcus* and *Streptococcus* form the core of the

group. Recent taxonomic revisions of these genera suggest that lactic acid bacteria comprise the following: *Aerococcus, Carnobacterium, Enterococcus, Lactobacillus, Lactococcus, Leuconostoc, Pediococcus, Streptococcus, Tetragenococcus* and *Vagococcus*. The classification of lactic acid bacteria into different genera is largely based on morphology, the mode of glucose fermentation, growing at different temperatures, the configuration of the lactic acid produced, ability to group at high salt concentrations, and acid or alkaline tolerance. For some of the newly described genera, additional characteristics such as fatty acid composition and motility are used in classification. The measurements of true phylogenic relationships with rRNA sequencing have aided the classification of LAB and clarified the phylogenic of the group. Most genera in the group form phylogenetically distinct groups, but some, in particular *Lactobacillus* and *Leuconostoc* are very heterogeneous and the phylogenetic clusters do not correlate with the current classification based on phenotypic characters. New tools for routine use are nucleic acid probing techniques, partial rRNA gene sequencing using the polymerize chain reaction and soluble protein patterns.

Two main sugar fermentation pathways can be distinguished among lactic acid bacteria. Glycolysis (Embden-Meyerhof pathway) results in almost exclusively lactic acid as end product under standard conditions and the metabolism is referred to as homolactic fermentation. The 6-phosphogluconate/phosphoketolase pathway results in significant amounts of other end products, such as ethanol, acetate and carbondioxide in addition to lactic acid, and the metabolism is referred to as heterolactic fermentation. Various growth conditions may significantly alter the end-

product formation by some lactic acid bacteria. These changes can be attributed to an altered pyruvate metabolism and/or the use of external electron acceptors such as oxygen or organic compounds (19).

2.4.1 Characteristics of *Lactobacillus acidophilus*

Lactobacillus acidophilus was first isolated in 1900 by the Australian Moro from the feces of bottle fed infants and named *Bacillus acidophilus*. At the present time *Lactobacillus acidophilus*, *Lactobacillus salivarius*, *Lactobacillus leichmanii* and *Lactobacillus fermentum* are recognized as possible resident species in the human digestive tract; these are called resident species to distinguish them from transient species, which can only pass live through the digestive tract. Transient strains include strains of *Lactobacillus brevis*, *Lactobacillus plantarum* and *Lactobacillus casei*.

Lactobacillus acidophilus is found resident from the lower small intestine and through the large intestine and is detected particularly in the lower small intestine. Bifidobacteria (*Bifidobacterium* spp.); on the other hand are present mainly in the large intestine. *Lactobacillus acidophilus* shows as the following characteristics.

1. It does not grow at 15°C and does not ferment ribose.

2. Optimum temperature range for growth is 35-38°C and optimum pH range is 5.5-6.0.

3. In cow's milk it produces 0.3-1.9% D- L- lactic acid. The acid producing capability differs among strains.

4. Generally speaking it has strict nutritional requirements; it requires acetates (or mevalonic acid), riboflavin, patothenic acid, calcium, niacin and folic acid.

5. It is resistant to bile acids.

6.It is produced threonine aldolase and alcohol dehydrogenase which influence aroma (34).

2.4.2 Characteristics of *Lactobacillus delbruckii* subsp. *bulgaricus*.

The stains of *Lactobacillus delbruckii* subsp. *bulgaricus* are strictly fermentative, aero tolerant or anerobic aciduric acidophilie and have complex nutritional requirements (e.g. for carbohydrates amino acids peptides, fatty acid esters, salts, nucleic acid derivatives and vitamins). These cells are gram-positive and non-spore-forming, usually catalase negative, non-motile and facultatively anaerobic. They do not synthesis porphyrinoids and thus are devoid of heme-dependent activities. With glucose as a carbon source *Lactobacillus delbruckii* subsp. *bulgaricus* may be either homofermentative, producing more than 85% lactic acid or heterofermentative, producing lactic acid, carbondioxide, ethanol (and/or acetic acid) in equimolar amounts. In the presence of oxygen or other oxidants increased amounts of acetate may be produced out the expense of lactate or ethanol whereby one additional mole of ATP is gained via the acetate kinase reaction. Thus, variations in the metabolic and products may occur. Various compounds (e.g. citrate, malate, tortrate, quinolate, nitrite, nitrate, etc.) may be metabolized and used as energy sources (e.g. via building up a proton motive force) or electron acceptors (13).

2.4.3 Characteristics of *Streptococcus thermophilus.*

The only streptococcal species associated with food technology is *Streptococcus thermophilus.* It is used in the manufacture of yogurt (in coculture with *Lactobacillus delbruckii* subsp. *bulgaricus* (35). *Streptococcus thermophilus* is a gram-positive cocci which may be spherical or ovoid in shape. The cells are are usually arrarrged in chains or pairs. Cell is 0.7-1 μm in diameter, spherical or ovoid, forming chains or occurring in pairs. It is non-motile and does not form endospore and is facultatively anaerobic. It requires carbondioxide for growth. It is chemo-organotroph, ferment carbohydrates with the production of lactic and other acids and has complex nutritional requirements. Growth at 45°C can give rise to irregular cells and segments. Strains are either α-haemolytic or non-haemolytic on blood agar and are isolated from yogurt (36).

2.4.4 Antimutagenic Activity of Lactic Acid Bacteria

It is well known that carcinogenesis is initiated by mutation induced in animal cells with carcinogen. Recently, publication has begun of evidence for the suppression by lactic acid bacteria of carcinogen-induced mutations (37). Hosono, *et al.* (38) were the first to report that milk fermented with *Lactobacillus delbrueckii* subsp. *bulgaricus* IFO3533, *Lactobacillus lactis* subsp. *lactis* IFO12546 or *Enterococcus faecalis* IFO12964 exhibited antimutagenic activity against 4-nitroquinoline-N-oxide, a typical mutagen, and the water extract of dog faeces, a faecal mutagen, *in vitro* using the *Escherichia coli* B/rWP2trp^{-}hcr^{-}strain.

Since lactic acid possessed no anti-mutagenic effect on the mutagenicity of the mutagen, the antimutagenic activity seemed to arise from some metabolites other than lactic acid and/or cellular component from the starter bacteria. Morotomi and Mutai (39) reported almost the same time that all gram-positive bacteria (*Bifidobacterium breve, Bifidobacterium adolescentis, Bifidobacterium bifidum, Lactobcillus acidophilus, Lactobacillus casei* and *Lactobacillus delbrueckii* subsp. *bulgaricus*) and some Gram-negative bacteria (*Bacteroides fragilis* and *Enterobacter cloacae*) isolated from human faeces were effective to bind to 3-amino-1,4-dimethyl-5H-pyrido (4,3-b) indole (Trp-P-1) and 3-amino-1-methyl-5H-pyrido (4,3-b)-indole (Trp-P-2), well known strong mutagens from pyrolised beef and fish meat. The binding was somewhat inhibited by a simulated electrolyte solution of the terminal ileum. The experimental facts seemed to suggest an antimutagenic function of some intestinal bacteria against certain mutagens originating from ingested food *in vivo* (39).

From the results of an *in vitro* assay with streptomyocin dependent strains of *Salmonella typhimurium* TA98 and TA100, and 2-(2-furyl)-3-(5-nitro-2-furyl) acryl-amide (AF-2), 4-nitroquinoline-N-oxide (4NQO) and faecal mutagenic extracts from cats, monkeys, dogs and other animals, Hosono, *et al.* (37) described the anti-mutagenic activity of milk fermented with *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus salivarius* subsp. *thermophilus* against all the mutagens. The antimutagenic activities against the mutagenic chemicals increased with incubation time but were lost rapidly at temperatures higher than 55°C (40).

2.4.5 Antitumour activity of Lactic Acid Bacteria

Bogdanov, *et al.* (40) found that the antitumour activity of a crude preparation from *Lactobacillus delbrueckii* subsp. *bulgaricus* 51 against sarcoma - 180 in mice was due to glycopeptides present in the preparation. This is the first time that specific antitumour activity of glycopeptides derived from bacteria cell wall pointed out, though immuno-adjutant effects was described for a bacterial peptidoglycan and its fragments. The anti-tumour activity of cell wall peptidoglycan of *Bifidobacterium infantis* studied by Sekine, *et al.* (41). They demonstrated that purified cell walls, which consisted essentially of two hydrophilc polymers of polysaccharide and peptidoglycan, is responsible for the antitumour activity of heat-killed organism.

Essentially, peptidoglycan consists of linear heteropolysaccharide chains that are cross-linked (by peptides) to form a three-dimensional net-like structure (the 'sacculus') which envelops the protoplasm. Covalently bound to peptidoglycan are compounds such as teichoic acids: typically, substituted polymers of glycerol phosphate or ribitol phosphate. In some bacteria (e.g. Mycobacterium) the wall contains lipids, while in the others (e.g. strains of Streptococcus) it contains carbohydrates. (Figure 2 and 3) The composition of the wall can vary with growth conditions; for example, in Bacillus, the availability of phosphate affects the amount of cell wall teichoic acids (42).

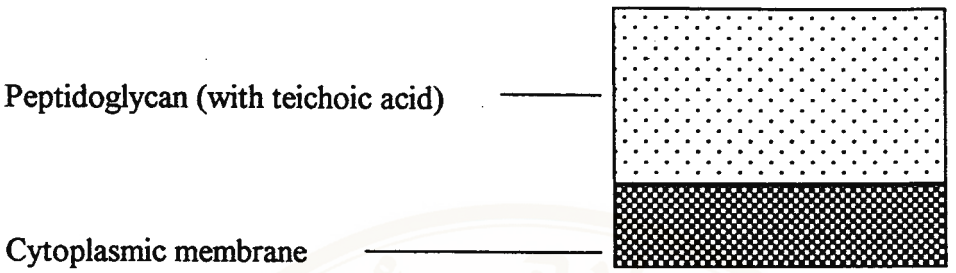

Figure 1 Diagram showing the structure of Gram-positive bacterial cell walls (42).

