CHAPTER 1

INTRODUCTION

1.1 Statement of problem

Colon cancer incidence in Thailand has been increasing in recent years (Parkin, 1997). The incidence of colon and rectal cancer is the highest in both males and females in Bangkok, followed by Lampang, Chiang Mai and Khon Kaen, respectively. Most cases of colorectal cancer were diagnosed at an advanced stage, especially in the provinces outside Bangkok (Martin *et al.,* 1999). The overall five-year survival from colorectal cancer is only 30.5% in Chiang Mai (Marin *et al.,* 1998). A report of cancer statistics for the year 1997 of National Cancer Institute indicated the new patients of colon cancer was increased about 50% from year 1996 (National Cancer Institute, 1996,1997). Vogelstein and colleague (1988) reported specific mutations in colorectal cancer and defining their relationship to the adenoma-carcinoma sequence (Vogelstein *et al.,* 1988). This report led to wide acceptance of the multistep hypothesis as the basis for malignant transformation and gave a genetic perspective to the processes of tumor initiation, promotion, and progression. It is estimated that 35% of all cancers may be related to diet (Doll and Peto, 1981). In Thailand, there has been strikingly changing in life style, especially with respect to eating and smoking habits. Ecological and migration studies indicate the importance of environmental factors in colon cancer. Diet appears to have a particularly strong association with occurrence of this cancer (Willett and MacMahon, 1984; Potter, 1992) and thus offers promise for intervention. The risk of developing colorectal cancer appears to be associated with a diet that is low fiber and high in calories, protein and fat. In additional, obesity, secondary life styles, and alcohol consumption have been implicated as potential risk factors (Kinsella, 1993; Potter *et al.,* 1996; Giovannucci *et al.,.*1995). Primary prevention of colon cancer is the identification and elimination of cancer causing environmental agent, but the success with exposure-based prevention strategies is limited. Up to date, the recommendations for colorectal cancer prevention are the modification of diet and life style, medication uses and adenoma screening and removal. Chemoprevention was introduced

into the strategy for colon cancer prevention. It refers to the science of utilization of specific pharmacological agents or nutrients to prevent, delay, or retard the development and progression of cancer (Greenwald, 1994). Prevention trials involve the administration of natural or synthetic substances such as vitamins, minerals and pharmaceutical drugs with anti-carcinogenic activities (Krishnan and Brenner, 1996). A large number of natural chemopreventive substances against colorectal carcinogenesis have been investigated (Chewonarin *et al.*, 1999; Suaeyun *et al.*, 1997; Intiyot, 1999). However, these compounds must be administered at high doses to obtain preventive effects (Suaeyun *et al.*, 1997; Intiyot, 1999). Although some crude extracts from medicinal plants showed inhibitory effect on the initiation stage of colon carcinogenesis, they also exhibit synergistically action on the promotion stage (Chewonarin *et al.,* 1999). Intestinal bacteria contain both pathogenic and non-pathogenic species were mentioned to be the harmful group, which enhanced the colon carcinogenesis processes (Gibson, 1998). They are also considered to be an important factor in colon carcinogenesis, since some might behave as promoters and others as anti-promoter on the development of colon cancer. (Van Tassel *et al.,* 1982; Zarkovic *et al.*, 1993; Ozawa *et al.*, 1983; Suzuki and Mitsuoka, 1987; Kulkarni and Reddy, 1994; Reddy and Revenson, 1993). Decrease of bacteria that produce substances promoting colon carcinogenesis, and increase of beneficial intestinal bacteria, are new proposed strategies for colon cancer prevention. Live microbial food supplements, called probiotics, provide the health benefit of consumers by maintaining or improving their intestinal microbial balance (Fuller, 1985). The use of probiotic bacterial cultures stimulates the growth of preferred micro-organisms, crowds out potentially harmful bacteria, and reinforces the body's natural defense mechanism. Bacterial anticarcinogenic properties are considered to represent one or more of the following types: binding and degradation of procarcinogens, production of antimutagenic compounds, modulation of procarcinogenic enzymes in the gut, and suppression of tumors by an immune response mechanism (Fernandes and Shahani, 1990; Lidbek *et al.*, 1992; Hirayama and Rafter, 1999). However, according to the current criteria, only few of these strains can be considered to represent probiotics such as Bifidobacterium and Lactobacilli. Some intestinal bacteria that acquire new functions by introducing genes for chemopreventive agents are candidates for prevention and treatment of colon cancer. Recently, *Escherichia coli*-producing lycopene, which is red-colored carotenoid mainly found in tomatoes, was successfully constructed (Arimochi *et al.*, 1999).

Lycopene-producing *E. coli* significantly decreased the number and multiplicity of aberrant crypt foci (ACF), intermediate markers for colon carcinogenesis, in the azoxymethane-treated rat colon. However, *E. coli* is not suitable as a host species to express chemopreventive substances since β-glucuronidase of this species is considered to be responsible for colon carcinogenesis (Kulkarni and Reddy, 1994). In addition, *E. coli* has less ability to colonize in the colon. Genus Bacteroides is a better candidate as a host bacterium because members of genus Bacteroides are the most predominant bacterial species in the colon and account for up to 30% of all isolates from feces (Macfarlane and Macfarlane, 1997). They are the most numerous members of the normal flora, representing nearly 10^{11} organisms per gram of feces (dry weight) (Finegold *et al.*, 1983). Gut organisms are involved in numerous metabolic activities in the colon, including fermentation of carbohydrates, utilization of nitrogenous substances, and biotransformation of bile acids and other steroids (Hentges, 1989). It is clear that the anaerobic members of this ecosystem play a fundamental role in the processing of complex molecules into simpler compounds, and through their metabolic activities the human microflora participate in the complex physiology of the host (Bry *et al.*, 1996). Since the Bacteroides occupy a significant position in the normal flora, they also are opportunistic pathogens, primarily in infections of the peritoneal cavity. Moore and Moore (1995) expected that *Bacteroides spp.* were associated with the high risk of colon cancer because consumption of red meat and a high fat-diet is reported to be associated with high risk colon cancer (Draser and Hill, 1974; Finegold, 1977) and fat stimulates bile flow, which in turn specifically stimulates *Bacteroides spp*. Only two *Bacteroides spp.*; *B. vulgatus* and *B. stercoris* showed significant association with high risk as opposed to low risk (Moore and Moore, 1995). Furthermore, *B. uniformis* is a non- or less-pathogenic species among intestinal bacteria. Therefore *B. uniformis* strain BU1001, isolated from human feces, was selected to use as a host for expression human lactoferrin. Then it was subsequently investigated for anti-colon carcinogenesis properties.

Lactoferrin is an 80 kD, iron-binding glycoprotein in milk, plasma and exocrine fluid such as saliva, bile, pancreatic fluid and tear (Lonnerdal and Iyer, 1995). The polypeptide consists of two globular lobes linked by an extended α-helix loop structure that is sensitive to proteolytic attack (Anderson *et al.*, 1987). Human lactoferrin (*hLF*) cDNA has been isolated from a mammary gland cDNA library and amino acid sequences have been deduced from the

nucleotide sequence (Rey *et al.,* 1990). Lactoferrin is known as a primary defense against pathogenic bacteria, fungi, protozoa and viruses (Vorland, 1999). Bezault and colleagues demonstrated that human lactoferrin suppressed growth of tumor cells, MCA4-P5 fibrosarcoma and v-*ras* transformed NIH3T3, and inhibited experimental metastasis of B16-F10 melanoma cells in mice (Bezault *et al.,* 1994). It was shown that the anti-tumor activity of lactoferrin could be explained by modulation of natural killer cell and lymphokine-activated killer (LAK) cell cytotoxicity (Damiens *et al.,* 1998). More recently, Damiens and colleague have demonstrated that lactoferrin inhibits epithelial cell proliferation by blocking the cell cycle progression (Damiens *et al.,* 1999). Therefore, hLF is considered to play an important role in anticarcinogenicity. Recombinant human lactoferrin has been expressed in baby hamster kidney cells (Stowell *et al.,* 1991). It also can be produced on large scale using microorganisms such as Saccharomyces (Liang and Richardson, 1993) and Aspergillus (Ward *et al.,* 1992a). Then, Ward and colleague demonstrated the successful production of large quantities of human lactoferrin using a recombinant *A. awamori* expression system (Ward et *al.,* 1992b). Therefore lactoferrin was initially selected as a beneficial substance to be expressed in *B. uniformis* BU1001.

