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Effect of Fermentation on Folate Bioavailability

by

Estatira Sepehr, B. Sc.

**A thesis submitted to
the Faculty of Graduate Studies and Research**

**In partial fulfillment of
the requirements for the degree of**

Master of Science

Department of Chemistry

Carleton University

Ottawa, Ontario

January, 2002

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Acceptance of the thesis

Effect of Fermentation on Folate Bioavailability

Submitted by Estatira Sepehr, B. Sc.
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The degree of Master of Science

Chair, Department of Chemistry

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CARLETON UNIVERSITY

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Abstract

The objective of this study was to assess the association between dietary fibre (DF) and fermentable substrates (FS) intake, colonic bacterial fermentation, and folate bioavailability.

Folate is important in human nutrition because folate deficiencies are associated with neural tube defects and anemia and excess folate intake may mask other disease states. Folate may also modulate hyperhomocysteinemia, an independent risk factor for cardiovascular disease. Current recommended intakes (400 μ g/d) are based solely on folacin intake but it has been suggested that folacin may also be obtained indirectly from colonic bacteria, stimulated by fermentation of DF and FS. In order to study this possibility, male weanling Sprague Dawley rats were folate depleted by feeding a low folacin AIN93G formulated basal diet for 28 d and then fed diets differing in DF or FS and folate (0.25 - 1.0 mg/kg diet) to repleat liver stores. During the repletion phase, the increase in liver folate was proportional only to the dietary folate content and did not vary with changing dietary DF or FS. An antibiotic (succinylsulfathiazole) was added to inhibit gut bacterial populations and bacterial fermentation. Addition of the antibiotic lowered total liver folate in controls and test diets equally suggesting an effect of succinylsulfathiazole on folate absorption /or metabolism. Changes in cecal volatile fatty acids, increases in fecal diaminopimelic acid excretion, DF or FS balance as well as increases in fecal folate content provided evidence for an increased bacterial fermentation, an increased bacterial excretion, and an increased production of folate in the colon. However, colonic folate produced by bacterial fermentation was not bioavailable.

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List of Abbreviations

DAPA	Diaminopimelic Acid
DF	Dietary Fibre
FS	Fermentable substrates
Hb	Hemoglobin
Hct	Hematocrit
MA	Muramic Acid
MCHC	Mean Cell Hemoglobin Concentration
MCV	Mean Cell Volume
NTDs	Neural Tube Defects
PABA	Paraaminobenzoic Acid
RDA	Recommended Dietary Allowances
SCFA	Short Chain Fatty Acid
SST	Succinylsulfathiazole

Chapter I

Review of the Literature

1.1 Folate Metabolism

1.1.1 Chemical Structure and properties

Folate is a water-soluble B-complex vitamin. The term folate is a generic term that refers to a family of related compounds. All of these compounds represent modifications of the simplest form of the vitamin, folic acid (Pteroylglutamic acid, Pte Glu) (Brody, 1999). The structure of folic acid consists of a pterin nucleus which is bound to paraaminobenzoic acid (PABA) to form pterioic acid. Glutamic acid is linked to the PABA portion of pterioic acid, forming folate or pteroylmonoglutamic acid or pteroylmonoglutamate (**Figure 1.1**). While humans can synthesize all three components of folate, they lack the conjugase enzyme that condenses these components to form folate. Therefore, for humans folate remains an essential dietary component (Wildman and Medeiros, 2000).

Folic acid is the completely oxidized form of folate, which is seldom found in nature in appreciable amounts, though it is readily assimilated by the body and converted to other biologically active cofactor forms of the vitamin (**Table 1.1**). Folic acid is the form of vitamin used in folate supplements. Folates are modified by reduction and by addition of a polyglutamyl chain or tail (Brody, 1999, Stipanuk, 2000).

Folate coenzymes function as acceptors and donors of one-carbon moieties in reactions of nucleotide and amino acid metabolism. To be converted to tetrahydrofolate, folic acid must be reduced by an electron donor to dihydrofolic acid by the enzyme folate reductase and then to tetrahydrofolic acid by the enzyme dihydrofolate reductase.

Nicotinamide adenine dinucleotide phosphate (NADPH) serves as the electron donor in the cell (**Figure 1.2**). One-carbon moieties may be covalently linked to tetrahydrofolate at either the N-5 position of the pteridine ring or the N-10 position of the PABA, or they may bridge at position 5 and 10 (Bailey, 1995, Herbert and Colman, 1988). Over 95% of tissue folates are polyglutamate species, primarily with glutamate chain lengths between 5 and 8 (Stipanuk, 2000).

Figure 1.1: Structural formula of folic acid (Pteroylglutamate)¹.

¹Adapted from Stipanuk, 2000.

Table 1.1: Various one-carbon substitution and related oxidation states of tetrahydrofolic acid¹.

FORM	One-Carbon Form	OXIDATION Level	Oxidation Number
N ⁵ -Formyl-THF	-CH=O	Formate	2
N ¹⁰ -Formyl-THF	-CH=O	Formate	2
N ⁵ -Formimino-THF	-CH=NH	Formate	2
N ⁵ , N ¹⁰ -Methenyl-THF	-CH=	Formate	2
N ⁵ , N ¹⁰ -Methylene-THF	-CH ₂ -	Formaldehyde	0
N ⁵ -Methyl-THF	-CH ₃	Methanol	-2

THF: Tetrahydrofolate

¹Adapted from Garrett and Grisham, 1995.

Figure 1.2: Schematic diagram of dihydrofolate reductase reaction¹.

¹ Adapted from Garrett and Grisham (1995).

1.1.2 Absorption, Transport, Storage and Excretion

As naturally found in foods, folate may have nine or more glutamate residues attached to paraaminobenzoic acid (PABA). However, only the monoglutamate form is found in human plasma. Thus, polyglutamates must be digested to the absorbable monoglutamate form. This process is performed by intestinal conjugases or γ -glutamylcarboxypeptidases, which are active in the jejunum (Wildman and Medeiros, 2000). Two separate folate hydrolase activities exist in the human jejunal mucosa: one soluble and intracellular, and the other membrane-bound and concentrated in the brush border (Reisenauer *et al.*, 1977). Folate conjugase is located in the intestine, pancreas, liver, plasma and bile (Halsted, 1979). Most of the folate is absorbed by a sodium-dependent, saturable, carrier-mediated active transport mechanism on the brush border. Transport is an energy dependent process stimulated by glucose. It is a zinc, temperature and pH-dependent process with maximum transport occurring at a pH of 6.0 (Shoda *et al* 1990, and Steinberg, 1984). Passive absorption may account for 20 to 30% of folate absorption (Bailey, 1995, Brody, 1999).

Folate may be transported in portal and systemic blood, either freely dissolved or bound to blood proteins. Some of the folate is bound to albumin and α -2-macroglobulin, which have a relatively low affinity for folate (Wildman and Medeiros, 2000). Additionally, a high affinity, low-capacity specific folic acid binding protein (FABP) is present in the serum that non-covalently binds folate and does not dissociate under physiological conditions (Bailey, 1995, Steinberg, 1984). Once folate is absorbed by the gut, dietary folates appear in the blood stream in the monoglutamyl form. The main form

of serum folate is N⁵-methyltetrahydrofolate monoglutamate (Table 1.1). The absorbed folate may be converted to the 5-methyl-form during passage through the enterocyte (Herbert and Das, 1994). In order for the folate coenzyme to function, and folate be available for DNA synthesis, the methyl group needs to be removed with the help of B₁₂ (Stipanuk, 2000). The liver represents a major site for the storage and processing of folate compounds for distribution to other mamalian tissues (Henderson, 1990). Folate compounds received by the liver from the portal vein or from the general circulation are taken up, metabolized to coenzyme forms, and then released back to the circulation or diverted into the bile (Henderson, 1990). Analysis of human liver samples from Canadian autopsies revealed mean levels of 8.8 µg/g (3.6-14.8 µg/g) in subjects 11-30 years old (Hoppner and Lampi, 1980).