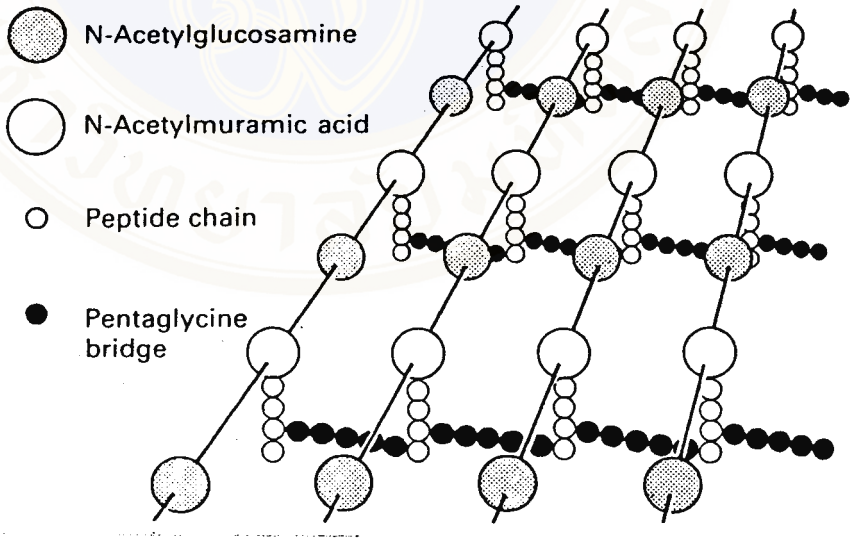

Figure 2 Diagram showing chemical structure of cross-linking in peptidoglycan component of cell walls (42).

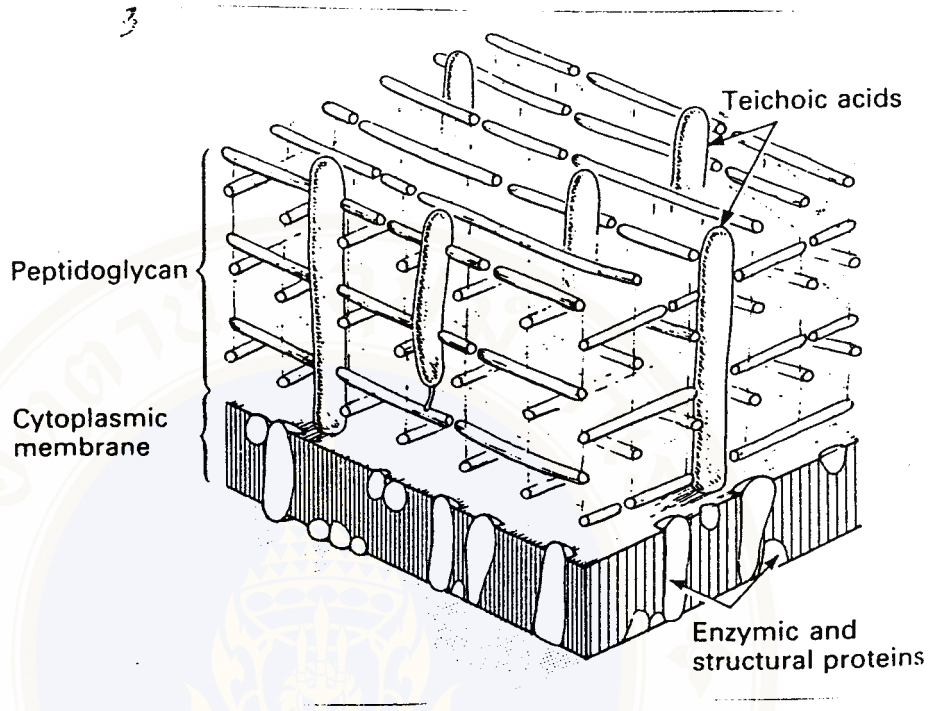

Figure 3 Three-dimensional representation of Gram-positive bacterial cell walls (42).

The whole peptidoglycan which has physical integrity of cell wall structure is much more effective than physically dirsurpted cell walls, that is cell wall skeleton or sonicated whole peptidoglycan against the progressively growing Meth-A solid tumour. These results seemed to suggest evidence that the physical form of cell wall preparation plays an important role in the induction of the antitumour activity.

An augmentation of local immunity with oral administration of peptidoglycan from *Streptococcus thermophilus*, an intestinal flora component of swine, was observed in the lamina propia of small intestinal mucosa of weaning piglets (43). It was emphasised that peritoneal nonspecific cytotoxicity stimulated by injection of peptidoglycans from gram-positive bacteria against tumours was basically different

according to the peptidoglycan structures, though the elicitation of NK cell populations by peptidoglycans was not (44). Consequently, the anti-tumour activity of lactic acid bacteria appears to depend on, at least in part, non-specific cytotoxicity induced by peptiglycan from their cell walls (44).

2.5 Somatic Mutation and Recombination Test (SMART)

The somatic mutation and recombination test of *Drosophila melanogaster* is developed as a rapid and inexpensive in vivo assay for identifing genotoxicants in the somatic tissue of a higher eukaryote (45, 46, 47). The assay found to sensitive to a broad array of chemical mutagens (48). The capability of identifying both gene mutation/deletion and mitotic recombination makes the wing spot assay is an extremely informative short-term assay for potential carcinogens because the development of several human cancers is to involve mitotic recombination (49).

2.5.1 Principle of Somatic Mutation and Recombination Test

Drosophila melanogaster is a dipteran insect develops through successive developmental stages of different duration. *Drosophila* undergoes complete metamorphosis (duration 1 day at the optimal culture temperature of 25°C), 1st larval instar (L1, 1 day), 2nd larval instar (L2, 1 day), 3rd larval instar (L3, 2 days), metamorphosis in pupal stage (prepupa 4 h, pupa 4.5 days) and adult stage (imago, up to 40 days) (50, 51) During embryogenesis primarity larvae tissues (cuticle, gut, fat body, nervous system, etc.) are formed and during the larval period these tissues enlarge and finally form the body of a large L3 larva ready for pupation. The adult

structures (wings, legs, eyes, etc.) formed in the pupal stage from the so-called imaginal discs. These develop during larval life as proliferating entities derived from groups of cells set apart during embryogenesis.

For example, the cells determined to build the wings represent the imaginal discs. Such discs grow during the larval period by cell proliferation. The developmental parameters of this type of imaginal disc were described by Garcia-Bellido (52) as follows. The cells of the imaginal wing disc derive from a sample of about 50 nuclei of the primitive egg syncytium, which happen to migrate to a given region of the egg cortex. After these nuclei surrounded by cytoplasm and membranes, the corresponding cells become developmentally segragated from the neighboring ectodermal cells. They do not divide during embryonic development but can already be detected histologically, grouped in a discrete wing imaginal disc, in the newly hatched larvae. Proliferative growth starts in the first instar and continues throughout the larvae. Cell proliferation is logarithmic in all the presumptive adult cuticular cells, although the number of cells per clone deviates from 2^n. On average, cells divide every 8.5 hours and the growth is complete after 9-10 cell divisions. After pupation, mitoses are still detectable but somatic crossing over initiated at this time results in single marked cells. Twenty-four hours later, mitosis ceases altogether and visible cell differentiation begins. During differentation about 50,000 cells given rise to single identifiable cuticular processes organized in the typical adult pattern.

During disc growth in the larval stage a wing imaginal disc cell is genetically altered into a mutant form, a group of mutant cells will result from clonal expansion

during disc growth. After pupation, in the course of metamorphosis of the imaginal disc into an adult wing the mutant phenotype will become expressed. The mutant clone will be recognizable as a group of phenotypically altered wing blade structure (called a "spot"), e.g. showing multiple hairs instead of the single hair formed by each wild-type wing cells (52).

The number of pre-ommatidia cells is about 20 at the end of the first instar, increases to 100-150 cells in the second instar, and reaches the final number of 780-800 pre-ommatidia cell in the third after 2-3 more cell divisions (53, 54). Differentiation of primordial cells takes place during third instar. Primordial cells of the adult compound eye divide continuouslly throughout the larval period. Cell lineages producing pigment cells are established during the first wave of mitotic activity that sweeps across the eye in the third instar: most pigment cells are produced in the single division of the second wave. Genetic alterations in the differentiated cells can be recognized as mosaic spots (mutant clones) in the adult if appropriate genetic markers are used. Clones induced at the beginning of larvae life will be large in size while those produced in the second or third instar will successively be smaller. Hence, classification of mosaic spots into size classes and subsequent analysis of clone size distribution can be used to estimate the time point of the induction of the mutational events. According to Becker (53), the mosaic spots can be classified into 10 size classes, taking as class limits the power of 2. In most assay systems larvae heterozygous for suitable marker genes are used so that different genetic changes (gene mutations, deletions and mitotic recombination) detected as mutant spots on

adult structures. Referring to the different genetic endpoints detectable in the assays, they are called "**Somatic Mutation And Recombination Tests**" (SMART) (55).

2.5.2 Genetic Basis of the Effects Detected

The assay consists of exposing to a mutagen populations of cell that are destined to multiply in relatively fixed configurations so that an induced mutation in one of the exposed cell give rise to a detectable clone. To ensure the clone is identifiable on the surface of the adult fly. One chooses genetic markers that are expressed autonomously in wing cell. The exposed cell of the larvae wing imaginal discs is trans-heterozygous for two recessive markers located on the left arm of chromosome 3. Multiple wing hairs (*mwh*) are at position 0.0 cM and flare (flr^3) at 39.0 cM: while the centromere is located at 47.7 cM (56). The appearance of multiple wing hairs is a homozygous viable recessive mutation producing multiple trichomes per cell instead of the normally unique trichome. The second marker, flare (flr^3), are a zygotic recessive lethal but homozygous cells in the wing imaginal disc survive and lead to wing blade cells with short, and misshapen trichome. In stock cultures, the flr^3 alleles have to be balanced over inverted chromosomes such as TM1 or TM3 because it's a recessive zygotic lethal. In all the experimental series analyzed, the occurrence of the various types of spots was as follows: most frequent were single spots expressing the mwh phenotype, less frequent twin spots with both a *mwh* and a flr^3 sub-clone, and quite rare single spots with the flr^3 phenotype.