The hypothesis of this investigation is that recombinant bacteria, which can produce beneficial substances such as human lactoferrin, may be a promising candidate for prevention and treatment of colon cancer, and the lactoferrin-producing *B. uniformis* might behave as beneficial bacteria, instead of their harmful manner, that may reduce the colon cancer incidence.

1.2 Literature review

1.2.1 Colon cancer prevention

The epidemiology of colorectal cancer provides valuable insight into the cause of lower intestinal tumors and the way to prevent them. The most direct method of preventing colorectal cancer is by identification or removal of precursor adenomas. Because colorectal neoplasia is linked to dietary intake of various chemicals with cancer preventing properties in animal studies, an intense effort is also underway to develop effective, safe chemopreventive agents. The ideal chemopreventive agent is simple to administer with extremely low toxicity, as it would likely require almost life long use. A number of promising agents have been identified that modulate basic determinants of carcinogenesis such as cellular activation and differentiation. Some of these

agents, described in the following section, are presently under investigation in both animal and human colon tumor studies.

Evaluating the efficacy of chemopreventive agents is difficult in human because the time required for development of sporadic colorectal cancer is long, in the range 20-40 years. Unlike cancer treatment studies, where the end points of disease-free survival and overall survival are measured in a few years, most chemoprevention analyses must rely upon measurement of intermediate biomarkers for cancer development. The most commonly utilized intermediate biomarkers for colorectal neoplasia are intestinal adenoma formation, aberrant crypt formation, and alteration in colonic epithelial differentiation. It may one day be possible to follow the structure and expression of genes responsible for tumor development as predictors of tumor development and progression.

1.2.1.1 Endoscopic screening and adenoma removal

Over the past several years, there has been a gradual decline in colorectal cancer incidence and mortality, and this trend may at least in part be attributable to endoscopic polypectomy (Winawer *et al.*, 1997). It is now widely accepted that most colorectal carcinomas arise from preexisting adenomas. The sequence from normal mucosa to adenoma to carcinoma provides clinicians with a unique opportunity to prevent colorectal carcinoma by removing its premalignant precursor. A growing body of literature supports the efficacy of polypectomy in reducing colorectal cancer-associated mortality.

In a unique study performed during the pre-endoscopic era, longitudinal follow-up was provided on 226 patients who were discovered to have a polyp during barium studies of the colon (Stryker *et al.*, 1987). In untreated patients, 21 invasive cancers developed at the site of the index polyp after a mean follow-up of 9 years. Additional 11 invasive cancers developed at sites remotely located to the index polyp. Cumulatively, these data reaffirm the adenoma-carcinoma sequence, and highlight the concepts of synchronous and metachronous colorectal neoplasm, the potential importance of neoplasm that arise in the proximal colon, and the potential inadequacies of barium studies to direct early, relatively small tumors within the colon. Just over two-decade ago, Gilbertsen suggested that polypectomy reduced the incidence of colorectal cancer (Gillbertsen, 1974). This study employed rigid sigmoidoscopy to detect and remove distal large

bowel adenomas. As a result, 85% reduction in cancer incidence was noted in the portion of the colon that was examined and cleared of adenomas annually. This observation has been confirmed by numerous case-control studies (Atkin *et al.*, 1992; Selby *et al.*, 1992; Newcomb *et al.*, 1992). The subsequent development of fiber optic and video-endoscopic equipment has had a dramatic impact on the diagnosis and treatment of colorectal adenomas.

1.2.1.2 Chemoprevention of colorectal cancer

Chemoprevention of colorectal cancer is defined as the use of a specific chemical compound to prevent, inhibit, or reverse carcinogenesis. Chemopreventive intervention can occur throughout the long process of carcinogenesis--from its starting point in epithelial cells with normal morphology, to the development of early and advanced adenomatous polyps, and finally to the formation and eventual metastases of invasive adenocarcinoma. Epidemiological and animal studies suggest that a number of vitamins, minerals, and drugs may be protective.

A. Dietary intervention.

Although the exact role of individual dietary constituents in colorectal cancer development is unknown, a large body of cumulative evidence supports some specific cancer-preventing dietary recommendations. A healthy diet should be high in vegetables and fruits, with carbohydrates consumed as whole grains added to diet should be low (approximately 20%-25% of total calories) and added fats should be unhydrogenated forms derived mainly from plants, such as olive oil. Saturated fats, red meats, salt, sugar, processed grain products, and excessive alcohol intake should be avoided. A diet such as this naturally contains many of the micro-ingredients found to inhibit intestinal carcinogenesis.

It is possible that humans can benefit from supplementation of the diet with many of the minor dietary components associated with colorectal cancer prevention in epidemiological and animal studies. Although not proven by prospective, randomized study, there is consistent evidence that adding additional calcium, vitamin D, and folate to the diet may help to prevent colorectal neoplasia. This supplementation should not exceed well-established recommended daily allowances (RDA) for these substances. Increased intake of ω-3 fatty acids, which are present in fish oils and certain vegetable oils, has been shown in clinical studies to decrease

proliferation of colonic epithelium, and may therefore be beneficial as a dietary additive (Singh, *et al.,* 1997). Studies to document the efficacy of these and other agents in colorectal cancer prevention are underway (Table 1.1)

Naturally occurring compounds with chemopreventive properties, such as the plant phenolics, isothiocyanates, indoles, tannin and organosulfur compounds, are consumed in human diets, high content in vegetables and fruits. Many of these natural substances demonstrate antitumor effects in carcinogen- induced animal models of colorectal cancer (Rao *et al.,* 1995, Deschner *et al.,* 1991). These agents exert a variety of activities associated with antitumor effects including inhibition of prostaglandin synthesis, mutagenesis, nitrosation, ornithine decarboxylase activity, and protein kinase C activity. As already mentioned, some dietary compounds may also protect against tumors as a result of natural antioxidant activity. Some plant phenolics are weak phytoestrogens that can bind to estrogen receptors, resulting in a mild antiestrogen effect (Aldercreult, 1990; Serrino and Thompson, 1992). Because these compounds are naturally occurring dietary components, they are potentially bettered tolerated during the long-term administration required of a chemopreventive agent.

B. Chemopreventive medication and colorectal cancer

Some of the most promising colorectal cancer prevention studies involve the administration of NSAIDs or aspirin. The regular use of aspirin or NSAIDs is associated with a decreased incidence of colon cancer in human studies (Waddel and Loughry, 1983; Waddel *et al.,* 1989; Labayle *et al.,* 1991). In patients with FAP, the NSAID sulindac is effective in mediation repression of colorectal adenomas. A prospective, randomized trial is presently underway to evaluate the efficacy of aspirin for prevention of colorectal adenomas in individual that have undergone curative treatment for colorectal cancer. These compounds share the ability to block the activity of cyclooxygenase, an enzyme responsible for conversion of arachidonic acid to prostaglandins. Prostaglandins have been implicated in epithelial carcinogenesis as stimulators of cell growth, inhibitors of apoptosis, and suppressors of immune surveillance. Widespread use of aspirin and NSAIDs for colorectal cancer chemoprevention is hampered by side effects of intestinal upset and increased bleeding tendency. New drugs, specific for inhibition of inducible cyclooxygenase (Cyclooxygenase-2) , may provide effective tumor prevention with reduced side

effects (Oshima *et al.,* 1996; Reddy *et al*, 1996). Several additional drugs are under investigation for their ability to mediate anti-tumor effects in animal models (Table 1.2). These agents demonstrate chemopreventive activity in animal tumor models but have yet to be tested in human studies.

Many compounds, both synthetic and diet derived, exhibit antitumor activity in cell line and animal models of colorectal carcinogenesis. Although most potential chemopreventive agents await study in human trials, some of them, such as dietary fiber, calcium, vitamin D and the anti-inflammatory drugs, show promise in limited human tumor studies. Table 1.3 summarizes the current recommendations for colorectal cancer prevention. In the future, the ability to better characterize both genetic and environmental determinants of colorectal cancer will undoubtedly lead to more effective methods of tumor prevention.