In the kidney folate passes easily across the glomerulus wall into urine and is then actively reabsorbed across the tubular wall. After reabsorption a normal adult excretes 5-40 µg (11-91 nmol) of folate in the urine daily (Bailey, 1995). Unabsorbed intestinal folate is also excreted in the feces and this approximates 200 µg/day (453 nmol). Fecal folate excretion is variable and is not a measure of folate availability due to folate biosynthesis by the intestinal microflora and folate contribution from the bile. Biliary excretion of folate has been estimated to be as high as 100 µg /day in humans (Bailey, 1995, Friedrich, 1988).

THF=Tetrahydrofolate

Figure 1.3: Folate metabolism¹.

¹Adapted from Journal of Nutritional Biochemistry, 1990, Vol. 1 (5), PP: 256.

1.2 Folate Bioavailability

The term folate bioavailability is used to describe the overall efficiency of utilization, including physiological and biochemical processes involved in intestinal absorption, transport, metabolism, clearance via catabolic or excretory process of dietary folates and retention (Krebs, 2001). The possible digestive or absorptive factors that influence the bioavailability of ingested folates include: 1) binding or entrapment in the food matrix, 2) altered pH at the jejunal mucosal surface, which could affect the extent of conjugase action or folate transport, 3) inhibition of jejunal brush border folate conjugase or transport by food components, and 4) effects on intestinal transit time (Bailey, 1995).

1.2.1 Chemical forms of folate

The bioavailability of polyglutamyl folates relative to monoglutamyl forms has been examined for many years. Polyglutamyl folates (chain length $n = 5-7$) are available for absorption and metabolic utilization only to the extent that they undergo enzymatic deconjugation in the small intestine (Gregory, 2001). Many investigators have shown that between 50 and 100% of polyglutamyl folates are utilized, with an average value of 75%. This is equivalent to monoglutamyl utilization (Gregory *et al*, 1992; Abad & Gregory III, 1988). Using an intestinal perfusion technique, Bailey *et al*, (1984) reported a bioavailability of 59% for [^{14}C] PteGlu₇ relative to [^3H] PteGlu on the basis of urinary recovery in young and elderly healthy human subjects. Keagy and coworkers (1988), after administering a diet containing monoglutamyl or heptaglutamyl folic acid to six men, reported 54-63% bioavailability of heptaglutamyl folic acid relative to monoglutamyl folic acid by following serum concentrations and urinary excretion, respectively. This question has not been decided since a number of well-conducted

studies indicate approximate equivalent bioavailability of monoglutamyl and polyglutamyl folates. This suggests that the human small intestine has sufficient pteroylglutamate hydrolase activity to deconjugate polyglutamyl folates fully in the absence of food materials (Wei et al, 1996; Bahandari & Gregory, 1992; Bailey et al, 1988; Abad & Gregory, 1987).

1.2.2 Intraluminal pH

Folate absorption exhibits a pH dependence with optimal transport into brush border membrane vesicles at pH 4.9-5.5, while the intact intestine exhibits maximal transport at pH 6.0-6.3 and a sharp reduction between 6.3-7.6 (Zimmerman *et al*, 1989; Said *et al*, 1986). Several investigators have shown that alterations in human upper jejunal pH, induced by diseases such as pancreatic insufficiency (Russell *et al*, 1976) and atrophic gastritis (Russell *et al*, 1986) or by drugs such as histamine H₂ blockers and antacids (Russell, 1988), decrease folate absorption in humans. Absorption in rats appears to be different since, Hoppner and Lampi (1988) showed that antacid ingestion and aspirin ingestion (1986) did not affect absorption of folates in rat small intestine.

1.2.3 Food Processing and Cooking

Food processing and cooking can alter folate stability, since folates are thermolabile. Boiling, steaming and pressure-cooking green vegetables can decrease folate content to approximately 10% of the original value. The majority of the loss occurs in the first minute (Ball, 1998). According to Leichter *et al*, (1978), loss of folate during the cooking of vegetables is caused mostly by leaching into the surrounding cooking water and not by actual destruction of the vitamin. Even the richest food sources are readily depleted of their folate by excessive cooking, especially with large amounts of water

(Williams *et al*, 1990). The addition of ascorbic acid, which is a reducing agent, prevents loss of folate content during subsequent storage, but little protective effect takes place during the heating process itself (Hawkes and Villota, 1989).

1.2.4 Folate conjugase inhibitors

Many food substances and drugs were found to have *in vitro* inhibitory effects on the brush border conjugase activity of human and pig intestine (Ball, 1998). Inhibition of the intestinal hydrolase is of interest because certain pathological conditions as well as treatment with a variety of drugs are associated with low serum folate levels and the risk of folate deficiency. Inhibition of intestinal hydrolase by diphenylhydantoin (Dilantin), an anticonvulsant, has been postulated to cause the occasional incidence of megaloblastic anemia and folate deficiency in these patients (Blakley and Benkovic, 1984).

Reisenauer and Halsted (1987) have calculated that the activity of human jejunal brush border conjugase is present in sufficient quantity so as not to limit folate absorption. However, the presence of dietary conjugase inhibitors and folate binders in foods could adversely effect the rate of deconjugation and absorption of folate in a meal. To test this, conjugase activity has been measured in the presence of various foods suspected to interfere with folate bioavailability. A possible explanation for this effect is the binding of folate to slowly digested legume starch. Bhandari and Gregory III (1990) showed that extracts of foods such as beans, banana and spinach caused (20-35%), tomato (46%), and orange juice (73-80%) inhibition in brush border conjugase activity in human and porcine intestine *in vitro*. Keagy *et al* (1988) reported that the bioavailability of monoglutamyl and polyglutamyl folates did not differ significantly when consumed

with Californian white beans. Foods that exhibited no significant inhibition included whole-wheat flour, wheat bran, whole egg, milk, cabbage, cauliflower and lettuce.

In conclusion these findings suggest that components of certain foods and drugs can specifically inhibit folate conjugase activity of human and pig intestine.

1.2.5 Dietary Fibre

The possible influence of dietary fibre on folate bioavailability is of interest, because of a recent requirement to fortify cereal based foods with folic acid to measure the folate intake of women of child bearing potential to decrease the risk of occurrence of neural tube defect pregnancy (Food and Nutrition board, Institute of Medicine, 1998). Differences in folate bioavailability of food differing in fibre composition may influence gut bacterial fermentation and folate synthesis and decrease dietary folate requirements. This may be of importance in terms of long-term exposure to higher dietary folate exposures to potential risk groups such as the elderly.

Russell *et al*, (1976) examined the effects of fibre in humans and reported that Iranian breads of high or low fibre content had little or no influence on the absorption of [^3H] folic acid, relative to fasting controls. Ristow *et al*, (1982) reported that diets containing various forms of dietary fibre (cellulose, pectin, lignin, sodium alginate, and wheat bran) exhibited equivalent bioavailability of added folic acid using a chick bioassay. Similar conclusions were reached by Keagy and Oace (1984) using a rat bioassay.