There exist several mechanisms that lead to genetically marked clones (see Figure 4). An important possibility is a mitotic recombination event between two non-sister

chromatids. Twin spots are expected if recombination occurs between *flr*^{3} and the centromere and if an X-type segregation follows so that each daughter cell

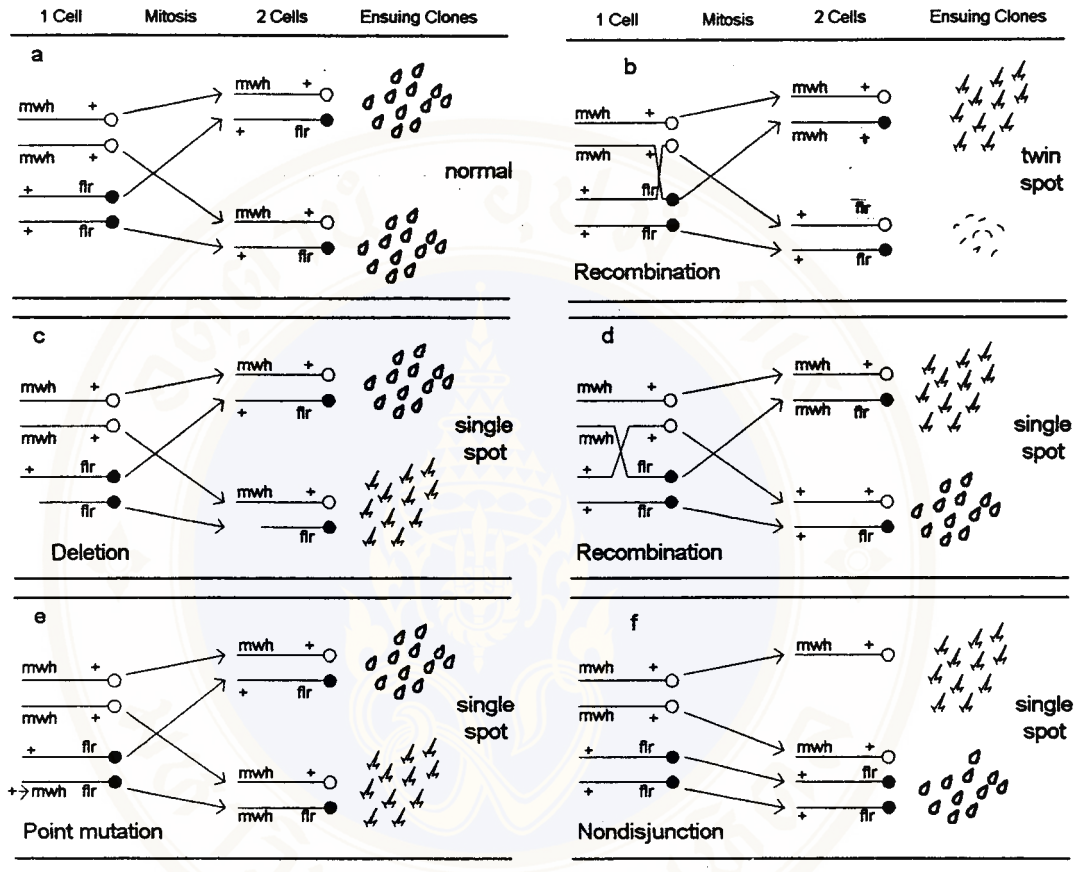

Figure 4 Genetic schemes illustraing various ways of spot formation in the somatic mutation and recombination test with the wing cell markers multiple wing hairs (*mwh*) and flare (*flr*^{3}). Twin spots are obtained by recombination proximal to the *flr*^{3} marker (b), wile more distal recombination produces *mwh* single spots only (d). Deficiencies (c), point mutations (e) and nondisjunction events (f) give rise to *mwh* single spots, or in analogous ways to *flr*^{3} single spots (not illustrated). Only the relevant left arms of chromosome 3 are shown in the panels (62).

receives and recombines and one nonrecombined chromosome (54). A recombination event between *mwh* and *flr*^{3} may result in a mwh single spot. If both types of recombination events (one between *flr*^{3} and the centromere and a second between *mwh* and *flr*^{3}) take place within the same cell, a *flr*^{3} single spot may result.

Selection against cells in one of the homozygous marked subclones in a growing twin spot with exinction of one and the persistence of the other of the two subclones results in a single spot. The same may result from lethal damage to one of the cell lineages in an original twin spot. A *mwh* single spot can also appear in a small twin spot *flr*^{3} is not phenotypically expressed (57).

2.5.3 Approach of SMART

All three crosses produce larvae which are heterozygous for the 2 marker genes *mwh* and *flr*^{3} on chromosome 3 and required for the wing spot test. The following 3 crosses of flies carrying markers on the left arm of third chromosome are:

1. **Standard cross:** *flr*^{3}*/ In (3LR) TM3, ri pp sep bx*^{34e} *es Ser* vergin females mated to *mwh* males. This is the reciprocal cross of the standard cross used previously

2. High bioactivation (HB) cross: *ORR; flr*^{3}*/TM3* females crossed with *ORR; mwh* males. This is the reciprocal cross of the one described by and Würgler (60). A number of promutagens showed increased genotoxicity when the HB cross was used, compared too standard cross (60, 61). However, the HB cross presents a

number of difficulties and disadvantages (60, 61). These are: (1) the presence of an irregular whirling in the pattern of wing hairs making spot classification difficult, especially for inexperienced scorers, (2) an undesirably high variation in results from repeated experiments, (3) the low egg production of the females used and the delay in development of the larvae of HB cross.

3. **Improved HB cross**: *ORR, flr*^{3} */TM3* females crossed with *mwh* males (63). The main advantage of the improved HB cross is to combine the high bioactivation capacity with the ease of scoring the wings using the same criteria as for the standard cross. The hybrid larvae of the improved HB cross show P450-dependent activation capacity equal to or even slightly higher than those of the original HB cross. In addition, the improved HB cross is more sensitive than the standard cross in evaluating the genotoxicity of promutagens (63).

2.5.4 Mutagen Treatment

Acute exposures. The initial idea was to treat young larvae that would develop large, easily recognizable wing spots. On the other hand, older larvae had the advantage that larger numbers of cells in the wing discs are exposed simultancously. To compromised these two reason, 72 h larvae.

All chemicals used for acute feeding can be fed as solutions mixed to powdered cellulouse without addition of yeast. In this way, possible influences of live yeast on the mutagen exposure of the larvac can be avoided. This is particularly important because Callan and Philpot (64) have shown that under certain growth

conditions *Saccharomyces cerevisiae* is capable of activating promutagens such as benzo (a) pyrene.

Some chemicals tested with acute feeding gave, in terms of a developmental analysis of spot formation, easily interpretable results with respect to the size of the induced spots. After an acute exposure of 72 h larvae, the wing disc cells are passing through about six to seven cell divisions until final differentiation occurs at metamorphosis. Those of the spot generating events that take place at the time of exposure mostly lead to large spots (65).

Chronic exposure. Theoretically, different spectra of spot size are expected after chronic feeding as compared with acute exposures. A continuous treatment during 48 h results in a distribution in which the small spots predominate and in/with the larger spots are represented with continuously decreasing frequencies. The imaginal discs are growing and thus increasingly more target cells become exposed, which increases the number of spots initiated in a disc. Yet, the number of cell divisions following a spot initiating event becomes less as development proceeds. This consequence leads to gradually smaller spots (65).

2.5.5 Statistical Consideration

In experiments designed to assess the mutagenicity of a chemical, most often treatment series is compared with a control series. One might like to decide whether the compound used in the treatment should be considered as mutagenic or nonmutagenic. The formulation of 2 alternative hypotheses allows one to distinguish

among the possibilities of a positive, inconclusive, or negative result of an experiment (66).

In the null hypothesis one assumes that there is no difference in the mutation frequency between control and treated series. The rejection of the null hypothesis indicates that the treatment resulted in a statistically increased mutation frequency. The alternative hypothesis postulates a priori that the treatment reaults in an increased mutation frequency compared to the spontaneous frequency.

This alternative hypothesis is rejected if the mutation frequency is significantly lower than the postulated increased frequency. The rejection indicates that the treatment did not produce the increase required to consider the treatment as mutagenic. If neither of the 2 hypotheses is rejected, the results are considered inconclusive as one cannot accept at the same time the 2 mutually exclusive hypotheses. In the practical application of the decision procedure, one defines a specific alternative hypothesis requiring that the mutation frequency in the treated series be m times that in the control series and used together with the null hypothesis. It may happen in this case that both hypotheses have to be rejected. This would mean that the treatment is weakly mutagenic, but leads to a mutation frequency which was significantly lower than m times the control frequency.

Testing against the null hypothesis (Ho) at the level and against the alternative hypothesis (Ha) at the level β led to the error probabilities for each of the possible diagnouse; positive, weak but positive, negative, or inconclusive. The following four decisions were possible; 1) accept both hypotheses; These cannot be true

simultaneously, so no conclusions can be drawn-inconclusive result; 2) accept the first gypothesis and reject the second hypothesis-negative result; 3) reject the first hypothesis and accept the second hypothesis-positive result; 4) reject both hypotheses weak effect.

Because small single spots and total single spots had high spontaneous frequncies, m is fixed at a value of 2 (testing for the exclusion of a doubling of the frequency to define a negative result). For the rare spontaneous large single and twin spots, m=5 is used in accordance with the practice followed with the mouse spot test (67).

2.6 Standard Mutagens for Mutagenicity Evaluation using of SMART

Cyclophosphamide and urethane are generally used as positive standard toxicants in evaluation genotoxicity of the unknown compounds in SMART (68, 69, 70). These two chemicals required metabolic activation to express their mutagenic activity (71, 72).

2.6.1 Cyclophosphamide

Cyclophosphamide is fine, white, odorless or near odorless, crystalline powder with a molecular weight of 279.1 ($C_7H_{15}Cl_2N_2O_2P{\cdot}H_2O$). It is a cyclic derivative of the alkylating agent nornitrogen mustard and consists of a phosphoramide ring linked to a bifunctional moiety containing two chloro-ethyl groups (Figure 5). It is soluble in water and slightly soluble in alcohol, benzene, ethylene glycol, carbon tetrachloride, and dioxane, and sparingly soluble in diethyl

ether and acetone. This drug is widely used as a cancer chemotherapeutic agent and as immunosuppressant. Cyclophosphamide is, however, a known carcinogen in humans and produces secondary tumors. It requires metabolism to produce pharmaco-logically active, but the metabolic activation of cyclophosphamide produces both the therapeutic and the toxic effect. Cyclophosphamide is also implicated recently in producing primary and secondary cancer in humans, including both leukemia and solid tumors.

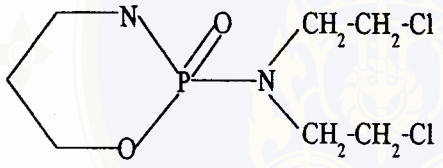

Figure 5 Cyclophosphamide

2.6.1.1 Genotoxic and Carcinogenic Effect of Cyclophosphamide

Cyclophosphamide, in the absence of a metabolic system, fails to bind to DNA. The genotoxic activity of cyclophosphamide results from metabolic activation to the highly reactive metabolite PAM. PAM (Figure 6B) is the major cytotoxic species of cyclophosphamide metabolism tract is responsible for the antineoplastic activity of cyclophosphamide. This metabolite binds to DNA forming labile covalent DNA adducts and intra/interstrand cross-links. Interstrand cross-likes, in particular, have been shown to result in a blockage of DNA replication and are thus widely considered to be responsible for the cytotoxic action of cyclophosphamide (73).