Table 1.1 Possible agents of colorectal cancer prevention: Dietary supplement

Dietary additive	Antitumor effect
High Fiber: wheat barn	Decrease fecal diacylglycerol (Reddy *et al.*, 1994) decrease protein kinase C activation (Albert *et al.*, 1992)
Calcium	Bind bile salts; direct antiproliferative effect on erythrocyte (Buset *et al.*, 1986; Appleton *et al.*, 1991)
Vitamin D	Normalized differentiation in colonic epithelium (Shabahang *et al.*, 1993)
Folic acid	Correct DNA methylation imbalances (Giovannucci *et al.*, 1995)
Selenium	Antioxidant activity (Robinson *et al.*, 1979)
Allyl sulfides, Isothiocyanate, Indoles	Induced glutathione-S-transferase and other detoxifying enzymes (Zhang and Talalay, 1994)
Vitamin C Vitamin E β-carotene, flavonoids	Scavenge oxygen radical, preventing DNA damage (Ames *et al.*, 1995)
Inositol, phytic acid	Modulate transmenbrane signaling (Salamoto *et al.*, 1993)
Caffeic acid, other plant phenolic	Inhibit nitrosation to form carcinogenic nitrosamines, antioxidants *in vitro*; reduce arachidonate metabolism (Newmark, 1984 and Rao *et al.*, 1993a)
ω-3 fatty acid	Reduce arachidonate metabolism, thereby reducing prostaglandin activity (Singh *et al.*, 1997; Anti *et al.*, 1992)
Conjugated linoleic acid	Alter membrane phospholipids, decrease enterocyte proliferation; induce epithelial differentiation (Belury, 1995)

Table 1.2 Possible agents of colorectal cancer: Chemoprevention drugs

Chemopreventive drugs	Anti-tumor effect
Sulindac and related nonsteroids anti-inflammatory drugs	Block prostaglandin activity, induce apoptosis (Waddel and Loughry, 1983; Waddel *et al.*, 1989; Labayle *et al., 1991)*
Aspirin	Block prostaglandin activity (Reddy *et al.*, 1993)
Specific cyclooxygenase-2 Inhibitors	Block arm of prostaglandin pathway responsible for cellular activation (Oshima *et al.*,1996; Reddy *et al*, 1996)
DFMC (difluoromethylcarnitine)	Inhibit ornitine decarcoxylase (Love *et al.*, 1993)
N-acetyl-L-cysteine	Increase DNA repair capability (Kennedy *et al.*, 1996)
Protease inhibitors	
Oltipraz	Induce glutathione-S-transferase and other detoxification enzyme (Rao *et al.*, 1993)

Table 1.3 Recommendation for colorectal cancer prevention

Prevention method	Recommendation
Dietary modification	20%-25% of calories as fat; Increase cereal bran fiber, fruits and vegetable
Life style modification	moderate, regular exercise; maintain ideal body weight low alcohol intake
Medication use	regular low dose aspirin use; supplementation with calcium and vitamin D
Adenoma screening and removal	Endoscopic examination beginning at age 50 with polypectomy if indicated; increase screening for those with identified genetic risk

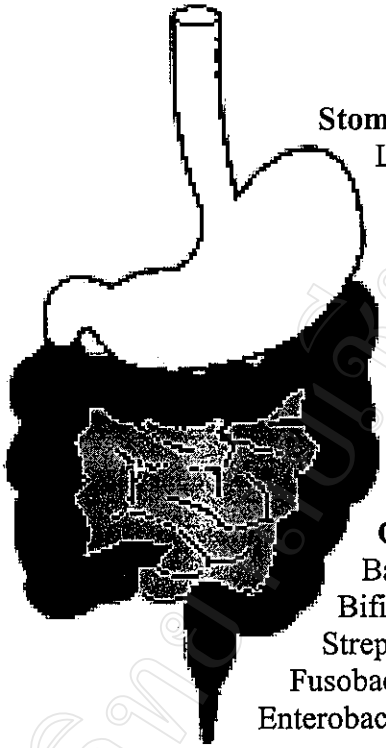
1.2.2 Roles of intestinal flora in the colon carcinogenesis

1.2.2.1 Microbial numbers and the ecosystem

It is estimated that the colon of healthy adults harbors about 300-400 different cultivable species belonging to more than 190 genera. A significant additional proportion of the colon microflora is, however, not cultivable by existing techniques. Among the known colonic microbial flora only a few major groups (Gedek, 1993) dominate at levels around 10^{10}-10^{11} g^{-1}, all of which are strict anaerobes such as Bacteroides, Eubacterium, Bifidobacterium and Peptostreptococcus (Figure 1.1). Facultative aerobes are considered to belong to the sub-dominant (intermediate) flora or `satellite flora' (Gedek, 1993), constituting Enterobacteriaceae, streptococci and lactobacilli.

Compared to the colonic flora, the fecal flora undergoes distinct quantitative variations and seems to be a good qualitative indicator of the distal colonic microflora. It does not reflect, however, the intestinal flora and most definitely not those of the small intestine. Furthermore, our current knowledge on the stability of the strains, species and even genera relationships is still extremely limited. While stability in species composition may be a feature of the `normal' microflora, stability of bacterial strains within the population may be less common (McCartney *et al.,* 1996). Genetic fingerprinting techniques indicated the presence of a collection of Bifidobacterium and Lactobacillus strains "unique of each human" (Tannock, 1997). It was also suggested that the composition of these populations may remain relatively constant for some individuals and may fluctuate considerably for others.

Minor groups of pathogenic and opportunistic organisms, the so-called `residual flora' according to Gedek (1993), are always present in low numbers (Gedek, 1993). In a healthy state, the quantity even of toxic metabolites seems insufficient to act detrimentally on the host. Ducluzeau (1989) proposed the action on the host to be considerable only when the number of particular bacteria exceeds 5×10^{7} g^{-1} (Ducluzeau, 1989). This may be the case for the colon, but lower numbers may be required to dominate in other regions and to promote significant pharmacokinetic interactions, e.g. in the jejunum. In these regions the concentration of metabolites is host mediated by active reabsorption pathways.



Stomach and Duodenum (10^1-10^3 CFU/ml)
Lactobacilli
Streptococci
Yeasts

Jejunum and Ilium (10^4-10^8 CFU/ml)
Lactobacilli Bacteroides
Enterobacteriaceae Bifidobacteria
Streptococci Fusobacteria

Colon (10^{10}-10^{12} CFU/ml)
Bacteroides Clostridia Psudomonas
Bifidobacteria Veillonella Yeast
Streptococci Lactobacilli Protozoa
Fusobacteria Proteus
Enterobacteriaceae Staphilococci

Figure 1.1. Microbial colonization of the human gastro-intestinal tract (modified after Simon and Gorbach, 1982).

1.2.2.2. Intestinal flora and health defects

It may be possible to categorize the gut microbiota components on the basis of whether they exert potentially pathogenic and health promoting aspects (Figure 1.2). By a number of physiological functions the intestinal flora contribute to overall health. Disturbance of the ecological balance in the gastro-intestinal system may therefore be detrimental to health. Bacteria typical of the 'normal' intestinal flora may possess a range of beneficial features, and are (e.g.) able to degrade certain food components, produce certain B vitamins, stimulate the immune system and produce digestive and protective enzymes. The normal flora is also involved in the metabolism of some potentially carcinogenic substances and may play a role in drug efficacy. These effects can be either beneficial or detrimental to health. Furthermore, the colon mucosa is dependent on short chain fatty acids (SCFA) produced by the colonic microflora. Products of polysaccharide metabolism, SCFA, are passively absorbed by the enterocytes (Hoverstad, 1989). Roediger (1982) estimated that 40-50% of the required energy has to be provided by the colonic microflora, suggesting one form of established mutualism in a region where SCFA are also partially responsible for bowel motility and circulation (Roediger, 1982; Kvietys and Granger, 1981). The influences of intestinal bacteria on the metabolism of potential carconogenic substances are as folllows;

A. Bacterial enzymes

Several bacterial enzymes have been implicated in the generation of mutagen carcinogens, and various tumor promoters: β-glucuronidase, β-glucosidase, β-galactosidase, nitroreductase, azoreductase, 7α-steroid dehydrogenase, and 7α-hydroxysteroid dehydrogenase (Goldin and Gorbach, 1976; Goldin *et al.*, 1980; Wilkins and Van Tassell, 1983)