In contrast to the lack of effect of dietary fibre on the bioavailability of monoglutamyl folate, a negative effect of wheat bran on the bioavailability of polyglutamyl folate has been reported in rats (Keagy, 1985). In this study wheat bran-fed

rats had lower liver folate levels when fed PteGlu₇ relative to PteGlu. Similarly, Bailey *et al*, (1988) found in studies of plasma folate kinetics in humans that bran cereal retarded the absorption of PG-7 but had no effect on the absorption of folic acid. Keagy *et al*, (1988) reported that the addition of wheat bran to the diet accelerated PteGlu absorption, but did not significantly alter the absorption of PteGlu₇. The possible factors that may explain the lower bioavailability of polyglutamyl folate compared with monoglutamyl folate when consumed with wheat bran are differences in the chemical structure of monoglutamyl folic acid ($M_w = 441$ g/mole) and heptaglutamyl folic acid ($M_w = 1216$ g/mole) and the six additional negative charges in the PteGlu₇ molecule that increase the potential for interactions with cations. In contrast, wheat bran has cation exchange properties and can decrease intragastric concentrations of hydrogen ions and pepsin. Wheat bran reaches the colon faster than other sources of fibre, thus causing the more slowly absorbed heptaglutamyl folic acid to be less completely absorbed. It may alter the pH of the medium, as well as the rate and extent of digestion of other diet components, resulting in reduced bioavailability (Keagy *et al*, 1988, Bailey *et al*, 1988).

1.3 Animal bioassay for folate bioavailability

There are no experimental laboratory species, identical to man in terms of digestive structure or metabolism. There are obvious resemblances and similarities in function between man and other animal species, but straightforward extrapolation from animal tests to man is not possible. Therefore, the choice of test species can be influenced by other considerations such as ease of breeding or purchasing, animal husbandry, speed of growth and handling under experimental conditions (OECD Guidelines for testing of chemicals, 1993).

For several decades, scientists used chicks as an animal model for studies of folate because of their sensitivity to, and rapid onset of, folate deficiency. In addition, their ease of handling, high growth rate, and lower incidence of coprophagy made them good animal models (Bailey, 1995, Keagy, 1990). The chick bioassay has been used by Graham *et al* (1980) to examine folate bioavailability in selected fortified foods. Ristow *et al*, (1982), used the chick bioassay to examine the effects of dietary fibre on the bioavailability of folic acid as well as to assess the folate bioavailability of various foods and the bioavailability of synthetic folates. More recently, the development of a folate bioassay with rats was reported (Keagy and Oace, 1982, Hoppner and Lampi, 1985 and 1986) that has provided an alternative to the avian procedure. Rat intestinal folate conjugase is a soluble enzyme like it is in chicken, and has a pH optimum of 4.5 and molecular mass of 80,000 Daltons. It acts as an endopeptidase yielding pteroylmonoglutamate as its product (Wang *et al*, 1985).

The human jejunum has two separate folate conjugase activities, one on the brush border surface of the cell, the other soluble and intracellular (Reisenauer *et al*, 1977). Brush border folate conjugase has a pH optimum of 6.5, has exo-peptidase like activity and is activated by zinc (Halsted *et al*, 1983, Reisenauer and Halsted, 1981). Intracellular folate conjugase is maximally active at pH 4.5 and is inhibited by p-hydroxymercuribenzoate (Divedi *et al*, 1983).

Wang *et al*, (1985), reported the lack of folate conjugase activity in the brush border of the rat mucosa. Commercial rat diets contain folic acid as pteroylmonoglutamate, which makes the brush border folate conjugase unnecessary. The microflora of the large intestine in rats can also synthesize folate. This may help rats meet

their folate requirement. Prevention of coprophagy significantly decreases the folate status of the rat (Abad and Gregory III, 1987).

1.4 Anatomy of the rat large intestine

The large intestine is 1.5 m long in man and 15cm in rats. This segment of the gut in humans includes the cecum, the appendix, the ascending, transverse and descending colon, the rectum and the anal canal (Rozman and Hanninen, 1986). At the entrance to the colon is a large, caudally projecting sac, the caecum, and a forward continuation of the colon proper, and the ascending colon. Anatomically, the colon loops caudally at the level of the stomach, and then descends to the rectum (Chiasson, 1990).

1.4.1 Cecum

The adult rat cecum is a comma shaped pouch 7-9 cm in length and 1cm in diameter located on the left side of the abdomen (Hebel and Stromberg 1986). Unlike that of other rodents, the rat cecum does not have internal septa, but instead has a constriction dividing the cecum into an apical and basal segment (Rozman and Hanninen, 1986, Hebel and Stromberg 1986). The human cecum lies at the ileocecal junction. Its average axial length is about 6cm and its breadth about 7.5 cm (Williams *et al*, 1995).

In the rat, the cecum wall is composed of single layer of columnar epithelial cells (Chiasson, 1990). The enlarged cecum absorbs sodium and potassium from food and may contain enzyme-producing microorganisms to digest cellulose. But the diet of the rat is not restricted to plant foods, and consequently the rat has less need for the specializations for digesting plants than do some other rodents (Chiasson, 1990).

1.4.2 Colon

In the rat, the ascending colon emerges from the cecum as a wide tube with a length of about 10cm and diameter of 1-3cm. In front of the right kidney, it slants toward the lateral abdominal wall behind the first flexure of the duodenum. In this part of the colon the viscous contents are formed into large and loose fecal balls (Hebel and Stromberg, 1986). Water is absorbed through both the cecum and colon, thus making the fecal mass more viscous and difficult to move. The epithelium of the colon has a greater population of mucous-secreting goblet cells than does in the ileum, which compensates for the loss of water by lubricating the fecal mass for extrusion (Chiasson, 1990). While the wall of the proximal part of the colon is similar to that of the cecum, it becomes thicker distally due to the increase of the submucosa and muscularis externa. A feature of the intestinal tract is the presence of eosinophilic leukocytes with an anular nucleus (Hebel and Stromberg, 1986). Rijke *et al* (1979) studied cell migration within the intestinal crypts of the descending colon. The number of cells per crypt was approximately 625, with 33 cells per column and 19 columns per crypt circumference. It takes 60 to 72 hours for a cell to migrate from the proliferation zone of the crypt to the luminal surface.

In the ascending colon, the columnar type of stem cells, are smaller compared to the other parts of the colon. When approaching the surface, these cells lose their vacuoles and become typical epithelial cells with microvilli. At the anorectal junction there is a transition from columnar to stratified squamous epithelium (Rozman and Hanninen, 1986).

1.5 The impact of intestinal micro flora on folate nutrition

1.5.1 Bacteria of the intestinal tract

The human intestinal tract is colonized by more than 400 species of bacteria from the small to the large intestine, but they are not uniformly distributed in number, species or metabolic activity (Metges, 2000). The organisms in the upper bowel are in low concentrations, from 10^2 at the proximal intestine up to 10^5 colony- forming units (CFU) per milliliter in the region of the distal ileum (Woods and Gorbach, 1993). The flora of the lower ileum is qualitatively similar to that of feces, with the latter showing a bacterial count in the range of 10^8 to 10^{12} per milliliter (Metges, 2000).

Among all anaerobic bacteria present in the large intestine, the predominant species are the non-sporing, obligate anaerobes but facultative anaerobic bacteria (*Enterobacteriaceae*) also exist in the colonic flora (Woods and Garbach, 1993). *Bacteroides*, *Eubacterium*, *Bifidobacterium*, *Peptostreptococcus*, and *Fusobacterium* are the five major genera of the colon anaerobes. The first four are saccharolytic, which means they can break down various complex carbohydrates present in the intestine and colon (Woods and Garbach, 1993). It has been shown that certain *Lactobacillus* and *Bifidobacteria* strains can adhere to the mucousal surface, in particular to the terminal ileum and cecum (Hendrickson *et al*, 1999, Macfarlane and Cummings 1999). Under normal conditions, intestinal epithelial cells remain free of adherent bacteria; the bacteria are attached to the mucous layer lining the intestinal walls (Metges, 2000). For pathogenic bacteria, adherence is a prerequisite for invasion (Hume 1996).

1.5.2 Folate Synthesizing Bacteria

Unlike mammalian tissues, certain intestinal microflora are capable of *de novo* synthesis of folate (Rong *et al*, 1991). Several researchers have reported the possible contribution of intestinal bacteria to the folate nutrition of the animal host. In these studies, differences in the folate status of the host animal were observed after perturbing the intestinal microflora by adding anti microbial agents (Shiota, 1984, Daft *et al*, 1963, Hutching *et al*, 1941).