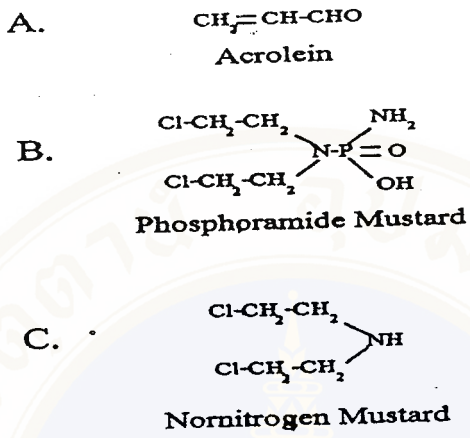

Figure 6 Cytotoxic metabolite of cyclophosphamide metabolism (72).

Other minor cytotoxic product, nornitrogen mustard (Figure 6C), is produced by enzymatic cleavage of the phosphoramide residue from carboxyphosphamide (73, 74). Acrolein (Figure 6A), the last cytotoxic metabolite of cyclophosphamide, is highly toxic. It induced DNA single strand breaks (73, 75), chromosome damage (76) and expressed its teratogenicity in rat (77).

2.6.1.2 Toxicity of Cyclophosphamide

Anderson, *et al.* (72) reviewed that cyclophosphamide was shown to produce gene mutations, chromosome aberrations, micronuclei and sister chromatic exchanges in a variety of cultured cells in the presence of metabolic activation as well as sister chromatic exchanges without metabolic activation. In germ cells,they were shown to induce genetic damage in mice, rat and hamster (72). Cyclophosphamide was shown to induce unscheduled DNA synthesis in a human cell line. It was also a

base-pair substituting mutagen and was shown to produce positive results in *Salmonella typhimurium* strain TA1535 (78, 79) but it was negative in the *E. coli* chromotest. It produced positive responses in *Drosophila melanogaster* for various endpoints (80, 81) and in *Anopheles stephensi* (82). Sufficient evidences for the carcinogenicity of cyclophosphamide in experimental animals (83, 84) and in humans (85, 86) were documented.

2.6.1.3 Mutagenic Activities of Cyclophosphamide in Drosophila

Zijlstra and Vogel (87) described the influence of changes in metabolic activity on the *in vivo* mutagenic effectiveness of cyclophosphamide in *Drosophila melanogaster*. A dose-dependent increase in mutagenicity was observed until a plateau value was reached which was increased only slightly after enzyme induction with Aroclor 1254, whereas induction with phenobarbital resulted in a decrease, especially when cyclophosphamide was applied by injection. In addition, treatment of the adult males with inhibitors of the monoamine oxidase (MAO), such as iproniazid (Ipr), benzimidazole or tryptamine, led to a marked increase of the mutagenic effectiveness of cyclophosphamide especially in spermatocytes. The principal active metabolite of cyclophosphamide, phosphoramide mustard, is extensively de-activated by enzymes that can be inhibited by 1-phenylimidazole, presumably cytochrome P-450, but not by those blocked by MAO inhibitors. The marginal mutagenicity of cyclophosphamide in Drosophila larvae was not increased either by cytochrome P-450 induction with phenobarbital or by MAO inhibition with Ipr.

The protective effects of coffee against somatic mutation and mitotic recombination induced by cyclophosphamide and urethane were evaluated (68, 88).

It was concluded that co-administration and pretreatment of instant coffee was effective in exerting significant dose-related inhibitory effects in the genotoxicity of cyclophosphamide and urethane (68).

2.6.2 Urethane (ethyl carbamate)

Urethane ($NH_2COOCH_2CH_3$), also known as ethyl carbamate, is the ethyl ester of carbamic acid (NH_2COOH). Urethane may occur as a colorless, odorless crystal or a white, granular powder. It is slightly soluble in olive oil and soluble in water, ethane, ether, glycerol, chloroform, and ethyl ether. In the 1940s, urethane was used as a hypnotic in man at doses of 1 g/person/day and as an anaesthetic for laboratory animals. In 1943, it was discovered that urethane had a carcinogenic effect in animals. It is regarded as an initiator, but also as a cocarcinogen and specifically as a promoter of radiation-induced cancer (89). Since 1948 it has been known that urethane is mutagenic in *Drosophila melanogaster*. Today, human is exposed to urethane in their food and mainly alcoholic beverages.

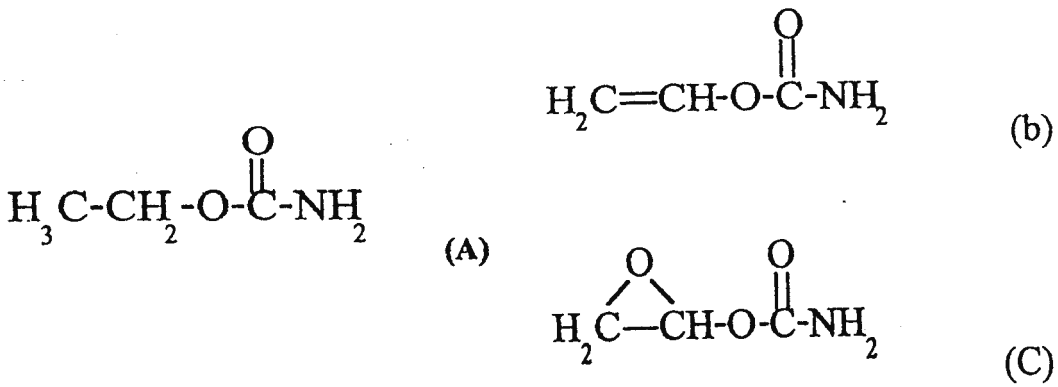

Figure 7 (a) Urethane (ethyl carbamate); (b) Vinyl carbamate; (c) Vinyl carbamate epoxide.

2.6.2.1 Mutagenicity of Urethane

Many studies were published concerning the mutagenicity of urethane in a wide range of organisms (90, 91). In tests with eukaryotic cells, positive and negative findings were about equal in frequency. It that positive result seems to be obtained only under conditions of appropriate metabolic activation. Urethane was genotoxic in the somatic mutation and recombination test in *Drosophila melanogaster* (number and shape of wing hairs after treatment of larvae), in a standard strain and in a strain in which genetic control of cytochrome P-450-dependent enzyme systems were altered (constitutively increased P-450 enzyme activities) (61, 92). The effects were dose-dependent and the modified strain was more sensitive to urethane by about one order of magnitude than the standard strain. This further suggests that the P-450 enzyme system be involved in the activation of urethane. More than 10 publications are available with quantitative data on DNA-adduct formation by urethane. Several authors proposed a metabolic pathway, which leads to the formation of vinyl carbamate and, after epoxidation, to DNA, and RNA adducts (93, 94, 95). Since 7-(2-oxoethyl)-guanine is also formed by vinyl chloride (96). In bacterial test systems the results were mainly negative. An explanation for this may be that in the standard Salmonella/microsome assay, using rat-liver S-9, there was insufficient oxidation of urethane to vinyl carbamate (the first step in the metabolic activation) to give positive results. In fact that vinyl carbamate gave positive results in the Ames test (97) strongly supports this hypothesis. In addition, there is a vast literature on urethane carcinogenicity (98, 99, 100, 101) and urethane is a pluripotent carcinogen with

respect to tumor induction in different species, organs, and the stages of development of the animals (102).

A dose-dependent increase in the genotoxic activity of urethane was observed in SMART (61). The frequency of induction of mutations in the modification strain with increased P-450 enzyme activities was increased by about one order of magnitude compared with the standard strain. The frequencies of spots per wing in high bioactivation cross were higher than those of standard cross (103). This might result from the constitutive expression of the enzymes required for the transformation of urethane into ultimate genotoxic metabolites.

Statement of Thesis Problem

The use of antimutagens in everyday life may be the most effective procedure for preventing human cancer and genetic diseases, and protection against mutations could be beneficial at late, as well as early stage of cancer development. Some epideomiologic studies showed a decreased incidence of colon cancer in people consuming fermented milk products or yogurt, whereas other studies either showed no effect or even pointed to an increased risk. The present study provides an additional information regarding the antimutagenic activities of yogurt as well as the lactic acid bacteria used in yogurt fermentation on standard mutagens using somatic mutation and recombination test.

Experimental Objective

1. To investigate the antimutagenic activity on cyclophosphamide and urethane of two types of yogurt namely, yogurt A (*Streptococcus thermophilus* TUA 196L and *Lactobacillus acidophilus* ATCC 4356) and yogurt B (*Streptococcus thermophilus* TUA 196L and *Lactobacillus bulgaricus* TUA 0963L) using somatic mutation and recombination test.

2. To investigate the antimutagenic activity on cyclophosphamide and urethane of three freezes dried bacterial cells namely, *Streptococcus thermophilus* TUA 196L, *Lactobacillus acidophilus* ATCC 4356 and *Lactobacillus bulgaricus* TUA 0963L using somatic mutation and recombination test.

CHAPTER III

MATERIALS AND METHODS

3.1 Chemicals and Medium

The genotoxic compounds used in this study were cyclophosphamide ($C_7H_{15}Cl_2N_2O_2P$; CAS No.50-18-0) and urethane ($H_2NCOOC_2H_5$, CAS No.51-79-6). They were purchased from Sigma Chemical Co. (Louis, USA). MRS (Man Rogosa Sharpe) (see Appendix A) and M17 (see Appendix B) brought from Merck (Germany). Glycerol was brought from Farmitalia Carlo Erba S.p.A. Arabic powder was purchased from BDH chemical Ltd., (Poole, England). Srichand United Dispensary Co., Ltd., (Thailand) (49, 63, 103), supplied chloral hydrate. Other chemicals were of laboratory grade.

3.2 Bacterial Culture and Manipulation

Lactobacillus acidophilus ATCC4356, *Lactobacillus bulgaricus* TUA093L and *Streptococcus thermophilus* TUA196L were purchased from Thailand Institute of Technology Research (Bangkok, Thailand). The first two cultures were grown in MRS broth. The pH was adjusted with glacial acetic acid (to be 5.4 after sterilization). While the last one was grown aerobically in a M17 broth.

3.2.1 Preparation of Bacterial Cells

One loopful of stock culture (*Lactobacillus acidophilus* ATCC4356 or *Lactobacillus bulgaricus* TUA093L) was inoculated to 10 ml MRS broth and incubated at 37°C for 24 h. *Streptococcus thermophilus* TUA196L was grown in 10 ml M17 broth at 45°C for 24 h. Cells were harvested by centrifugation and then were washed twice with 0.15 M phosphate buffer (pH 7.2) (104). The cells were lyophilized using a freeze-drier (FTB system, Inc., Stoneridge, New York, USA). Samples were stored at 4 °C.