The carcinogen potential of bacteria enzymes in the intestinal microflora has been illustrated in a series of studies involving experimental colon cancer induced by cycasin. Cycasin is a naturally occurring β-glucoside of methoxylazoxymethanol, extractable from the seeds and roots of cycad plants. Laqueur and Spatz (1975) discovered that cycasin fed to infant rats caused hepatomas, renal sarcomas, squamous cell carcinomas of the tear duct, and most frequently

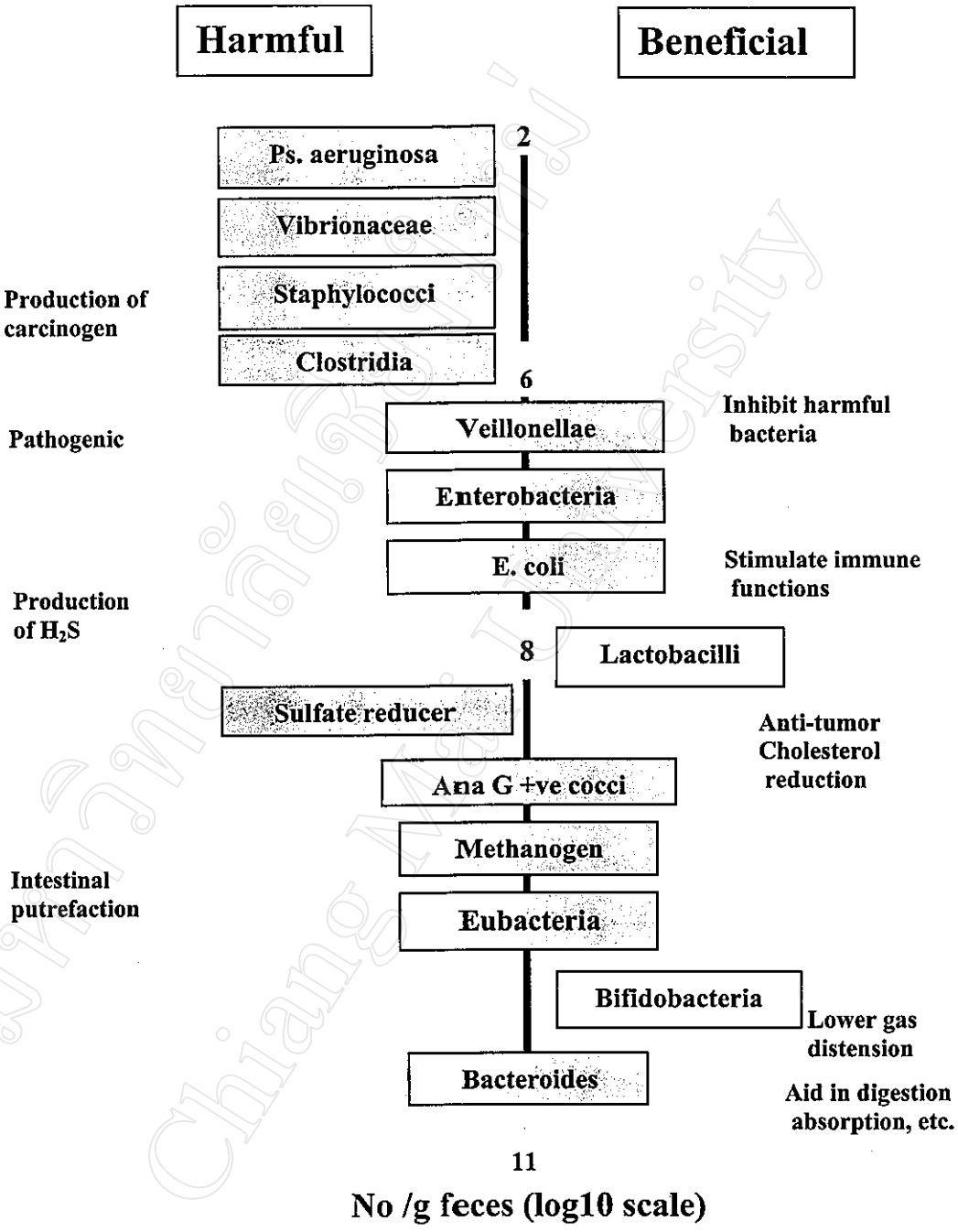


Figure 1.2 Predominant colonic micro-organism catagorized into potentially harmful or health-promoting groups (Modified from Gibson and Roberfroid, 1995)

intestinal adenocarcinomas that were almost exclusively located in the large bowel (Laqueur and Spatz, 1975). The genetic strain of the rat has a little influence on the carcinogenic effect of cycasin; similar tumors were induced in Osborne-Mendel, Sprague-Dawley, Fisher and Wistar rats. It was also noted that the intestinal flora was required for the carcinogenic activity of cycasin, since the compound was completely inactive when given orally to germ free rats (Laqueur *et al.*, 1967)

A tissue (host) or bacterial (microflora) β-glucosidase is required to hydrolyze the glycolytic bond in cycasin in order to release the active aglycone, methyl azoxymethanol (Laqueur and Spatz 1975). The obsevation that subcutaneous or intraperitoneal injection of cycasin caused tumors in infant rats but not in older rats supports the view that young animals have a tissue β-glucosidase that disappeared by the third week of life. The discovery of carcinogenicity of cycasin led Drukery (1967) to test the precusors axozymethane, azomethane and dimethylhydrazine (Drukery *et al.*, 1967). These three compounds were carcinogenic in conventional and germfree animals. The route of administration was not critical; tumors developed after oral or subcutaneous administration (Laqueur and Spatz , 1975).

Bacterial β-glucuronidase seems to be important in colon carcinogenesis. β-glucuronidase has wide substrate specificity and consequently can hydrolyze many different glucuronides. These reactions are potentially important in the generation of carcinogenic and toxic substances in as much as many compounds are detoxified by glucuronide formation in the liver and subsequently enter the bowel via the bile. Deconjugation in the intestine then generates the carcinogenic or toxic compound. Several studies have shown that intestinal β-glucuronidase can alter or amplify the biological activity of exogeneous and endogeneous compounds. For example, toxic aglycones can be regenerated in situ in the bowel by bacterial β-glucuronidase. Morotomi and colleague (1985) reported that cell free extracts of some strains of intestinal bacteria (including *B. fragillus*, *B.vulgatus, B thetaiotaomicron, Eubacterium eligens, Peptostreptococcus, and E. coli*) enhanced the mutagenicity of bile from rats given 1-nitropyrene via stomach tube (Morotomi *et al.*, 1985). These bacterial cell free extracts hydrolyzed the synthetic p-D-glucuronide of phenolphthalein and/or p-nitrophenol. Cell free extracts of bacteria incapable of increasing mutagenicity did not hydrolyze the glucuronides. These data indicate that the

glucuronides of 1-nitropyrene metabolites secreted in to bile can be hydrolyzed in the intestine by bacterial β-glucuronidases to form a potent glycone.

B. Nitrosamine

Bacteria have also been implicated in the formation of N-nitroso compounds. Nitrite is produced through the reduction of nitrate by many common microbial species. Certain leafly vegetable may accumulate high concentration of nitrate, which can be converted to nitrite by bacteria during storage. Tannenbaum and colleague (1974) reported that oral microbial flora of human can reduce nitrate to nitrite levels in saliva as high as 1-10 ppm (Tannenbaum *et al.*, 1974). Klubes and colleague (1972) reported that dimethylamine and sodium nitrite, when incubated with rat intestinal bacteria under anaerobic conditions at pH 7.0, gave rise to dimethyl nitrosamine (Klubes *et al.*, 1972). The formation was enhanced by the presence of riboflavin. The major implication of these findings is that nitrosamine could possibly be generated in the intestine, where the pH is nearly neutral and where the reaction could be expected to occur nonenzymatically at a very low rate. This reaction could be important because secondary amines can come from many dietary sources and nitrates are present as food additives. Although rapid transit in the stomach may prevent nonenzymatic nitrosamine formation to some extent, the bacterially catalyzed reaction in the large bowel could lead to significant *N*-nitroso formation.

C. Amino acid

Tyrosine and tryptophan can be convert to toxins and carcinogens by microflora. Tryptophanase catalyzes the conversion of tryptophan to the known carcinogen indole. Chung *et al.* (1975) showed that *B. thetaiotaomicron*, an organism commonly found in the intestine, has high level of tryptophanase when grown in the presence of tryptophan (Chung *et al.*, 1975). Rats fed a high-meat diet have higher levels of tryptophanase than animals maintained on grain diet. Tyrosine is converted to phenol by aerobic intestinal organisms and to p-cresol by intestinal anaerobes. Both of these metabolites are absent from the urine of germfree mice and are present in the urine from conventional mice. Phenol and cresol are tumor promoters in a mouse cancer model. Their role in etiology of colon cancer is not known.