It is currently believed that *bifidobacteria* form a major part of the human intestinal flora and are capable of synthesizing and releasing many kinds of vitamins, such as thiamine, folic acid, nicotinic acid, pyridoxine and vitamin B₁₂. Deguchi and coworkers (1985) reported that among the five strains of Bifidobacteria derived from human intestine, *B. bifidum* and *B. infantis* were higher producers and *B. breve*, *B. longum*, and *B. adolescentis* were lower producers of folic acid in the culture. Other intestinal bacteria, like *Escherichia coli*, are also capable of synthesizing folate. Folate levels in liver, muscle and brain of germ free rats and chickens were half as much as those found in similar models that had been inoculated with a strain of *Escherichia coli* (Coates *et al*, 1968, Miller and Luckey, 1963, Daft *et al*, 1963).

Although more recent studies have focused on *bifidobacteria* and *E. coli*, early work with *in vitro* cultures of *Lactobacillus gayonii*, *Bacillus lactis acidi*, *Lactobacillus arabinosus*, *Lactobacillus pentosus*, *Bacillus brassicae*, and *Leuconostoc mesentroides* showed that these organisms are also capable of synthesizing significant amounts of folic acid in folate free medium (Hutching *et al*, 1941). Folate synthesizing bacteria contain a precursor pterin, 6-hydroxymethyl-7, 8-dihydropterin pyrophosphate that is derived from

GTP and condenses first with p-aminobenzoic acid (PABA) to form dihydropteroate and then with glutamic acid to form dihydrofolic acid (Shiota, 1984).

1.6 Nutritional aspects

1.6.1 Food sources of folate in Canadian diets

Folate was originally identified in green leafy vegetables and rich sources (> 100 µg/serving) of this vitamin are brussels sprouts, asparagus, spinach, and kale. Other vegetables contain lower amounts (50-100µg/serving), including broccoli, spring greens, iceberg lettuce, cabbage and cauliflower (Report of the Committee on Medical Aspects of Food and Nutrition Policy, 2001). The best fruit sources are lemons, bananas, and melons. Folates are also found in liver, kidney, yeast, yeast extract, beer, and mushrooms (Report of the Committee on Medical Aspects of Food and Nutrition Policy, 2001, Williams *et al*, 1990).

1.6.2 Recommended Dietary Folate Equivalent (DFE)

The food and nutrition board institute of medicine (1998) edition of the dietary reference intakes concluded that for all adults the recommended dietary allowance (RDA) is 400 micrograms of folate per day. They also report the amount of folate as dietary folate equivalent (DFE), which is the difference in bioavailability between folic acid and the folate found in foods. The average daily dietary level that is sufficient to meet the nutrient requirement of nearly all (97 to 98 percent) healthy individuals in a particular life-stage and gender group. However, the amount of folate demonstrated to minimize NTD risk is 400 µg of synthetic folic acid/day in addition to food folate.

Table 1.2: Recommended Dietary Allowances (RDA) for folate (in Dietary Folate Equivalent (DFE)/day) across the life cycle¹.**

Different stages of life cycle		µg Folate/day
Infants	0-5months	65*
Infants	6-11months	80*
Children	1-3yr	150
Children	4-8yr	200
Children	9-13yr	300
All	14yr and over	400
Pregnant Women		600
Lactating Women		500

¹Adapted from Food and Nutrition Board, Institute of Medicine (1998) Dietary Reference Intakes: Folate. National Academy press, Washington, D.C

*Adequate intake (AI), since insufficient information is available to set an RDA

**1DFE= 1microgram food folate=0.6microgram folic acid consumed by food

1.7 Folic acid and Risk

Folic acid in food has a structural and metabolic function in the body. It is possible to either be deficient or to ingest too much folate. The risk of getting too little is the basis of the derivation of population Dietary Reference Values for folate and folate fortification of common foods. These policies, however, increase the possibility of exposing individuals to undesirably high levels of micronutrients (Report of the Committee on Medical Aspects of Food and Nutrition Policy, 2001). Both folate and B₁₂ deficiencies in humans share certain similar clinical symptoms. B₁₂ deficiency is common in the elderly. Increased dietary folate may mask preliminary symptoms of B₁₂ deficit in the elderly and lead to irreversible nerve damage, which is the end result of B₁₂ deficiency. The intimate involvement of folate in DNA metabolism suggests that increased long-term dietary intakes of folate could also have detrimental effects in growing children.

1.7.1 Neural tube defects

Several research studies have confirmed the importance of folate in reducing the risks of neural tube defects (Whitney and Rolfes, 1999). The term neural tube defect (NTD) applies to any malformation of the embryonic brain and /or spinal cord, which is formed 20 to 28 days after fertilization (Butterworth and Bendich, 1996). NTDs can be either open lesions like anencephaly, or closed lesions such as encephalocele and meningocele (Fleming, 2001). The rate of NTD occurrence with the first pregnancy is about 0.5-8 in 1000 births; the rate can be 10 to 20 times higher in mothers of a previously affected child (Fleming, 2001, Butterworth and Bendich, 1996). The observation that periconceptual folate supplementation with folic acid reduces the

incidence of these defects by about two thirds has led to fortification of the food supply with folic acid in United States (Shane, 2000). The Food and Drug Administration (February 29, 1996) has mandated that grain products (bread products, flour, corn grits, cornmeal, farina, rice, macaroni and noodles) be fortified (150 micrograms per 100 grams of grain) to deliver folate to the U.S. population (Whitney and Rolfes, 1999). Most Canadian manufacturers of flour and pasta began adding folic acid effective January 1, 1998 (Food and Nutrition Board Institute of Medicine, 1998). In the United Kingdom, fortification of foods with folic acid, as with other nutrients, is well established. It is estimated that between 80 and 90% of breakfast cereals consumed are fortified with folic acid. Most folic acid fortified cereals claim to contain between 125 and 200µg/100g (Report of the Committee on Medical Aspects of Food and Nutrition Policy, 2001).

1.7.2 Cardiovascular Disease

Homocysteine is an amino acid byproduct of methionine metabolism (Bendich and Deckelbaum, 2001). Remethylation of intracellular homocysteine to methionine requires folate (Ueland *et al*, 1993). Few recent studies have examined the relationship between intake of folate and vascular diseases. Elevated homocysteine values have been associated with endothelial dysfunction and injury followed by platelet activation and development of thrombosis (Lentz and Sadler, 1991 and Bendich and Deckelbaum, 2001). High plasma homocysteine concentrations are also considered a risk factor for cardiovascular diseases (Brouwer *et al*, 1999).

Several investigators have conducted studies examining the effect on homocysteine levels in response to changes in folic acid intake. Ueland *et al*, (1993), and Lindenbaum *et al*, (1994), found that total homocysteine levels markedly increased as

serum folate decreased. O'Keefe *et al*, (1995), studied 17 healthy, non- pregnant women between the ages of 21-27 years, with healthy blood chemistry profiles, randomized into three groups. The groups consumed 200, 300 or 400 $\mu\text{g/d}$ of total folate for 70 days. The level of total plasma homocysteine in the group consuming 200 $\mu\text{g/d}$ was significantly higher ($p<0.05$) than that of the 300 or 400 $\mu\text{g/d}$ groups. Therefore, they concluded that 200 $\mu\text{g/d}$ folate was not sufficient to maintain the folate states of those women. Brouwer and colleagues (1999) studied the effect of low-dose folic acid administration (250 or 500 $\mu\text{g/d}$) for 4 weeks on plasma homocysteine concentrations and folate status in 144 women aged 18-40 years. Their results indicated that administration of folic acid for four weeks significantly increased folate concentrations in plasma and red blood cells, but total homocysteine concentrations decreased significantly in women who took 500 μg folic acid/day compared with the group receiving 250 μg folic acid/day. These studies provide support for an inverse association between total homocysteine levels and plasma/serum folate levels.