3.3 Preparation of Yogurt

Lactobacillus acidophilus ATCC4356 or *Lactobacillus bulgaricus* TUA093L was inoculated into 1 ml sterilized MRS broth while *Streptococcus thermophilus* TUA196L was inoculated into 1 ml sterilized M17 broth. Active cultures were prepared initially by inoculating a small volume of autoclaved medium and kept at 37°C (except *Streptococcus thermophilus* kept at 45°C) for 24 h. UHT milks was inoculated with starter cultures and incubated at 45°C for 4-6 h (see Figure 8) (21). Yogurt A was prepared by inoculating milk with a mixture of *Lactobacillus acidophilus* ATCC4356 and *Streptococcus thermophilus* TUA196L. Yogurt B was prepared by inoculating milk with a mixture of *Lactobacillus bulgaricus* TUA093L and *Streptococcus thermophilus* TUA196L (see Figure 8).

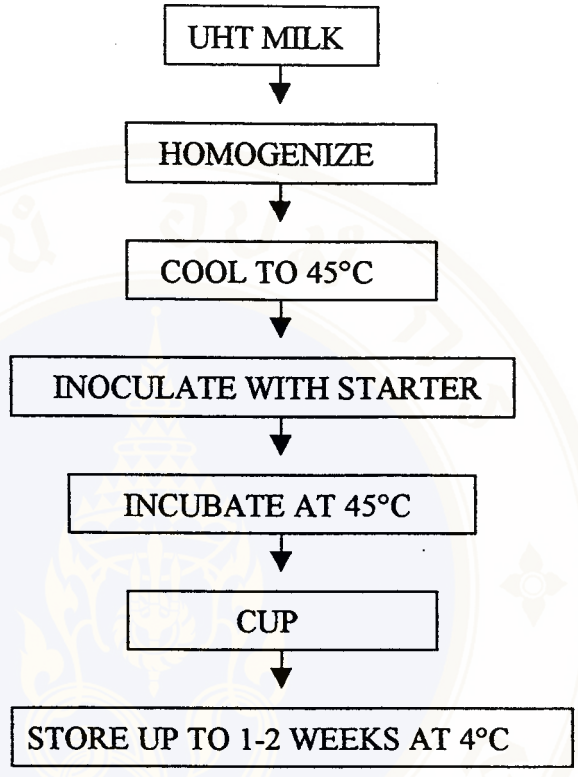

Figure 8 Steps involved in the preparation of yogurt

3.4 Experimental Design

Lyophilized bacterial cells (0.0005, 0.005 or 0.05 mg containing 10^7, 10^8 or 10^9 cells, respectively), freshly prepared yogurt (2 g) or milk was mixed with 2 ml cyclophosphamide (2.791 mg/ml) or 2 ml urethane (4.445 mg/ml). The mixture was incubated at 37°C for 1 h in a shaking water bath (104). Somatic Mutation and Recombination Test in *Drosophila melanogaster* was used to estimate the antimutagenic activity of each mixture. A concurrent run of each sample alone was

also performed as shown in Figure 9. The 3-day-old larvae were transferred in equal batches into glass vials containing 2 g each of Drosophila medium mixed with 2 ml of the sample. Two vials were used for each control or treatment. Both control and treated larvae were allowed to feed on the culture medium until pupation (48 h). The adult flies from the treatment vials were collected on days 7-10 after pupation. The transheterozygous flies were stored in ethanol. Subsequently, the wings were removed, mounted and scored under a compound microscope for recording the three different categories of wing spots (see Figure 9) as described by Graf, *et al.* (49).

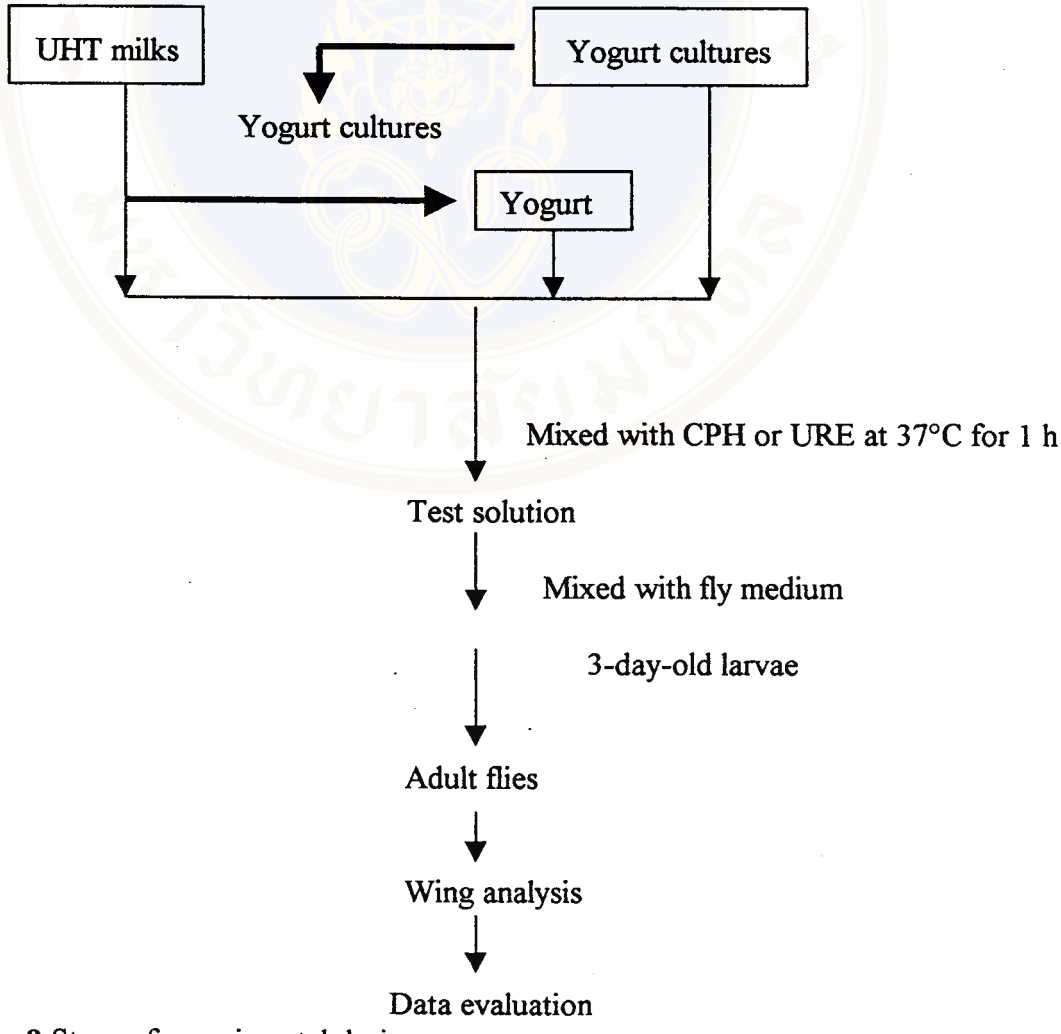

Figure 9 Steps of experimental design

3.5 Somatic Mutation and Recombination Test

The test was carried out as described by Graf, *et al.* (49, 63). Virgin females of the genotype ORR/ORR;*flr*^{3}/ *In (3LR) TM3, ri pp sep bx*^{34e} *e*^{s} *Ser* were allowed to mate with the males of the genotype *mwh/mwh.* After this the females were transferred to new culture bottles containing a fresh medium. Yeast-glucose-agar Drosophila medium was prepared as decribed by Robert (103). Sugar (0.20g), agar (0.028g), yeast (0.10g) and corn flour (0.25g were blended and boiled in a 15 ml test tube containing 2 ml water until became sticky. The inseminated females were allowed to lay eggs for 8 h. Subsequently, these females were removed from the culture bottles. Three days after the end of the eggs laying period, the larvae were collected and transferred to glass vials containing 2 g medium prepared with 2 ml of the treatment solution instead of water. The larvae were fed on this medium until pupation (48 h). Trans-heterozygous adults indicated with round wings from the vials were collected and stored in 70% alcohol. The wings were subsequently removed and mounted on slides in Faure's solutions. The wings were scored under a compound microscope at 400×magnification for recording of the spot (65).

3.5.1 Preparation of Wings

Flies stored in 70% alcohol were first washed in water and then transferred into a drop of Faure's solution. It was blended and boiled in a 100 ml flask containing 50 ml distilled water until became sticky. The wings were first separated from the body and were then, with forceps, lined up on a clean slide, and allowed to spread out. The arranged wings on the slide were kept in a dust-free atmosphere for at least 24 h to become firmly glued to the slide. A droplet of Faure's solution was put on a cover slip,

which was then, with the hanging drop, lowered on top of the wings. The wings (both the dorsal and ventral surface) were analyzed under a compound microscope at 400× magnification. During the microscopic analysis of the wings, the position of the spots was noted according to the sector of wing (see Figure 10). Different types of spots namely, single spots showing either the multiple wing hairs (*mwh*) or the flare (flr^3) phenotype and twin spots showing adjacent *mwh* and flr^3 areas were recorded separately. The size of each spot was determined by counting the number of wing cells (hairs) exhibiting the mutant phenotype. The spots were counted as two spots if they were separated by three or more wide type cell rows. Multiple wing hairs (*mwh*) were classified when a wing cell contained three or more hairs instead of one hair per cell as in wide type (see Figure 11). Flare wing hair exhibited a quite variable expression, ranging from pointed, shortened and thickened hairs to amorphic, sometimes balloon like extrusions of melanolic chitinous material (see Figure 11).

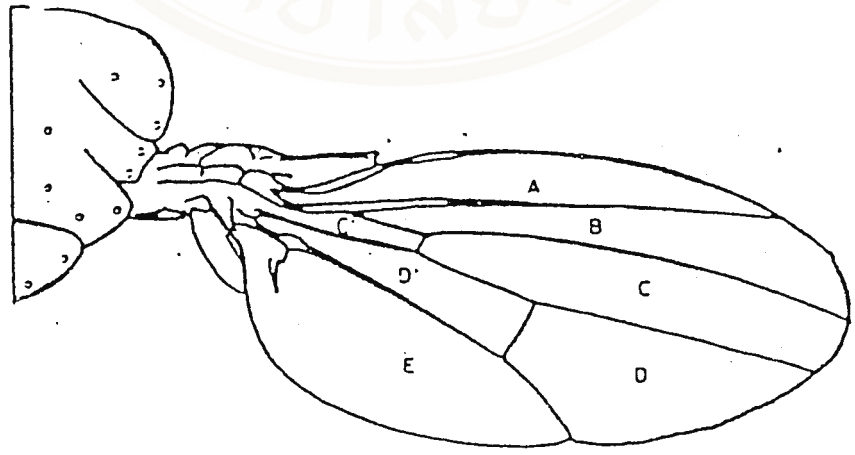

Figure 10 Normal half mesothorax showing the regions A-E of the wing surface scored for spot (49).