D. Bacterial production of fecal mutagen

The presence of mutagenicity in ether extract of freeze-dried feces was first described in 1977 by Bruce and colleagues (Bruce *et al.,* 1977). The mutagen is very stable under strictly anaerobic conditions. The amount of mutagenic activity increases dramatically if a fecal specimen already containing mutagen is incubated anaerobically at 37 °C for several days. Mutagen production is inhibited by exposure of the feces to oxygen, low temperature, autoclaving, or radiation (Lederman *et al.,* 1980). Van Tassel and colleaguee (1982a), then was demonstrated that a precursor was present in feces containing mutagen (Van Tassel *et al.,* 1982a). *In vitro* studies, when this precursor was added to bacteriological media containing bile, incubated with fresh feces, and incubated anaerobically at 37 °C, mutagen was produced. Studies on pure cultures revealed that five species of Bacteroides (*B. fragilis, B. ovatus, B. uniformis, B. thetaiotaomicron,* and *Bacteroides* strain 3452A) produced the mutagen. These strains are commonly found in human feces. Fermentable carbohydrates such as glucose, starch, or dextran inhibited the *in vitro* production of mutagen. Some 40 other species of intestinal anaerobes tested for production of the mutagen all proved negative (Van Tassel *et al.,* 1982). The fact that *Bacteroides spp.* are common in the human colon and that only a relatively small percentage of individual produce the mutagen indicate that the precursor is the determining factor in its production. This was confirmed by demonstration that bacteria from feces that did not have mutagen were capable of producing it in the presence of the precursor compound. The precursor is either the product of other bacteria in colon, a result of the diet, or a metabolite derived from the host. It is necessary, in combination with *Bacteroides spp.,* for mutagen production. It has subsequently been demonstrated that cell-free extract of Bacteroides in the presence of bile could produce the mutagen. The bacterial enzyme system is not oxygen sensitive; however, the mutagen is only produced anaerobically (Wilkins *et al.,* 1981). The structure of fecal mutagen was first announced in 1982 (Harai *et al.,* 1982), and was confirmed independently the following year (Gupta, *et al.,* 1983). The characteristic UV spectra and shift on oxidation implied that the compound was a pentane. Chemical ionization, mass spectrometry, and NMR analysis elucidated the structure of the compound, called (S)-3-(1,3,5,7,9-dodecapentaenyloxy)-1,2-propanediol.

The role of the gut flora as a barrier against pathogenic and opportunistic microorganisms is surprisingly effective, considering the large amounts of allochronous ('non-resident') bacteria

entering the gastrointestinal tract. Most of them have no chance to establish within the system. One approach to understanding aspects of microbial interactions related to *in situ* barrier effects was to study restoration effects in simplified two-strain models, using a continuous flow system (Du Toit, *et al.,* 1998a). It could be shown that the probiotic *Enterococcus faecium* SF68 promoted restoration of sublethally damaged *Lactobacillus reuteri*, a representative of the autochthonous gut flora. More complex *in vitro* models and *in vivo* experiments need to be conducted for further elucidating the complex microbial interactions and host dependent factors involved in restoration.

1.2.3 Biomarkers for colon cancer evaluation

The ultimate proof of efficacy of a putative chemopreventive agent is derived from a randomized trial comparing the incidence of cancer in a group of treated subjects with a matched control group who are treated with a placebo. Because cancer is an uncommon event and the duration of time until sufficient subjects develop cancer is long, it is not feasible to perform such an observational trial in the many potential chemopreventive compounds that are becoming available for clinical trial. Modulation of earlier biochemical, cytogenetic, proliferative, immunologic, or differentiation markers of premalignant cells from individuals at risk reduces the size of population and time required for study. Such studies with many putative chemoprevention agents now become feasible (Boone and Kelloff, 1993; Lipkin, 1994; Lipkin *et al.,* 1992; Lippman *et al.,* 1990).

Biomarker is a biomolecule for the targets of assays that monitor critical aspects of the relevant tumor biology in a defined tumor system. Abnormalities of cell proliferation, differentiation, and gene structure or expression have been identified that appear to enhance the susceptibility to cancer development (Lipkin *et al.,* 1992). Measurements of these alterations are called *intermediate endpoints biomarkers* and have the potential use as markers of premalignant change. A SEB is defined as a measurable and modulatable biologic or chemical property that is highly correlated with cancer incidence and that may serve as an indicator of the likely progression to cancer (Boone and Kelloff, 1993). An ideal intermediate biologic endpoint for an at-risk population must be readily expressed in tissues that are accessible to biopsy or in the plasma, is related in some way to the process of neoplastic transformation, must be easily

measured from small quantities of tissue, should be quantifiable as a continuous variable, and must be modulated by a chemopreventive intervention (Lippman *et al.,* 1990). Modulation of such markers may serve as surrogate indicators of drug effect and may be used to follow and document the response to a putative agent in a trial. Use of biomarkers as endpoints in chemoprevention trials may permit completion of a clinical trial in a shorter period of time.

Potential biomarkers include genetic markers (e.g., nuclear aberrations such as micronuclei and gene amplifications or mutations) (Sidrabsky *et al.,* 1992), epigenetic markers (e.g., cellular markers of proliferation and differentiation) (Yamada *et al.,* 1992), histologic surrogates (e.g., leukoplakia, colon polyps, premalignant lesions such as aberrant crypt foci) (Bird, 1987; Boone and Kelloff, 1993; Petrow *et al.,* 1992), and biochemical markers (e.g., ornithine decarboxylase activity, cyclooxygenase activity, and prostaglandin levels) (Heckbert *et al.,* 1992; Karmali, 1980;Kraus *et al.,* 1994; Ruffin *et al.,* 1994).

1.2.3.1 Animal models of chemical carcinogenesis

Models of carcinogenesis arise primarily from studies of cancer induction or its modulation in experimental animal systems. The Chemoprevention Branch of the National Cancer Institute has developed the following model to test putative chemopreventive agents for colon cancer. 1,2-Dimethylhydrazine (1,2-DMH) when administered intraperitoneally produces adenocarcinomas of the colon in both rats and mice. The compound is activated initially to azoxymethane (AOM) and then to methoxymethane (MAM). Low –dose and high-dose animal cancer models are used. In the rat model, a single subcutaneous dose of AOM administered to 7-week-old Fisher 344 male rats produces adenomas and adenocarcinomas in 40 weeks. In female CF_1 mice, MAM acetate administered intraperitoneally four times in 11 days (low dose) and eight times in 22 days (high dose) produces colonic tumors in 38 weeks (Reddy and Maeura, 1984a, 1984b).

In rodents treated with chemical carcinogens, increased epithelial cell production and increased rate and size of the proliferative compartment are early changes. Uptake of tritiated thymidine or bromodeoxyuridine and expression of the proliferating nuclear cell antigen (PCNA) have been used as potential biomarkers in such rodent models (Craven *et al.,* 1988). Observations in the 1970s suggest that aspirin and other nonsteroidal antiinflammatory drugs (NSAIDs) may

inhibit carcinogenesis when higher concentrations of prostaglandin (PGE_2) were detected in human tumors of the colon, lung, and breast compared to normal tissue (Craven and DeRubertis, 1992). Several animal experiments in the 1980s showed definite evidence that NSAIDs suppressed chemically induced colon carcinogenesis in the rat (Moorghen *et al.,* 1990; Polland and Luckert, 1981a, 1981b; Polland *et al.*, 1983; Reddy *et al.,* 1993).

1.2.3.2 Aberrant crypt focus in pathogenesis of colon cancer

Aberrant crypt foci (ACF) were first identified in the colon of carcinogen treated rodents by a simple methodological approach (Bird, 1995). The method involved the microscopic examination of the mucosal surface of whole rodent colons that had been stained with methylene blue. Examination of carcinogen treated rodent colon revealed the presence of a single, or cluster of, morphologically altered crypt(s). These crypts termed aberrant crypts were present only in carcinogen treated rodent colon. It was hypothesized that aberrant crypts represented preneoplastic lesion. Thus, their number and growth features could be used to identify modulators of colon carcinogenesis, to identify the underlying cellular and molecular event leading to tumor development and to quantify the stepwise development of colon cancer. Topographic examination of colonic mucosa harboring adenomas and the surrounding tissue has demonstrated the presence of ACF exhibiting morphologic and growth heterogeneity in close vicinity to adenomas and adenocarcinomas. Occasionally, an adenoma is seen in direct association with an ACF.