1.7.3 Red blood cell maturation failure

Folic acid is an important factor for the final maturation of red blood cells. It is required for the formation of thymidine triphosphate, one of the essential building blocks of DNA (Guyton and Hall, 1996). Inadequate folate intake leads to decreased serum folate concentrations, which are detected after two weeks of consumption of a low folate diet. However, it is not until 8 to 9 weeks have passed before the first hematological sign of abnormalities in the bone marrow and hyper-segmentation of the neutrophils in peripheral blood are detected. Lack of folic acid causes the erythroblast cells of the bone marrow to produce larger cells (macrocytes) with consequent depression of the red blood

cell count (Wildman and Madeiros, 2000). Macrocytes have flimsy membranes and are irregular, large, and oval instead of the usual biconcave disc form. This results in a reduced oxygen capacity of the blood and a one half to one third normal life span (40-60 vs. 120 day). In addition, they are not be able to travel through the capillaries as efficiently as normal red blood cells (Guyton and Hall, 1996, Food and Nutrition Board Institute of Medicine, 1998, Whitney and Rolfes, 1999).

1.7.4 Cancer

It has been hypothesized that poor folate status itself is not carcinogenic but may enhance an underlying predisposition to cancer (Mason and Levesque, 1996). However folate deficiency has been associated with colon cancer (Kim *et al*, 1997). The mechanism behind this is not clear but according to Stipanuk (2000), uracil misincorporation arising from defective thymidylate synthesis has been hypothesized as one possibility (Mason and Levesque, 1996). Increased chromosomal fragility or diminished DNA repairs are other possibilities (Kim *et al*, 1997).

1.8 Hazard Identification

Manipulating the diet through fortification and dietary supplements increases the possibility of unwitting exposure to undesirably high levels of folic acid. However, according to Butterworth and Tamura, (1989), no adverse effect has been associated with the consumption of excess natural forms of dietary folate. Therefore, the main concern is the intake of synthetic folic acid (Food and Nutrition Board Institute of Medicine, 1998). Although evidence from animal as well as *in vitro* tissue and cell culture studies suggests that excess consumption of synthetic folic acid is neurotoxic and epileptogenic in animals, there is no clear evidence of folic acid induced neurotoxicity in humans (Baxter *et*

al, 1973; Loots *et al*, 1982; Kehl *et al*, 1984; Weller *et al*, 1994). Large doses of folic acid will mask the diagnosis of vitamin B₁₂ deficiency and a delayed diagnosis can result in an increased risk of progressive, unrecognized neurological damage (Stipanuk, 2000). A positive association between folic acid intake and total cancer incidence, as well as an increased incidence of oropharynx and hypopharynx cancer was observed by Selby *et al*, (1989). In their study, they used computerized pharmacy records of cancer patients from 1969 to 1973 to look at the effect of different drugs with incidence of cancer. The authors believed that the observed association between cancer and folate was related to unmeasured variables like alcohol and smoking. According to Herbert, (1994), folate deficiency protects against malaria, but there is no data to support an increased susceptibility to malaria in humans who receive folic acid supplementation.

Weakness, tiredness, diarrhea, and anorexia are also associated with folate deficiency (Devries, 2001).

1.9 Influences of Dietary Fibre on the Ecology of the Intestinal Flora

1.9.1 Definition of dietary fibre

Several definitions of dietary fibre have been used over the past 50 years. The diversity in the definition arises because of the multiplicity of concepts involved in dietary fibre and the difficulties associated with its measurement. The term “dietary fibre” first appeared in a 1953 publication by Hipsley and referred to edible fibre (hemicelluloses, cellulose and lignin) but not to the digestible constituents of the plant cell wall. He observed a relationship between the consumption of edible fibre components and the absence of pregnancy toxemia in Sydney, Australia and eclampsia in the Fiji Island (Prosky, 2000; Mongeau *et al*, 1999; Hipsley, 1953). In 1972, Trowell detected differences in the incidence of non-infective diseases in Africa and Western

countries. The rural Africans who ate diets very high in dietary fibre suffered from few Western-type diseases (Jones, 2000). In 1976, Trowell *et al*, defined dietary fibre (DF) as hemicellulose, cellulose, lignin and pectin in the diet that are not digested by the endogenous secretions of the human digestive tract. In 1985, Health and Welfare Canada adopted the following definition of dietary fibre: “Endogenous components of plant food material from the diet which are resistant to human digestive secretions but which are generally substantially fermented in the colon. They are predominantly nonstarch polysaccharides (NSP) and lignin and may include, in addition, associated substances”(Health and Welfare Canada, 1985). The American Association of Cereal Chemists (AACC) has recently adopted a newer definition of dietary fibre. On June 1, 2000, the AACC Board of Directors approved the following definition for dietary fibre as developed by the AACC Committee to Define Dietary Fibre. “Dietary fibre is the edible parts of plants or analogous carbohydrates that are resistant to digestion and absorption in the human small intestine with complete or partial fermentation in the large intestine. It includes polysaccharides, oligosaccharides, lignin, and associated plant substances. Dietary fibres promote beneficial physiological effects including laxation, and/or blood cholesterol attenuation, and blood glucose attenuation”. This definition does maintain some of the links to physiological function but also includes the term “analogous carbohydrates” to permit the acceptance of some resistant carbohydrates in the newer definition.

1.9.2 Dietary Fibre composition

Dietary fibre is composed of soluble and insoluble components (Prosky and Devries, 1992). Soluble fibre comprises between 25-33% of the dietary fibre consumed in

the human diet and consists of noncellulosic polysaccharides like pectin, gums, mucilages, and glucans (Cho *et al*, 1999). Good sources are whole grain oats and barley, oat bran, some fruits, dried beans and other legumes (Cho *et al*, 1999). Soluble dietary fibre, is not digestable by human pancreatic enzymes and is soluble in hot water (Cho *et al*, 1999).

Insoluble fibre makes up between 66-75% of the dietary fibre in foods and consists of cellulose, lignin and some hemicellulose, cutin and plant waxes (Cho *et al*, 1999; Prosky and Devries, 1992). Good sources are cereal brans and whole grain cereals, dried beans, peas, nuts and vegetables (Cho *et al*, 1999). Chemical components of dietary fibre are shown in (Table 1.3).

1.9.3 Adverse effects and benefits of dietary fibre consumption

In Western diets, low intakes of dietary fibre are associated with obesity, hyperlipidemia, coronary heart disease and large bowel disorders, including constipation, irritable bowel syndrome, diverticular disease and colon cancer (Gestel *et al*, 1994). Based on the epidemiological studies by Burkitt (1971), Reddy *et al* (1978), and Bingham *et al* (1985) there is an inverse relationship between high-fibre diets and colon cancer. Some scientists suggest that the protective effect of dietary fibre against cancer is due to dilution of carcinogens in the large level and decreased contact with the colonic mucosa (Reddy *et al*. 1989 and Bingham, 1993).

High fibre consumption improves laxation, which is a response to many factors including increased fecal bulk and higher water content. Blood glucose and insulin levels change in response to high soluble, viscous fibre intake (Prosky and Devries, 1992) because the increased luminal viscosity inhibits diffusion and slows absorption.

1.9.4 Dietary fibre intake recommendation in North America

Recommended dietary fibre intakes in North America are between 25 to 35 g/day (Proskey and Dreher, 1999). Current nutrition data suggests that Canadians consume far less than this (eating only 12-14 g/day, McCleary, 1999). Health and Welfare Canada, Canada's Food Guide to Healthy Eating (1992) recommends increased intake of cereals, breads and whole grain products from 3-5 servings /day to 5-12 servings. For most children over the age of 2 years, the recommended dietary fibre range is years of age+5 and years of age +10 g/day (Williams *et al*, 1995).