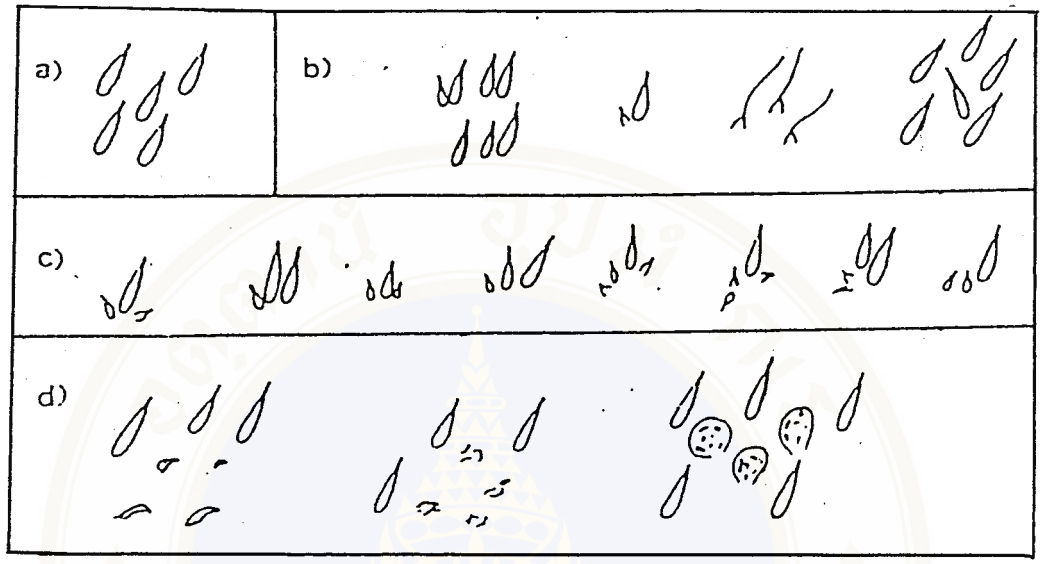

Figure 11 Trichomes on the wing blade a) normal, b) deviate Trichomes not counted as *mwh* or *flr*^{3}, c) configurations indicative of mwh, d) typical manifestations of *flr*^{3} (49).

3.6 Data Evaluation and Statistical Analysis

The wing spots data is evaluated as described by Frei and Würgler (67). Briefly, induction frequencies of wing spots of color treatment groups were compared with those of negative control group (water). The spots were grouped accordingly to the following 3 types: (1) small single spots of 1 or 2 cells in size (2) large single spots of 3 or more cells, and (3) twin spots. The estimation of spot frequencies and confidence limits of the estimated mutation frequency were performed with significance level of $\alpha = \beta = 0.05$. A multiple-decision procedure was used to decide whether a result was positive, weakly positive, inconclusive or negative according to Frei and Wü

rgler (67). Statistical considerations and calculation step by step are shown in Appendix C.

CHAPTER IV

RESULTS

4.1 Role of Milk and Yogurt on Mutagenicity of Toxicants

Cyclophosphamide or CPH (10 mM) and urethane or URE (50 mM) were used as standard mutagens in chronic feeding for 72 h of larvae derived from improved high bioactivation cross. Each of mutagen was incorporated alone and together with milk or yogurt (A or B). The data presented in Table 3 show that the frequencies (2.27-2.40) of total sport per wing induced by CPH are in the normal range as observed previously by Abraham (68, 86). The addition of yogurt A or yogurt B together with cyclophosphamide greatly reduced the frequencies of wing spots induction (more than 90% reduction). The data obtained from the fly received only yogurt were statistically insignificant with respect to that of the water control group. As shown in Table 4, urethane is a very powerful genotoxic. It induced the frequencies of total spots per wings to be higher than 14 at a concentration of 50 mM. Both yogurts A and B showed their antimutagenicity on the toxicants. Percents of inhibition are higher than 94%. The number of total spots per wing induced by each genotoxin in the present of each yogurt a closed to the value of water control but still significantly different.

Feeding cyclophosphamide or urethane in the presence of milk to larva also showed statistically significant reductions on the frequencies of wing spots compared to that of the distilled water group. The antimutagenicity of milk on CPH induced wing spots (approximate 73-74%) is less than that of yogurt A or yogurt B (more than

90%). However, the antimutagenic effect of milk is similar to that of each yogurt when an experiment was done on the mutagenic activity of URE. The data obtained from the studies on both milk and yogurts are shown to be higher than 94% inhibition.

4.2 Role of Lactic Acid Bacteria (LAB) on Mutagenicity of Toxicants

In order to discover whether the LAB used in yogurt production had any activity on the toxicity of both mutagens, separate experiments were done twice for each LAB (0.0005, 0.005 or 0.05 mg per tube). The data presented in Tables 5 and 6 are the results of the simultaneous feeding of *Lactobacillus acidophilus, Lactobacillus bulgaricus* or *Streptococcus thermophilus* on the frequencies of different categories of wing spots induced by cyclophosphamide or urethane. Induced frequencies of total spots per wing of flies treated with 10 mM CPH are 1.92-2.23 and those of flies treated with 50 mM urethane are 11.61-15.21. Statistically significant reductions on the frequencies of wing spots induced by CPH of LAB were again detected (more than 36%). The addition of LAB together with URE reduced the frequencies of wing spots induction (more than 39%). The results showed that each LAB culture exhibited antimutagenic effect on CPH or URE to a lesser extent than that of yogurt or milk.

Table 3 Frequencies of different categories of wing spots induced by 5.56 mg/ tube of cyclophosphamide combined with yogurt A (2 g/ tube) or yogurt B (2 g/ tube).

Treatment	Number of wings	Spots/ wing (number of spots); Statistical diagnosis				Inhibition (%)*
		Small single Spots (1-2 cell) m=2.0	Large single Spots (> 2 cell) m=5.0	Twin spots m=5.0	Total spots m=2.0	
Trial 1.						
H_2O	58	0.10(6)	0.07(4)	0.00(0)	0.17(10)	
Milk	59	0.05(3) I	0.02(1) -	0.00(0)	0.07(4) -	
CPH+H_2O	58	1.86(108) +	0.48(28) +	0.05(3)	2.40(139) +	
CPH+milk	59	0.42(25) +	0.15(9) -	0.03(2)	0.61(36) +	74.55
CPH+A	56	0.04(2) -	0.04(2) -	0.00(0)	0.07(4) -	97.04
CPH+B	59	0.19(11) I	0.03(2) -	0.00(0)	0.22 (13) -	90.82
Trial 2.						
H_2O	59	0.07(4)	0.05(3)	0.00(0)	0.12(7)	
Milk	58	0.05(3) -	0.03(2) -	0.00(0)	0.09(5) -	
CPH+H_2O	55	1.85(102) +	0.38(21) +	0.04(2)	2.27(125) +	
CPH+milk	60	0.50(30) +	0.07(4) -	0.03(2)	0.06(36) +	73.60
CPH+A	58	0.12(7) I	0.00(0)	0.00(0)	0.12(7) -	94.68
CPH+B	58	0.19(11) I	0.02(1) -	0.00(0)	0.21(12) -	90.89

A = yogurt prepared with a mixture of *Streptococcus thermophilus* and *Lactobacillus acidophilus.*

B = yogurt prepared with a mixture of *Streptococcus thermophilus* and *Lactobacillus bulgaricus.*

CPH = Cyclophosphamide.

Statistical diagnosis according to Frei and Würgler (1988) for comparison with distilled water: + = positive; I = inconclusive and - = negative. Confidence limit test, one-sided. Probability levels:$\alpha=\beta=0.05$.

*Percentage of inhibition = $\frac{(a-b) \times 100}{a}$

when a = total spots per wing obtained from standard mutagens, b = total spots per wing obtained from treatment of sample and mutagen.

Table 4 Frequencies of different categories of wing spots induced by 8.89 mg/ tube of urethane combined with yoghurt A (2 g/ tube) or yoghurt B (2 g/ tube).

Treatment	Number of wings	Spots/ wing (number of spots); Statistical diagnosis				Inhibition (%)*
		Small single Spots (1-2 cell) m=2.0	Large single spots (> 2 cell) m=5.0	Twin spots m=5.0	Total spots m=2.0	
Trial 1.						
H_2O	57	0.09(5)	0.05(3)	0.00(0)	0.14(8)	
Milk	60	0.07(4) I	0.02(1) -	0.00(0)	0.08(5) -	
URE+H_2O	55	12.36(680) +	1.91(105) +	0.15(8)	14.42(793) +	
URE +milk	59	0.64(38) +	0.10(6) I	0.08(5)	0.83(49) +	94.24
URE+A	58	0.26(15) +	0.03(20) I	0.00(0)	0.29(17) w	97.97
URE+B	58	0.41(24) +	0.12(7) I	0.03(2)	0.57(33) +	96.05
Trial 2.						
H_2O	53	0.09(5)	0.06(3)	0.00(0)	0.15(8)	
Milk	60	0.12(7) I	0.20(1) -	0.00(0)	0.13(8) -	
URE+H_2O	57	12.44(709) +	1.63(93) +	0.18(10)	14.25(812) +	
URE +milk	59	0.59(35) +	0.15(9) +	0.03(2)	0.78(46) +	94.53
URE+A	58	0.17(10) I	0.07(4) -	0.00(0)	0.24(14) -	98.31
URE+B	60	0.45(237) +	0.12(7) +	0.03(2)	0.60(36) +	95.79

A = yogurt prepared with a mixture of *Streptococcus thermophilus* and *Lactobacillus acidophilus.*

B = yogurt prepared with a mixture of *Streptococcus thermophilus* and *Lactobacillus bulgaricus.*

URE = Urethane.

Statistical diagnosis according to Frei and Würgler (1988) for comparison with distilled water: + = positive; I = inconclusive, w = weak positive and - = negative. Confidence limit test, one-sided. Probability levels:$\alpha=\beta=0.05$.

*Percentage of inhibition = $\frac{(a-b) \times 100}{a}$

when a = total spots per wing obtained from standard mutagens, b = total spots per wing obtained from treatment of sample and mutagen.

Table 5 Frequencies of different categories of wing spots of larva fed on cyclophosphamide (5.58mg/tube) and various amount of lactic acid bacteria.