The methodological approach that led to the identification of ACF was based on the promise that preneoplastic changes occur in single crypts. The changes must accompany aberrant growth and instability within the crypts, leading to alter crypt morphology as shown in Figure 1.3. This includes changes in crypt width, height and presumably the thickness of the cell wall lining in the crypt. It was reasoned that early changes affecting the luminal opening and epithelial lining of the crypts could be identified topographically, and ACF with increasing crypt multiplicity are more resistant to apoptotic cell death induced by AOM (Magnuson *et al.,* 1994). This information supports the notion that ACF are preneoplastic lesions and provides a basis for further studies pertinent to understanding the pathogenesis of colon cancer.

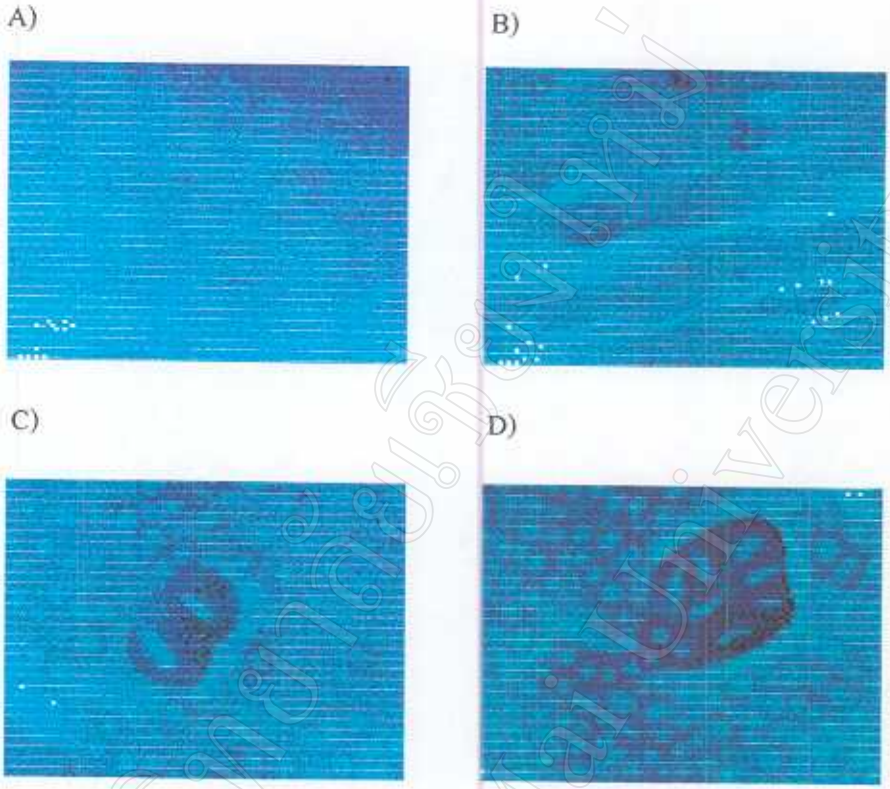


Figure 1.3 Aberrant crypt foci in rat colon stained with 0.2% methylene blue A) Normal crypt (10X) B) aberrant crypt foci (10X) C) 2 crypt/focus(40X). D) multiple crypt, magnetude 40X.

1.2.3.3 Aberrant crypt foci in the human colon

Following the observation of ACF in rodent colons it was reasonable to suggest that if ACF are preneoplastic lesions, similar lesions should be seen in human colons (Bird, 1987). Subsequently, two laboratories reported the presence of ACF-like lesions in human colons associated with high risk of developing colonic cancer (Pretlow *et al.,* 1991; Pretlow *et al.,* 1992; Roncucci *et al.*, 1991, 1994; Roncucci, 1992). The lesion exhibited morphologic atypia as reported for those present in the rat colons. Recently, it has been demonstrated that ACF harbor K-*ras* and APC mutation (Jen *et al.*, 1994; Petrow *et al.,* 1993; Smith *et al.,* 1994). Jen and colleagues examined the occurrence of K-*ras* and APC mutations of ACF and adenomas from patients with spontaneous colorectal adenocarcinomas (Jen *et al.,* 1994). It was demonstrated that K-ras mutation were present in 19 of 20 ACF examined. The only ACF which exhibited dysplasia lacked K-ras mutations but had APC mutations. Adenomas with dysplasia had K-ras and APC mutations. This finding alluded to the fact that dysplasia and abnormal growth are independently regulated. Smith and colleagues examined ACF for APC and K-ras mutations, and found that 13% of total ACF had K-ras codon 12 mutations and 4.6% had APC mutations (Smith *et al.,* 1994). These studies provided evidence those earliest detectable genetic changes occurring during colon tumorigenesis was also present in a population of ACF. This further supports the notion that some ACF are precursor lesions of colon cancer. It has been proposed that ACF are preneoplastic lesions in rat colons and in human colons. The preceding discussion on the induction specificity, growth, morphological and genotype features of ACF generally support the notion that ACF are preneoplastic lesions. Key features of ACF, which significantly strengthened the contention, that they are preneoplastic lesions are several ACF exhibit dysplasia, a precancerous cytological feature of several organs; and ACF exhibit proliferative and genotypic atypia in common with feature of colonic cancer. This scheme proposes that once ACF be formed, only selected ACF go through the multistep process leading to the formation of microadenomas. Only a select number of microadenomas develop into adenomas, some of which subsequently develop into adenocarcinomas. It also includes the concepts that precursor lesion regress, remodel or stay indolent. A colon harboring carcinoma is expected to also have precancerous lesion.

1.2.4 Introduction of lactoferrin

1.2.4.1 Lactoferrin metabolism

Synthesis: The cDNA for human lactoferrin encode a protein of 711 amino acids, of which 19 amino acids comprise a signal peptide and 692 residues the mature protein (Powell and Ogden, 1990; Rey *et al.,* 1990). There is 99.7% agreement between the partial cDNA sequence for neutrophil human lactoferrin and the overlapping cDNA sequence from human mammary gland (Rey *et al.,* 1990, Rado *et al.,* 1987). Lactoferrin transfers to its storage granules seem to be dependent on acidification mechanism, and occurs through the medial and transcisternae of the Golgi apparatus (Olsson *et al.,* 1988). Lactoferrin present in milk is synthesized by the epithelial cells of mammary gland (Campbell *et al.,* 1992). Lactoferrin present in tear, saliva, bile and pancreatic fluid is synthesized by epithelial cells of lachrymal gland (Janssen and Van Bijsterveld, 1983), salivary gland (Masson and Heremans, 1996), biliary tract (Saito and Nakanuma, 1992), and pancreas (Janssen and Van Bijsterveld, 1983). Lactoferrin in plasma is predominant neutrophil derived (Iyer and Lonnerdal, 1993). Lactoferrin is synthesized during the transition of neutrophil from promyelocyte, and is stored in the secondary granules (Rado *et al.,* 1984). It is released upon stimulation of the neutrophils.

Degradation of lactoferrin: Lactoferrin may be removed by receptor-mediated endocytosis into phagocytic cells such as macrophages, monocytes and other cells of the reticuloendothelial system (Olofsson *et al.,* 1977; Van Snick *et al.,* 1974; Van Snick and Masson, 1976; Ismil and Brock, 1993). An alternative way of lactoferrin removal is direct uptake by the liver endothelial cells and hepatocytes (Hu *et al.,* 1993). Lactoferrin is shown to be remarkably resistant to proteolytic degradation *in vitro* by trypsin and chymotrypsin, rendering it at least partially resistant to digestion in the gut (Iyer and Lonnerdal, 1993; Brines and Brock, 1983, Brock *et al.,* 1976). Studies in infants have shown that both intact lactoferrin and large fragments can be found in significant quantities in the stool, irrespective or whether the infants are breast- or formula-fed (Spik *et al.,* 1982).