Table 1.3: Chemical components of dietary fibre¹

Classical Nomenclature	Chemical components		Solubility Characteristics	Nomenclature used In Dietary fibre Litretaire
	Main chain	Side chain		
Pectic Substances	Galacturonic acid	Rhamnose Arabinose Xylose Fucose	Water Soluble	Included in NCP *and NSP*
Hemicellulose	Xylose Mannose Glucose	Arabinose Galactose	Insoluble in Water, Soluble in dilute alkali	Included in NCP and NSP
Cellulose	Glucose	None	Insoluble in alkali	Included in NSP
Lignin	Sinapyl alcohol Coniferyl alcohol p-Coumaryl alcohol	3-D structure	Insoluble in 12M H ₂ SO ₄	Lignin, and in total dietary Fibre
Gums	Galactose Glucuronic acid-mannose	Xylose	Water soluble	Included in NCP and NSP Soluble Fibre
Mucilages	Galactose-mannose Glucose-mannose	Galactose	Water soluble	Included in NCP and NSP Soluble Fibre

¹ Adapted from Slavin, 1987, Southgate, 2001.

*NCP: Non- Cellulosic Polysaccharide

*NSP: Non-Starch Polysaccharide

The human diet contains two major classes of polysaccharides: starch and the polysaccharides of the plant cell wall, which are referred to as nonstarch polysaccharides (NSP). Nonstarch polysaccharides are the non- α -glucan polysaccharides of the plant which are resistant to α -amylase. They are principally cell-wall structures and include a mixture of substances such as cellulose, pectin, and hemicellulose, which contain hexose and pentose sugars and uronic acids (Englest and Cummings, 1985). The resistance of dietary fibre and NSP to digestion in the small intestine has been demonstrated by Englyst and Cummings (1985, 1986, and 1987) who used human ileostomy patients as a model and obtained complete recovery of all NSP.

1.10 Fermentation in large intestine

Although dietary fibre is not digested and absorbed in the small intestine, a large proportion of it is broken down by the microorganisms in the colon (Southgate, 1998). The human large intestine contains different species of bacteria which belong to the genera: *Bacteroides*, *Bifidobacterium* and *Eubacterium* in large quantity and *Lactobacillus*, *Clostridia*, *Entrobacteria* and gram positive cocci to a lesser extent (Wang and Gibson, 1993). Nutritious materials for the growth of these bacteria are derived from human diets. The principle substrates are NSP, starch and protein (Macfarlane and Cummings, 1999, Cumming and Englest, 1987). Most of the non-digestible oligo or polysaccharides, when they reach the ceco-colon, are hydrolysed to small oligomers and monomers, which will be fermented by anerobic bacteria (Delzenne and Roberfroid, 1994). In humans, the major end products of non-absorbed carbohydrates are short chain fatty acids (SCFA); mainly acetate (C2), propionate (C3), and butyrate (C4), which compose more than 80% of SCFAs (Kobayashi and Fleming, 2001; Cumming and

Macfarlen, 1991). In addition to NSP, about 10% of other dietary polypeptides (digested incompletely in the small intestine) are available to the large intestine for bacterial fermentation. Bacterial fermentation produces many types of SCFA, including branched-chain fatty acids like isobutyrate, isovalerate and valeric acid. In addition, fermentation gases such as H_2 and CO_2 , phenols, ammonia, amines, indoles and energy are also produced. Bacteria use energy for growth and maintenance of cellular function (Nordgaard and Mortensen, 1995). In herbivores, the absorption of SCFA contributes between 60 and 90% of all their energy requirements. In humans, it contributes only 5-10% of total energy requirements (Bergman, 1990, Royall *et al*, 1990). Absorbed SCFAs are ionized at intracellular pH values to give hydrogen (H^+) and free fatty acids (FA^-). Absorption is electro-neutral since hydrogen ions exchange with Na^+ ions and the fatty acids exchange for Cl^- ions. Therefore, fermentation prevents diarrhea by removal of sodium and water from the colon (Royall *et al*, 1990).

Figure 1.4: Schematic Representation Of the bioavailability of digestible and non digestible oligo/polysaccharides¹.

¹Adapted from Delzenne and Roberfroid (1994).

1.11 Effect of Dietary Fibre on Protein Digestibility

Nitrogen enters the intestinal tract from two main sources: an endogenous source, which is derived from the salivary, gastric, pancreatic, plasmatic leakage, intestinal cell secretions and from cell desquamation (Low, 1990; LaRusso, 1984), and an exogenous source which comes from ingested animal and vegetable nitrogenous compounds (Mahe *et al*, 1994).

In the digestive tract, much of the exogenous nitrogen is absorbed so that excreted nitrogen arises mostly from endogenous sources and colonic bacteria. Several studies examined the effect of rich dietary fibre sources on nitrogen excretion. For example Shetty *et al* (1986) used healthy human subjects as a model for their study, giving them a diet containing 100g corn starch. They observed a 30 % increase in fecal weight and fecal nitrogen. Fecal nitrogen is an indirect measure of bacterial cell proliferation.

Other studies have compared the rat and human. These studies have shown that the rat is a good model for examining the effect of dietary fibre on protein digestibility. Bach Knudsen *et al* (1994) compared protein digestibility in both rats and humans by giving them identical mixed diets with low and high dietary fibre. Low dietary fibre diets were used as control diets and the level of dietary fibre was increased by adding fruits, and vegetables, citrus fibre concentrate, and insoluble barley fibre. Their results indicated a similar extent of protein digestibility by the two species.

1.12 Muramic acid and Diaminopimilic acid

Muramic acid (MA) is a constituent of the peptidoglycan (PG) backbone of gram positive and gram-negative bacteria, and is mostly present in an acetylated form

(Kozar *et al*, 2000; Hoijer *et al*, 1995). Peptidoglycan is a mucopolysaccharide consisting of a repeating disaccharide unit attached to chains of four or five amino acids. The component monosaccharides are N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM), which are related to glucose and have amino acid groups attached (**Figure 1.5**) (Tortora *et al*, 1989). The various components of peptidoglycan are assembled in the cell wall as follows, the NAG and NAM alternate in rows, each row containing from 10- 65 sugars and forming a carbohydrate backbone (Tortora *et al*, 1989). A tetrapeptide side chain is attached to each NAM molecule, forming a large complex macromolecule, which surrounds the cells like a basket around the cell membrane (Hoijer, *et al*, 1995). In gram-positive organisms, peptidoglycans form a large portion of the wall, where they may make up to 50% or more of the wall's dry weight. In gram-negative bacteria, on the other hand, they form usually less than 10% of the wall, and sometimes as little as 1%. These differences are major reason behind the difference in the proportion of amino sugars in the walls of gram-positives (O'Leary, 1989).

Although over 400 species of anaerobic bacteria are present in the large intestine of a human being, they only account for 35 to 50% of the volume of the content of the human colon (Woods and Gorbach, 1993).

Muramic acid is not synthesized by mammalian enzyme systems and, therefore, can be used as a marker to indicate the presence of both viable bacteria and their nonviable cell wall remnants in tissues and body fluids (Gilbert and Fox, 1987; Fox *et al*, 1980).

Diaminopimilic acid (DAPA) is also a component of bacterial cell wall mucopeptides; the rest of the cell contains only traces (El-Shazly, 1966). Since it is not found in plant or animal tissues, it can also be used as a marker of bacterial matter.