CPH (mg per tube)	LAB (mg per tube)	Number of wings	Spots/ wing (number of spots); Statistical diagnosis				Inhibition (%)*
			Small single spots (1-2 cell) m=2.0	Large single Spots (> 2 cell) m=5.0	Twin spots m=5.0	Total spots m=2.0	
Lactobacillus acidophilus							
Trial 1.							
0	0	59	0.05(3)	0.02(1)	0.00(0)	0.07(4)	
5.58	0	59	1.61(95) +	0.30(18) +	0.12(7)	2.03(120) +	
5.58	0.0005	59	0.81(48) +	0.22 (13) +	0.03(2)	1.07(63) +	47.49
5.58	0.005	56	0.55(31) +	0.16(9) +	0.02(1)	0.73(41) +	64.01
5.58	0.05	41	0.39(16) +	0.01(4) +	0.00(0)	0.49(20) +	76.01
Trial 2.							
0	0	60	0.07(4)	0.02(1)	0.00(0)	0.08(5)	
5.58	0	58	1.52(88) +	0.53(31) +	0.33(19)	2.23(129) +	
5.58	0.0005	58	0.09(40) +	0.29(17) +	0.09(5)	1.07(62) +	51.93
5.58	0.005	60	0.58(35) +	0.17(10) +	0.02(1)	0.77(46) +	65.51
5.58	0.05	57	0.53(30) +	0.14(8) +	0.02(1)	0.68(39) +	69.24
Lactobacillus bulgaricus							
Trial 1.							
0	0	60	0.07(4)	0.02(1)	0.00(0)	0.08(5)	
5.58	0	58	1.76(102) +	0.38(22) +	0.07(4)	2.21(128) +	
5.58	0.0005	50	1.06(53) +	0.22(11) +	0.04(2)	1.32(66) +	40.19
5.58	0.005	48	0.56(27) +	0.08(4) I	0.02(1)	0.67(32) +	69.64
5.58	0.05	55	0.20(11) +	0.09(5) I	0.04(2)	0.33(18) +	85.18
Trial 2							
0	0	59	0.05(3)	0.03 (2)	0.00(0)	0.08(5)	
5.58	0	60	1.52(91) +	0.37(22) +	0.03(22)	1.92(115) +	
5.58	0.0005	60	1.01(61) +	0.13(8) +	0.07(4)	1.22(73) +	36.88
5.58	0.005	58	0.55(32) +	0.02(1) +	0.02(1)	0.59(34) +	69.43
5.58	0.05	60	0.13(8) +	0.03(8) +	0.03(2)	0.30(18) +	84.35

Table 5 (cont.) Frequencies of different categories of wing spots of larva fed on cyclophosphamide (5.58mg/tube) and various amount of lactic acid bacteria.

CPH (mg per tube)	LAB (mg per tube)	Number of Wings	Spots/ wing (number of spots); Statistical diagnosis				Inhibition (%)*
			Small single spots (1-2 cell) m=2.0	Large single Spots (> 2 cell) m=5.0	Twin spots m=5.0	Total spots m=2.0	
Streptococcus thermophilus							
Trial 1.							
0	0	59	0.10(6)	0.02(1)	0.00(0)	0.12(7)	
5.58	0	59	1.52(90) +	0.46(27) +	0.12(7)	2.10(124)+	
5.58	0.0005	47	1.19(56) +	0.11(5) +	0.00(0)	1.30(61) +	38.25
5.58	0.005	59	0.41(24) +	0.15(9) +	0.07(4)	0.63(37) +	70.17
5.58	0.05	59	0.27(16) +	0.12(6) +	0.00(0)	0.37(22) +	82.25
Trial 2.							
0	0	58	0.09(5)	0.02(1)	0.00(0)	0.10(6)	
5.58	0	60	1.60(96) +	0.35(21) +	0.08(5)	2.03(122)+	
5.58	0.0005	59	0.68(40) +	0.27(16) +	0.03(2)	1.07(63) +	47.39
5.58	0.005	56	0.36(20) +	0.27(15) +	0.05(3)	0.68(38) +	66.60
5.58	0.05	60	0.18(11) W	0.07 (4) W	0.00(0)	0.25(15)w	87.68

CPH = Cyclophosphamide

Statistical diagnosis according to Frei and Würgler (1988) for comparison with distilled water: + = positive; I = inconclusive and w = weak positive. Confidence limit test, one-sided. Probability levels:$\alpha=\beta=0.05$.

*Percentage of inhibition = $\frac{(a - b) \times 100}{a}$

when a = total spots per wing obtained from standard mutagens, b = total spots per wing obtained from treatment of sample and mutagen.

Table 6 Frequencies of different categories of wing spots of larva fed on urethane (8.89 mg/tube) and various amount of lactic acid bacteria.

URE (mg per tube)	LAB (mg per tube)	Number Of Wings	Spots/ wing (number of spots); Statistical diagnosis				Inhibition (%)*
			Small single spots (1-2 cell) m=2.0	Large single Spots (> 2 cell) m=5.0	Twin spots m=5.0	Total spots m=2.0	
Lactobacillus acidophilus							
Trial 1.							
0	0	60	0.03(2)	0.03(2)	0.00(0)	0.07(4)	
8.89	0	57	9.16(522) +	1.86(106) +	0.60(34)	11.61(662) +	
8.89	0.0005	45	5.22(235) +	1.22(55) +	0.33(15)	6.78(305) +	41.62
8.89	0.005	59	2.98(176) +	0.81(48) +	0.00(0)	3.93(232) +	66.61
8.89	0.05	56	2.14(120) +	0.25(14) +	0.00(0)	2.39(134) +	79.40
Trial 2.							
0	0	59	0.08(5)	0.02(1)	0.00(0)	0.10(6)	
8.89	0	55	8.96(493) +	1.96(108) +	0.41(23)	11.34(624) +	
8.89	0.0005	54	4.37(236) +	1.18(64) +	0.31(17)	5.87(317) +	48.24
8.89	0.005	45	2.42(109) +	1.11(50) +	0.18(8)	3.71(167) +	67.28
8.89	0.05	56	1.64(92) +	0.48(27) +	0.00(0)	2.12(119) +	81.26
Lactobacillus bulgaricus							
Trial 1.							
0	0	60	0.08(5)	0.03(2)	0.00(0)	0.12(7)	
8.89	0	59	8.83(521) +	1.91(113) +	0.47(28)	11.39(672) +	
8.89	0.0005	60	3.45(207) +	1.62(97) +	0.50(30)	5.57(334) +	51.12
8.89	0.005	60	2.57(154) +	1.04(62) +	0.17(10)	3.77(226) +	66.93
8.89	0.05	54	1.59(86) +	0.33(18) +	0.18(10)	2.11(114) +	81.47
Trial 2.							
0	0	60	0.05(3)	0.00(0)	0.00(0)	0.05(3)	
8.89	0	57	3.58(204) +	1.88(107)	0.68(39)	13.16(750) +	
8.89	0.0005	59	3.41(201) +	1.54(91)	0.54(32)	5.49(324) +	58.27
8.89	0.005	55	3.05(168) +	1.42(78)	0.38(21)	4.85(267) +	63.11
8.89	0.05	51	2.35(120) +	0.88(45)	0.157(8)	3.39(173) +	74.22

Table 6 (cont.) Frequencies of different categories of wing spots of larva fed on urethane (8.89 mg/tube) and various amount of lactic acid bacteria.

URE (mg per tube)	LAB (mg per tube)	Number of Wings	Spots/ wing (number of spots); Statistical diagnosis				Inhibition (%)*
			Small single spots (1-2 cell) m=2.0	Large single Spots (> 2 cell) m=5.0	Twin spots m=5.0	Total spots m=2.0	
Streptococcus thermophilus							
Trial 1.							
0	0	59	0.07()	0.00(0)	0.00(0)	0.07(4)	
8.89	0	54	9.24(499) +	1.57(85)	0.81(44)	11.63(628)+	
8.89	0.0005	46	4.93(227) +	1.41(65)	0.65(30)	7.00(322) +	39.81
8.89	0.005	57	3.81(217) +	0.91(52)	0.46(26)	5.18(295) +	55.50
8.89	0.05	47	2.11(99) +	0.15(7)	0.06(3)	2.32(109) +	80.06
Trial 2.							
0	0	59	0.05(3)	0.05(3)	0.00(0)	0.10(6)	
8.89	0	57	11.72(668)+	3.03(173) +	0.46(26)	15.21(867)+	
8.89	0.0005	60	6.58(395) +	2.00(120) +	0.30(18)	8.88(533) +	41.62
8.89	0.005	55	5.05(278) +	1.49(82) +	0.27(15)	6.82(375) +	55.17
8.89	0.05	59	2.63(155) +	0.73(43) +	0.02(1)	3.37(199) +	77.84

URE = Urethane

Statistical diagnosis according to Frei and Würgler (1988) for comparison with distilled water: + = positive. Confidence limit test, one-sided. Probability levels:$\alpha=\beta=0.05$.

*Percentage of inhibition = $\frac{(a-b) \times 100}{a}$

when a = total spots per wing obtained from standard mutagens, b = total spots per wing obtained from treatment of sample and mutagen.

CHAPTER V

DISCUSSION

Studies on conditions that enhance or decrease activation or inactivation of environmental mutagens are important to control their risk to man. Fermented milk products and LAB were stated to promote health (8, 21) since the bacteria cells were demonstrated to possess antimutagenicity (104, 105). Consumption of fermented milk products or the lactic acid bacteria (LAB) used in their productions was observed to inhibit growth of certain types of tumors in mice and rats (108, 109). The present report, providing an evidence that yogurt is antimutagenic against the two standard mutagens, is significant because such food is now being favorite in Thailand. Two explanation are proposed for this inhibitory effect.

The first possible explanation may be due to the component of yogurt and cultured milk besides LAB act as quenching substances with hydrophobic pockets that decrease the availability of the genotoxic agents to the imaginal disc cells of larva by scavenging activity. From the study, both milk and yogurt inhibited the mutagenicity of standard mutagens more than 90%. It is suggested that milk components are important in binding the mutagens. The antimutagenicity on 1-methyl-1-nitroso-3-nitroguanidine in the sister chromatid exchange test using Chinese hamster cells (V79) of casein and pepsin hydrolyzed products were demonstrated to protect the cells against the genotoxic compound (110). Van Boekel, *et al.* (109) working on Salmonella/microsome and *Escherichia coli* DNA-repair tests also reported that casein was antimutagenic towards a number of mutagens including benzo[a]pyrene, N-

methylnitrosourea and nitrosated 4-chloroindole, and was less effective towards sodium azide and N-nitroquinoline-1-oxide and that antimutagenicity on sodium azide and NQO increased as a function of pepsin mediated hydrolysis. Antimutagenic activities on heterocyclic amines namely; 3-amino-1, 4-dimethyl-5H-pyrido[4,3-b] indole, 3-amino-1-methyl-5H-pyrido[4,3-b]indole and 2-amino-6-methyldipyrido[1,2-a:3',2'-d]-imidazole of whole casein, α-casein, β-casein, and κ-casein were shown to be varied from 60 to 97% by Yoshida (110).