1.2.4.2 Antimicrobial properties of lactoferrin

Lactoferrin is known as a primary defense against pathogenic bacteria fungi, protozoa and viruses (Brock, 1985; Arnold *et al.,* 1980; Matthews *et al.,* 1976; Hasekawa *et al.,* 1994). Lactoferrin exerts its antibacterial effects by means of different mechanism.

A. Iron binding

Efficient iron acquisition is an important virulence factor for pathogenic bacteria (Finkelstein *et al.*, 1983). Lactoferrin is mostly secreted from the secondary granules in its iron-deficient form. This enables it to scavenge iron from the physiological milieu. Eventually, such processes may deprive the microorganism of iron, thereby inhibiting their metabolic activities *in vivo*. This hypothesis elucidated the bacteriostatic effect caused by apolactoferrin with a variety of bacteria . However, other studies indicated that the mechanism of lactoferrin-mediated anti-microbial action was more complex than simple iron deprivation (Arnold *et al.,* 1982, Stuart *et al.,* 1984).

B. Destabilizing the outer membrane of gram-negative bacteria

Lactoferrin has a direct effect on bacteria. It destabilizes the outer membrane of Gram-negative bacteria, which results in the liberation of lipopolysaccharide (LPS) (Ellison *et al.,* 1988). It is avidly bound to OmpF and OmpC in *E. coli* and weakly bound to PhoE (Erdei *et al.,* 1994). Furthermore, lactoferrin seems able to bind directly to the lipidA part of LPS (Appelmelk *et al.,* 1994). It is possible that the direct binding of lactoferrin to LPS, followed by withdrawal of LPS from the outer membrane, accounts in part for its antibacterial effect. The increasing antibacterial activity of lactoferrin with increasing core lengths of LPS would be in agreement with an involvement of LPS-binding in this process (Naidu *et al.,* 1993). Elass-Rochard and colleague (1995) found two LPS-binding sites on human lactoferrin using *E. coli* O55B5 LPS (Elass-Rochard *et al.,*1995). They found that the residues 28-34 participated in the high affinity LPS-binding, in addition to the N-terminal basic stretch 1-5 which is located in the vicinity of residues 28-34. The results strongly suggest that both sequences are required for the high-affinity binding of lactoferrin to LPS.

C. Liberation of bactericidal peptides

Tomita (1991) discovered an antimicrobial peptide much more effective than lactoferrin, which generated upon gastric pepsin cleavage of this protein (Tomita *et al.,* 1991). The isolated and sequenced peptide from human lactoferrin, lactoferricin H (Lf-cin H), and bovine lactoferrin, lactoferricin B (LF-cin B), are derived from the N-terminal region of lactoferrin (Bellamy *et al.,* 1992a). Recently, several other peptides have been co-purified from the N-terminal region. Lactoferricin B consists of 25 amino acid, whereas lactoferricin H was found to consist of 47 amino acid residues, including a region homologous to lactoferricin B (Bellamy *et al.,* 1992b).

Lactoferricin B is active against a wide range of Gram-negative and Gram-positive bacteria (Bellamy *et al.,* 1992b), fungi (Bellamy *et al.,* 1993), and protozoa (Isamida *et al.,* 1998). The amino acid sequence of murine and caprine lactoferrin has been determined, and has the antibacterial efficacy (Vorland *et al.,* 1998; Rekdal *et al.,* 1999). They found that lactoferricin B was the most efficacious, and that the other peptides only had a very limited effect on the bacteria test. The acting mechanisms are not known in detail. From there it is assumed that it is brought to the cytoplasmic membrane, where it exerts its effect by disintegrating the cytoplasmic membrane (Bellamy *et al.,* 1992a; Yamauchi *et al.,* 1993; Vorland *et al.,* 1999).

D. Glycans of bovine lactoferrin

Wada and colleague (1999) recently showed that orally administered bovine lactoferrin B can significantly decrease the number of *Helicobacter pylori* colonizing the stomach (Wada *et al.,* 1999). Treatment of *H. pylori* with bovine lactoferrin can inhibit the attachment of the bacterium to the gastric epithelial cells. This was interpreted such that bovine lactoferrin binds to some of adhesin(s) expressed on the surface of *H. pylori*, and thus partly interferes with the attachment of the bacterium to the epithelial cells. Some Enterobacteriaceae express type-1 fimbriae-derived adhesins, which recognize oligomannoside-type glycan (Abraham *et al.,* 1988). Bovine lactoferrin, which bears these glycans, can strongly inhibit the hemagglutination activity of these bacteria. Such interaction between type-1 fimbriae and oligomannoside-type glycans of bovine lactoferrin may therefore also be involved in the binding of bovine lactoferrin of *H. pylori.*

1.2.4.3 Lactoferrin and modulation of inflammatory response

A. Influence of lactoferrin on the production of cytokines.

Lactoferrin can suppress *in vitro* the release of interleukin-1, interleukin-6 and TNF from monocytes in response to LPS (Crouch *et al.,* 1992; Mattsby-Balzer *et al.,* 1996). Shinoda and colleague (1996) showed that lactoferrin has the ability to stimulate the release of neutrophil activating polypeptide interleukin-8 from human polymorphonuclear leukocytes. The peptide derived from lactoferrin of bovine and human origin all have the ability to stimulate the release of interleukin-8 (Shinoda *et al.,* 1996), while only lactoferricin B was found to suppress interleukin-6 in a monocytic cell line (THP-1), when stimulated by LPS (Mattsby-Balzer *et al.,* 1996).

B. Influence of lactoferrin on complement activation

Human lactoferrin has been shown to inhibit complement-mediated lysis of antibody-coated red blood cells by blocking the formation of the classical pathway C3 convertase (Kijstra and Jeurissen, 1982). Adding iron can reverse the effect of lactroferrin on serum complement.

C. Influence of lactoferrin on immune cells

Lactoferrin is likely to flavor the rapid recruitment of polymorphonuclear monocytes from blood to inflammatory site (Boxer *et al.,* 1982; Kurose *et al.,* 1994), though the mechanism remains unclear. Iron saturated lactoferrin inhibits myelopoesis. The effect is probably exerted through suppression of interleukin-1 production, which in turn reduces granulocyte-monocyte-stimulating factor (GM-CSF) production (Zucali *et al.,* 1989). Lactoferrin has been shown to increase the cytotoxicity of natural killer cell *in vitro*, but the mode of action is not clear (Damiens *et al.,* 1998).

1.2.4.4 Lactoferrin and anti-tumor properties

Population studies in man have indicated a protective influence of milk and other diary foods against various tumors including colorectal cancer (Rider, *et al.,* 1984). Bezault and colleague (1994) demonstrated that human lactoferrin suppressed the growth of tumor cells, MCA4-P5 fibrosarcoma and v-*ras* transformed NIH3T3, and inhibited experimental metastasis of B16-F10 melanoma cells in mice (Bezault *et al.,* 1994). They suggested that the antitumor activity

of human lactoferrin might be mediated by NK cells and be dependent on the iron-saturation level. Yoo and colleague (1997) found that iron free bovine lactoferrin and lactoferricin B, but not iron bound bovine lactoferrin, inhibited experimental tumor metastasis, whereas iron-free human lactoferrin did not exhibit any activity in their experimental model (Yoo *et al.,* 1997). They indicated that the effect of iron free bovine lactoferrin on tumor resulted from iron chelation, while the effect of lactoferricin B probably resulted from direct disruption of cell membrane functions, much the same as observed for microorganisms. Ushida and colleague (1999) showed that bovine lactoferrin exerted a beneficial effect on esophagus and lung carcinogenesis in the rat. The modes of action remain unknown (Ushida *et al.,* 1999).

1.2.4.5 Molecular biology of lactoferrin

The complete cDNA for human lactoferrin has been isolated from a mammary gland cDNA library, and the amino acid sequence has been deduced from the nucleotide sequence as shown in Figure 1.4 (Powell and Ogden, 1990; Rey *et al.,* 1990). The cDNA encodes a protein with a signal peptide of 19 amino acids followed by a mature protein of 622 residues (Figure 1.5). There is 99.7% agreement between the partial cDNA sequence for neutrophil human lactoferrin and the overlapping cDNA sequence for human mammary gland (Rado *et al.,* 1987; Rey *et al.,* 1990)

Mead and Tweedie (1990) reported the cDNA and amino acid sequence of bovine lactoferrin using a combination of cDNA and protein sequencing techniques (Mead and Tweedie, 1990). The mRNA sequence of bovine lactoferrin has also been reported (Goodman and Schanbacher, 1991). The mRNA code for a 708-amino acid protein with a 19 amino acids signal peptide immediately preceding a sequence identical to N-terminal 40 amino acids reported for bovine lactoferrin (Rejman *et al.,* 1989). The nucleic acid sequence and deduced amino acid sequence of mature protein of bovine lactoferrin are homologous with published sequences for human lactoferrin (77 and 68% respectively) and to a lesser degree, with mouse lactoferrin (72 and 64%), human transferrin (68 and 60%), and porcine transferrin (67 and 61%).