Figure 1.5: NAG and NAM joined together as in peptidoglycan.¹
¹Adapted from Tortora *et al.*, 1989

Chapter II

Introduction and Objective

Folate has a critical role in the metabolism of nucleic and amino acids. Folate deficiencies are associated with anaemia and neural tube defects, which constitute an important public health problem in terms of mortality, morbidity, social cost, and human suffering. Recent Canadian surveys show that adults consume much less than the recommended 400 µg/d. There is now a requirement for folate fortification of cereal-based products to improve folate intake in women of childbearing ages and reduce the risk of neural tube defects. While this is beneficial from the view-point of reducing neural tube problems during pregnancy as well as for lowering circulating homocysteine levels (an independent risk factor for cardiovascular disease). It may also increase the masking of B₁₂ deficiency with resultant irreversible neural damage in risk groups such as the elderly and people who eat few or no foods of animal origin.

Many investigators have demonstrated that the bioavailability of folate depends on the form of folate used to fortify foods (Abad and Gregory 1988; Keagy *et al*, 1988). According to the standing committee on the scientific evaluation of dietary reference intakes (1998), endogenous folate is approximately 50% bioavailable and added folic acid, when consumed with food, is 85% bioavailable. In addition to endogenous and added folate, other sources of folate may exist. For example, experimental evidence in rats suggests that synthesis of folate by intestinal bacteria may influence folate status in folate deprived rats (Krause *et al*, 1996; Keagy and Oace, 1989). Rong and colleagues (1991) reported that some of the folate synthesized by the microflora in the rat large intestine is incorporated into the tissue folate of the host. They used [³H] PABA as a

tracer to label the folates synthesized by rat intestinal bacteria. Radioactive tracer was injected in the rat cecum to maximize the exposure of the label to the microflora, and to minimize potential loss through intestinal absorption. Because mammalian cells are not capable of *de novo* folate synthesis, any [^3H] folate found in the tissues of the rat must be derived from bacterial synthesis from [^3H] PABA. During the experimental period, the use of a sling-suit restricted the movement of the rats, to prevent coprophagy. The results indicated that a significant portion of the [^3H] PABA was taken up by the intestinal bacteria and converted to [^3H] folates. Synthesized [^3H] folates were released from the bacteria, transported across the intestinal wall and incorporated into peripheral tissues. Therefore according to their results the large intestine is also capable of some folate absorption. By analogy, folate bioavailability in humans may also be influenced through changes in colonic fermentation. Thus, high fibre foods, which promote fermentation, may increase folate bioavailability. This idea is supported by Houghton and coworkers (1997) who reported a positive association between dietary fibre intake and serum folate concentration in humans. Experimental data do not support the hypothesis that dietary fibre, reduces folate bioavailability (Gregory, 1989; Bailey *et al*, 1988). Therefore, using the rat as an animal model, the objectives of the present investigation were to assess the association between the dietary fibre intake, colonic bacterial fermentation and folate bioavailability. The experimental protocol was divided into two separate phases. The first phase was designed to provide data on the kinetics of folate repletion in the rat model and the potential influences of fermentation. This phase yielded the time course of repletion. In the second phase, the repletion time was based on the optimal time point defined in the first phase of the experiment. This phase used different sources of dietary fibre,

fermentable substances and antibiotics in order to better define the relationship between fermentation and folate reproduction. The role of the antibiotic was to inhibit folate synthesis by killing intestinal bacteria to provide another method for controlling folate bioavailability.

Chapter II

Introduction and Objective

Folate has a critical role in the metabolism of nucleic and amino acids. Folate deficiencies are associated with anaemia and neural tube defects, which constitute an important public health problem in terms of mortality, morbidity, social cost, and human suffering. Recent Canadian surveys show that adults consume much less than the recommended 400 µg/d. There is now a requirement for folate fortification of cereal-based products to improve folate intake in women of childbearing ages and reduce the risk of neural tube defects. While this is beneficial from the view-point of reducing neural tube problems during pregnancy as well as for lowering circulating homocysteine levels (an independent risk factor for cardiovascular disease). It may also increase the masking of B₁₂ deficiency with resultant irreversible neural damage in risk groups such as the elderly and people who eat few or no foods of animal origin.

Many investigators have demonstrated that the bioavailability of folate depends on the form of folate used to fortify foods (Abad and Gregory 1988; Keagy *et al*, 1988). According to the standing committee on the scientific evaluation of dietary reference intakes (1998), endogenous folate is approximately 50% bioavailable and added folic acid, when consumed with food, is 85% bioavailable. In addition to endogenous and added folate, other sources of folate may exist. For example, experimental evidence in rats suggests that synthesis of folate by intestinal bacteria may influence folate status in folate deprived rats (Krause *et al*, 1996; Keagy and Oace, 1989). Rong and colleagues (1991) reported that some of the folate synthesized by the microflora in the rat large intestine is incorporated into the tissue folate of the host. They used [³H] PABA as a

data using the factors of 4 kcal per g of protein or carbohydrate, 9 kcal per g of fat and 0 kcal per g of fibre. The diets were made up to 0.25% folate based on the measured folate content of HRW Bran and Oat Bran. During the depletion and repletion periods, food consumption and body weights were recorded weekly. Any changes in physical appearance were recorded. Feces samples were collected during the repletion phase. A total of seven animals from each diet group were sacrificed every week. This number is based on previous data of folate depletion/repletion in rats (Hoppner and Lampi, 1986). Using their data, significant differences between the basal (depleted) group and the repletion groups can be observed when $N \geq 7$ (One way ANOVA followed by a Student-Newman-Keuls comparison between all groups). From the sacrificed rats, livers, cecal contents, feces and blood samples were collected.

Table 3.1: Composition of the diets from phase 1.

g/kg diet mix					
	Depletion	standard 0.25	standard 1	Wheat bran	Oat Bran
Diet ingredient	0	1	3	4	5
Vitamin-free Casein	200.0	200.0	200.0	181.8	150.7
Corn Starch	393.3	393.3	393.3	379.5	320.6
Dextrinized Corn Starch	127.8	127.8	127.8	114.0	55.1
Sucrose	95.8	95.8	95.8	82.0	23.1
Soybean oil	70	70	70	70	70
AIN-93 minerals	35	35	35	35	35
AIN-93 vitamins (Nofolate)	10	10	10	10	10
Choline Bitartrate	2.5	2.5	2.5	2.5	2.5
DL Methionine	3	3	3	3	3
Alphacel	62.5	62.5	62.5	5.1	45.1
Di-t-butylhydroquinone	0.014	0.014	0.014	0.014	0.014
Extras					
Extra "fibre" g/kg diet	0.0	0.0	0.0	117.0	284.9
Sulfa drug	0	0	0	0	0
Totals (g/kg diet)					
Calculated folate (mg/kg diet)	0.00	0.25	1.00	0.25	0.25
Measured folate (µg/g)	0.397±0.032	0.521±0.044	1.395±0.202	0.866±0.195	0.521±0.052
Measured dietary fibre (g/100g)	7.246±0.094	6.891±0.130	7.539±0.632	6.757±0.862	9.639±0.038
Total Protein %	20.0	20.0	20.0	20.0	20.0
Total carbohydrate %	61.7	61.7	61.7	60.1	55.7
Total Fat %	7.0	7.0	7.0	7.5	9.0
Estimated Energy content (kcal/kg)	3904.4	3904.4	3904.4	3904.4	3904.4

*In the experimental diets, di-t-Butylhydroquinone (di-t-BHQ, Molecular weight =222.33, ICN Biomedicals Inc, 1263 South Chillicothe Road Aurora, Ohio 44202, U.S.A) at 0.014 g/kg (0.014 mg/g) was used as an antioxidant in the oil (7%). Diets with 7% oil should contain $(0.014\text{mg/g}) \times (100/7) = 0.2 \text{ mg/g}$ of antioxidant.