Moreover, varied ability of anti-urethane and anti-cyclophosphamide activities of yogurts in this experiment suggests that more antimutagenic compounds may be present. Acetone extracts of yogurt (milk fermented by *Lactobacillus bulgaricus* and *Streptococcus thermophilus*) were shown to exhibit dose-dependent antimutagenic activity against a number of direct acting and indirect-acting mutagens (113). Modified lipids in the yogurt sample may also have a role in antimutagenicity of yogurt. The addition of lipase to milk significantly increased its antimutagenicity and that the lipase activity of LAB during milk fermentation resulted in a significant increase in free fatty acids including palmitic acid (113). Nadathur (114) showed that free palmitic acid and isopalmitic acid, in particular which may result from lipolysis of milk, were antimutagenic towards N-Methyl-N'-nitro-N-nitrosoguanidine in the *Samonella typhimurium* mutagenicity assay.

The results showed that each LAB cultures exhibited antimutagenic effect on CPH or URE to a less extent than that of yogurt or milk (more than 36%). Binding of mutagens to cell wall of LAB may be the other explanation that decreased the mutagenicity of both urethane and cyclophosphamide. Since it was found that the incubation of each mutagen with each of LAB before subjected to the SMART

significantly decreased the incidence of all mutant spots. Literature review showed that the cell wall fraction of *Streptococcus faecalis* 12965 was especially effective in suppressing the induction of mutagenic pyrolyzates (37). Zhang and Ohta (104) studied the possible role of LAB as a vital factor in suppressing the process of mutagenesis thus reducing the risk for cancer development. LAB may play a vital role in providing a low risk to the population by removal of mutagens. Ohta and Zhang (104) suggested that the binding activities of LAB were possibly associated with the peptidoglycans and polysaccharides of cell wall. There are many reports on antitumor activity of peptidoglycans isolated from bacterial cell wall skeleton. Ohta and Zhang (11) found that peptidoglycans and polysaccharides isolated from *Streptococcus cremosus* Z-25 bound significantly with Trp-P-2. The peptidoglycan showed stronger binding of Trp-P-2 than cell wall skeleton and polysaccharide indicated that the site of binding of mutagenic pyrolyzates to cells was mainly peptidoglycan of cell wall skeleton. Peptidoglycan liberated from *Rhodococcus lentifragmentus* was reported to show tumor-suppressive activity (115).

Increasing public awareness of the probiotic concept of LAB is being promoted by the commercial addition of LAB to milk products, not to achieve fermentation in the traditional sense, but for the putative health-related benefits (116). The health benefits claimed include well being and stimulation of immune defense. In Thailand, there has been considerable interest in these products. In light of these presumed health benefits, it is expected that consumption of LAB may increase in the near future.

CHAPTER VI

CONCLUSION

Mutagenicity of cyclophosphamide or urethane studied in the wing spot somatic and recombination test was inhibited by the action of yogurt. The antimutagenic effect of yogurts brought the activity of the two mutagens closed to the effect produced in water control group. Subsequent experiments found that freeze-dried cultures of lactic acid bacteria namely; *Lactobacillus acidophilus*, *Lactobacillus bulgaricus* or *Streptococcus thermophilus* as well as the UHT milk used in yogurt production effectively decreased the incidence of wing spots induced such mutagen. The results of this study suggested that 1) both milk and yogurt decreased the availability of the genotoxic agents to the imaginal disc cells of larvae by scavenging activity, 2) binding of mutagens to cell wall of the lactic acid bacteria should decrease the mutagenicity of both standard mutagens.

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APPENDIX A

MRS (Man Rogosa Sharpe)

$MnSO_4$ $4H_2O$	0.05 g
Peptone	10.0 g
Meat extract	5.0 g
Yeast extract	5.0 g
Glucose	20.0 g
K_2HPO_4	2.0 g
Polyoxyethylene sorbitan monooleate	1.0 ml
Di-Ammonoum hydrogen citrate	2.0 g
Sodium acetate $3H_2O$	5.0 g
$MgSO_4$ $7H_2O$	0.1 g

APPENDIX B

M17

Trytone	5.0 g
Soya peptone	5.0 g
Peptone	5.0 g
Yeast extract	2.5 g
Ascorbic acid	0.5 g
$MgSO_4$ $7H_2O$	0.25 g
Disodium-β-glycerophosphate	19.0 g
Lactose	5.0 g

APPENDIX C

Statistical Consideration (65)

In experiments designed to assess the mutagenicity of a chemical, most often a treatment series were compared with a control series. One might like to decide whether the compound used in the treatment should be considered as mutagenic or non-mutagenic. The formulation of 2 alternative hypotheses allowed one to distinguish among the possibilities of a positive, inconclusive, or negative result of an experiment (64).

In the null hypothesis one assumes that there was no difference in the mutation frequency between control and treated series. Rejection of the null hypothesis indicated that the treatment resulted in a statistically increased mutation frequency. The alternative hypothesis postulated a priory that the treatment results in an increased mutation frequency compared to the spontaneous frequency.

The alternative hypothesis was rejected if the mutation frequency was significantly lower than the postulated increased frequency. Rejection indicates that the treatment did not produce the increase requires to consider the treatment as mutagenic. If neither of the 2 hypotheses was rejected, the results were considered inconclusive as one could not accept at the same time the 2 mutually exclusive hypotheses. In the practical application of the decision procedure, one defines a specific alternative hypothesis requiring the mutation frequency in the treated series be *m* times that in the control series and used together with the null hypothesis. It might

happen in this case that both hypotheses had to be rejected. This should mean that the treatment was weakly mutagenic, but led to a mutation frequency which was significantly lower than *m* times the control frequency.

Testing against the null hypothesis (H_O) at the level α and against the alternative a hypothesis (H_A) at the level β led to the error probabilities for each of the possible diagnoses: positive, weakly but positive, negative, or inconclusive. The following four decisions were possible; 1) accept both hypotheses; these can not be true simultaneously, so no conclusions can be drawn--inconclusive result; 2) accept the first hypothesis and reject the second hypothesis--negative result; 3) reject the first hypothesis and accept the second hypothesis--positive result; 4) reject both hypotheses --weak effect.

Calculation step by step

Estimation of spot frequencies and confidence limits of m_e

Particularly in the case that both hypotheses, H_O as well as H_A, had to be rejected, one might be interested in knowing the confidence interval of m_e, i.e., of the estimated multiple by which the mutation frequency in the experimental series was larger than the spontaneous frequency. The estimated value was

$$m_e = \frac{(n_t / n)\, N_c}{(n_c / n)\, N_t}$$

where N_c and N_t represented the respective sample sizes in control and treatment series, n_c and n_t the respective numbers of mutations found, and n the total of mutations in both series together. Exact lower and upper confidence limits p_l and p_u for the proportion n_c/n on one hand, as well as q_l and q_u for the proportion n_t/n on the

other hand, may be determined according to Sachs (117). He gave an easy method to calculate these values using an F-distribution table. To determined q_1 and p_0 one-sidedly at the level α, and q_u and p_1 also one-sidedly at the level β. In this way and in agreement with the foregoing section, a confidence limit $m_1 > 1$ led to rejection of H_o, while a confidence limit $m_u < m$ led to rejection of H_A.

In the first step, F-distribution according to Sachs (118) were used to determine the value $F_{\nu 1, \nu 2}$ at the level $\alpha = 0.05$, where the degrees of freedom ($\nu 1$, $\nu 2$) were given by the equations

$$\nu 1 = 2 (n - nt + 1) \text{ and } \nu 2 = 2n_t$$

In the second step, the F-value so obtained was used to calculate the lower confidence limit (q_1) for the proportion of spots in the experimental series

$$q_1 = n_t / [n_t + (n-n_t + 1 F_{\nu 1,\nu 2}]$$

This gave a lower confidence limit for the frequency of spots per wing in the control which was equal to

$$f_{t,1} = q_1 n/N_t$$

This was the following complementarity, namely that the lower confidence limit for the number of spots in the experimental series ($q_1 n$) plus the upper confidence limit for the number of spots in the experiment ($p_u n$) was equal to the total number of spots (n) found in experimental and control series together, i.e.,

$$P_u n = (1-q_1) n$$

This gave an upper limit for the frequency of spots per wing for the control which is

$$f_{c,u} = q_u n/N_c$$

The lower confidence limit m_1 of the multiple m_e was determined as the ratio between the lower confidence limit for the frequency in the treated series and the upper confidence limit for the frequency in the control, i.e.,

$$m_1 = \frac{f_{t,l}}{f_{c,u}} = \frac{q_1 n/N_t}{p_u n/N_c}$$

Only in the case that m1, the lower confidence limit of me, was larger than 1.0 would reject H_0. Since this was not the case, H_0 remain accepted.

In the same way, the lower confidence limit of the spot frequency may be determined in the control $f_{c,1}$ which will give $f_{t,u}$, the upper confidence limit of the spot frequency in the experimental series. This is also done one-sidedly, at the level β = 0.05. The inverse ratio of these values will provide the upper 5% confidence limit m_u for the multiple m_e.

Again, the F-distrivution according to Sachs () was used and detemined the value $F_{v1, v2}$ at the level $\beta = 0.05$, where the degrees of freedom $(v1, v2)$ were this time given by the equations

$$v1 = 2(n-n_c + 1) \text{ and } v2 = 2\, n_c$$

The F-value so obtained was used to calculate the lower confidence limit (p1) for the proportion of spots in the control

$$P_1 = n_c / [n_c + (n-n_c + 1)\, F_{v1, v2}]$$

This gave a lower confidence limit for the frequency of spots per wing in the control which equal to

$$f_{c,1} = p_1 n/N_c$$

Again, There was complementarily, in that the lower confidence limit for the number of spots in the control (p_1n) plus the upper confidence limit for the number of spots in the experiment (q_un) was equal to the total number of spots (n), so that

$$q_un = (1-p_1)n$$

This gave an upper limit for the frequency of spots per wing for this series, which is

$$f_{t,u} = q_un/N_t$$

The upper confidence limit m_u of the multiple m_e can be determined as the ratio between the upper confidence limit for the frequency in the treated series and the lower confidence limit for the frequency in the control, i.e.,

$$m_u = \frac{f_{t,u}}{f_{c,1}} = \frac{q_un/N_t}{p_1n/N_c}$$

H_A was rejected if m_u, the upper confidence limit of m_e, was less than m (m=2 for the total of all spots and for the small single spots, and m=5 for the large single spots as well as for the twin spots). Substitution of m_e by m_1 or m_u in the above formulas provided the respective exact upper and lower confidence limits for the frequencies estimated.

BIOGRAPHY

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