1 atgaaacttg tcttcctcgt cctgctgttc ctcggggccc tcggactgtg tctggctggc
61 cgtaggagaa ggagtgttca gtggtgcacc gtatcccaac ccgaggccac aaaatgcttc
121 caatggcaaa ggaatatgag aagagtgcgt ggccctcctg tcagctgcat aaagagagac
181 tcccccatcc agtgtatcca ggccattgcg gaaaacaggg ccgatgctgt gacccttgat
241 ggtggtttca tatacgaggc aggcctggcc ccctacaaac tgcgacctgt agcggcggaa
301 gtctacggga ccgaaagaca gccacgaact cactattatg ccgtggctgt ggtgaagaag
361 ggcggcagct ttcagctgaa cgaactgcaa ggtctgaagt cctgccacac aggccttcgc
421 aggaccgctg gatggaatgt ccctataggg acacttcgtc cattcttgaa ttggacgggt
481 ccacctgagc ccattgaggc agctgtggcc aggttcttct cagccagctg tgttcccggt
541 gcagataaag gacagttccc caacctgtgt cgcctgtgtg cggggacagg ggaaaacaaa
601 tgtgccttct cctcccagga accgtacttc agctactctg gtgccttcaa gtgtctgaga
661 gacggggctg gagacgtggc ttttatcaga gagagcacag tgtttgagga cctgtcagac
721 gaggctgaaa gggacgagta tgagttactc tgcccagaca acactcggaa gccagtggac
781 aagttcaaag actgccatct ggcccgggtc ccttctcatg ccgttgtggc acgaagtgtg
841 aatggcaagg aggatgccat ctggaatctt ctccgccagg cacaggaaaa gtttggaaag
901 gacaagtcac cgaaattcca gctctttggc tcccctagtg ggcagaaaga tctgctgttc
961 aaggactctg ccattgggtt ttcgagggtg cccccgagga tagattctgg gctgtacctt
1021 ggctccggct acttcactgc catccagaac ttgaggaaaa gtgaggagga agtggctgcc
1081 cggcgtgcgc gggtcgtgtg gtgtgcggtg ggcgagcagg agctgcgcaa gtgtaaccag
1141 tggagtggct tgagcgaagg cagcgtgacc tgctcctcgg cctccaccac agaggactgc
1201 atcgccctgg tgctgaaagg agaagctgat gccatgagtt tggatggagg atatgtgtac
1261 actgcaggca aatgtggttt ggtgcctgtc ctggcagaga actacaaatc ccaacaaagc
1321 agtgaccctg atcctaactg tgtggataga cctgtggaag gatatcttgc tgtggcggtg
1381 gttaggagat cagacactag ccttacctgg aactctgtga aaggcaagaa gtcctgccac
1441 accgccgtgg acaggactgc aggctggaat atccccatgg gcctgctctt caaccagacg
1501 ggctcctgca aatttgatga atatttcagt caaagctgtg cccctgggtc tgacccgaga
1561 tctaatctct gtgctctgtg tattggcgac gagcagggtg agaataagtg cgtgcccaac
1621 agcaatgaga gatactacgg ctacactggg gctttccggt gcctggctga gaatgctgga
1681 gacgttgcat ttgtgaaaga tgtcactgtc ttgcagaaca ctgatggaaa taacaatgac
1741 gcatgggcta aggatctgaa gctggcagac tttgcgctgc tgtgcctcga tggcaaacgg
1801 aagcctgtga ctgaggctag aagctgccat cttgccatgg cccgaatca tgccgtggtg
1861 tctcggatgg ataaggtgga acgcctgaaa caggtgttgc tccaccaaca ggctaaattt
1921 gggagaaatg gatctgactg cccggacaag ttttgcttat tccagtctga aaccaaaaac
1981 cttctgttca atgacaacac tgagtgtctg gccagactcc atggcaaaac aacatatgaa
2041 aaatatttgg gaccacagta tgtcgcaggc attactaatc tgaaaaagtg ctcaacctcc
2101 cccctcctgg aagcctgtga attcctcagg aagtaa

Figure 1.4 Nucleotide sequence of human lactoferrin (GenBank)

/translation="MKLVFLVLLFLGALGLCLAGRRRRSVQWCTVSQPEATKCFQWQR
NMRRVRGPPVSCIKRDSPIQCIQAIAENRADAVTLDGGFIYEAGLAPYKLRPVAAEVY
GTERQPRTHYYAVAVVKKGGSFQLNELQGLKSCHTGLRRTAGWNVPIGTLRPFLNWTG
PPEPIEAAVARFFSASCVPGADKGQFPNLCRLCAGTGENKCAFSSQEPYFSYSGAFKC
LRDGAGDVAFIRESTVFEDLSDEAERDEYELLCPDNTRKPVDKFKDCHLARVPSHAVV
ARSVNGKEDAIWNLLRQAQEKFGKDKSPKFQLFGSPSGQKDLLFKDSAIGFSRVPPRI
DSGLYLGSGYFTAIQNLRKSEEEVAARRARVVWCAVGEQELRKCNQWSGLSEGSVTCS
SASTTEDCIALVLKGEADAMSLDGGYVYTAGKCGLVPVLAENYKSQQSSDPDPNCVDR
PVEGYLAVAVVRRSDTSLTWNSVKGKKSCHTAVDRTAGWNIPMGLLFNQTGSCKFDEY
FSQSCAPGSDPRSNLCALCIGDEQGENKCVPNSNERYYGYTGAFRCLAENAGDVAFVK
DVTVLQNTDGNNNDAWAKDLKLADFALLCLDGKRKPVTEARSCHLAMAPNHAVVSRMD
KVERLKQVLLHQQAKFGRNGSDCPDKFCLFQSETKNLLFNDNTECLARLHGKTTYEKY
LGPQYVAGITNLKKCSTSPLLEACEFLRK"

Figure 1.5 Deduced amino acid sequence of human lactoferrin

Recombinant human lactoferrin

Recombinant human lactoferrin has been expressed in baby hamster kidney cells (Stowell *et al.,* 1991) using cDNA synthesized by a reverse transcriptase from total RNA from human bone marrow. The expressed protein was shown to be virtually identical to that isolated from human milk when migration pattern on SDS-PAGE and the presence of glycan chains were compared. Interestingly, all the recombinant lactoferrin purified from the cell culture medium was in a fully iron-saturated form, and yield range from 20-30 mg/liter. Large-scale production of human lactoferrin for clinical trials is now feasible using transgenic animal (Krimpenfort, 1993). Studies are well under way to produce transgenic cows that carry human lactoferrin in their milk. Recombinant human lactoferrin can also be produced on large scale using microorganisms such as Saccharomyces (Liang and Richardson, 1993) and Aspergillus (Ward *et al.,* 1992). They demonstrated the successful production of large quantities of human lactoferrin using a recombinant *A. awamori* expression system (Ward *et al.,* 1992). The recombinant protein was biologically active and indistinguishable from native human breast milk lactoferrin by a number of independent criteria. Because of its bactericidal activity of lactoferrin, the large-scale production by *E. coli*, gram negative bacteria were rarely reported. However, Sitaram and colleague (1998) reported the expression of bovine lactoferrin deletion mutant in *E. coli* BL21-DE3 and binding property of recombinant protein on isolated rat hepatocytes (Sitaram *et al.,* 1998).

1.3. Objectives

1. To construct recombinant human lactoferrin gene into intestinal bacteria in the genus of *B. uniformis*.
2. To study the effect of lactoferrin-producing *B. uniformis* on the growth of *E. coli in vitro*.
3. To evaluate the anti-colon carcinogenesis effects of this transconjugant bacterium by investigation its modulating effect on the formation of aberrant crypt foci in azoxymethane exposed rat.