Table 3.2a: Composition of the drug-free diets from Phase 2.

g/kg diet mix										
	Depletion	Std 0.25	Std 0.5	Std 1	Wheat bran	Oat Bran	Ground Corn	Wheat germ	Inulin	Poly- dextrose
Diet ingredient	0	1	2	3	4	5	6	7	8	9
Vitamin-free Casein	200.0	200.0	200.0	200.0	181.8	150.7	176.3	153.7	200.0	200.0
Corn Starch	393.3	393.3	393.3	393.3	379.5	320.6	327.4	353.2	385.2	385.2
Dextrinized Corn Starch	127.8	127.8	127.8	127.8	114.0	55.1	61.9	87.8	119.7	119.7
Sucrose	95.8	95.8	95.8	95.8	82.0	23.1	29.9	55.8	87.7	87.7
Soybean oil	70	70	70	70	70	70	70	70	70	70
AIN-93 minerals	35	35	35	35	35	35	35	35	35	35
AIN-93 vitamins (Nofolate)	10	10	10	10	10	10	10	10	10	10
Choline Bitartrate	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5
DL Methionine	3	3	3	3	3	3	3	3	3	3
Alphacel	62.5	62.5	62.5	62.5	5.1	45.1	32.5	29.0	36.8	36.8
Tert-butylhydroquinone	0.014	0.014	0.014	0.014	0.014	0.014	0.014	0.014	0.01	0.014
Extras										
Extra "fibre" g/kg diet	0.0	0.0	0.0	0.0	117.0	284.9	251.5	200.0	50.0	50.0
Sulfa drug	0	0	0	0	0	0	0	0	0	0
Totals (g/kg diet)										
Measured dietary folate(μ g/g)	0.370 $\pm$ 0.023	0.498 $\pm$ 0.006	0.582 $\pm$ 0.022	0.894 $\pm$ 0.123	0.488 $\pm$ 0.038	0.486 $\pm$ 0.010	0.370 $\pm$ 0.008	0.762 $\pm$ 0.046	0.505 $\pm$ 0.001	0.631 $\pm$ 0.020
Measured dietary fibre (g/100g)	7.380 $\pm$ 0.259	6.796 $\pm$ 0.522	7.079 $\pm$ 0.344	7.179 $\pm$ 0.479	6.406 $\pm$ 0.433	9.923 $\pm$ 1.143	11.689 $\pm$ 0.178	8.469 $\pm$ 0.283	5.392 $\pm$ 0.591	6.096 $\pm$ 0.686
Percentages										
Total Protein %	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0
Total carbohydrate %	61.7	61.7	61.7	61.7	60.1	55.7	57.6	57.0	59.3	59.3
Total Fat %	7.0	7.0	7.0	7.0	7.5	9.0	8.2	8.9	7.0	7.0
Estimated Energy content (kcal/kg)	3904.4	3904.4	3904.4	3904.4	3904.4	3904.4	3847.5	3904.4	3904.4	3904.4

Table 3.2b: Composition of the drug-added diets from Phase 2.

g/kg diet mix									
	Std 0.25/drug	Std 0.5/drug	Std 1.0/drug	Wheat/ drug	Oat/ drug	Ground corn/ drug	Wheat germ/drug	Inulin/ drug	Polydextros/ drug
Diet Ingredient	10	11	12	13	14	15	16	17	18
Vitamin-free Casein	200.0	200.0	200.0	181.8	150.7	160.5	153.7	200.0	200.0
Corn Starch	393.4	393.4	393.4	379.5	320.6	283.5	353.3	385.3	385.3
Dextrinized Corn Starch	127.9	127.9	127.9	114.1	55.1	18.0	87.8	119.8	119.8
Sucrose	95.9	95.9	95.9	82.1	23.1	29.9	55.8	87.8	87.8
Soybean oil	70	70	70	70	70	70	70	70	70
AIN-93 minerals	35	35	35	35	35	35	35	35	35
AIN-93 vitamins (Nofolate)	10	10	10	10	10	10	10	10	10
Choline Bitartrate	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5
DL Methionine	3	3	3	3	3	3	3	3	3
Alphacel	57.3	57.3	57.3	0.0	40.0	27.3	23.9	31.7	31.7
Tert-butylhydroquinone	0.014	0.014	0.014	0.014	0.014	0.014	0.014	0.014	0.014
Extras									
Extra "fibre" g/kg diet	0.0	0.0	0.0	117.0	284.9	419.1	200.0	50.0	50.0
Sulfa drug	5	5	5	5	5	5	5	5	5
Totals (g/kg diet)									
Measured dietary folate (µg/g)	0.453± 0.024	0.553± 0.030	0.826± 0.039	0.447± 0.006	0.399± 0.032	0.453± 0.082	0.774± 0.058	0.537± 0.049	0.494± 0.034
Measured dietary fibre (g/100g)	8.538± 0.439	6.003± 0.310	6.363± 0.347	7.903± 0.641	8.723± 0.483	5.828± 1.065	9.209± 0.639	5.322± 0.551	6.822± 0.047
Total weight	1000.0	1000.0	1000.0	1000.0	1000.0	1000.0	1000.0	1000.0	1000.0
Percentages									
Total Protein %	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0
Total carbohydrate %	61.7	61.7	61.7	60.1	55.8	54.9	57.1	59.3	59.3
Total Fat %	7.0	7.0	7.0	7.5	9.0	9.0	8.9	7.0	7.0
Estimated Energy content (kcal/kg)	3904.4	3904.4	3904.4	3904.4	3904.4	3847.5	3904.4	3904.4	3904.4

3.3 Folate Analysis

Liver, feces and dietary samples were analyzed for total folacin in triplicate and on different days by modification of the American Association of Cereal Chemists/Association of Official Analytical Chemists AACC/AOAC (1999) method 86-47. This is a microbiological assay with *Lactobacillus casei rhamnosis* class I (*L.casei*). *L. casei* is a bacterium that requires folate for growth. A three-enzyme procedure was used to breakdown protein and carbohydrate in sample matrices and to hydrolyse polyglutamate form of folate to one, two and three glutamate forms that are available to the bacteria, prior to measurement with *L. casei*. In this procedure, the freed folate was diluted with Difco *L. casei* folic acid basal medium containing all required growth nutrients except folate. The folate-dependent bacteria were then added and allowed to grow. The turbidity of the *L. casei* (ATCC 7469) growth response of the samples was compared quantitatively to that of a known standard folate solution (Folic acid USP, Pteroylglutamic acid; Vitamin M, Sigma Chemical, St. Louis, MO, U.S.A).

3.3.1 Enzymes

All three enzymes were prepared fresh at the time of the experiment.

1. α - Amylase (EC 3.2.1.1 from *Aspergillus oryzae*; 20 mg/ml)

0.5 g α -amylase (Sigma Chemical Co., St Louis, MO, USA; No. A-6211) was suspended in 25 ml of double distilled water (ddH₂O).

2. Protease (Pronase E, Type XIV: Bacterial from *Streptomyces griseus*; 2mg/ml)

0.05g protease (Sigma Chemical Co., St Louis, MO, USA; P-5147) was suspended in 25ml ddH₂O.

3. Conjugase (5mg/ml)

Chicken pancreases conjugase (Difco Laboratories, Detroit, MI, USA; No 0459-12-2) was suspended in 1.42% Na₂ HPO₄ buffer, pH 7.8, containing 1% ascorbic acid and stirred vigorously for 10 minutes.

The suspension of each enzyme was treated with the anion exchange resin Dowex 1-X8-400 (Sigma Chemical Co., St Louis, MO, U.S.A). Dowex was used to remove excess folate and by-products that remained in a solution. Ratio of 1g Dowex: 10 ml liquid (2.5 g: 25 ml) was used and solution mixed on a rocking panel for 30 minutes. The solution was then centrifuged for 10 minutes at about 3000 rpm. The supernatant was decanted through a microfibre filter paper size 12.5 cm (VWR Scientific product West Chester, PA 19380, USA) into a beaker, in order to remove the nonionic impurities which may interfere with the measurement. Enzymes were then covered with parafilm and refrigerated. Based on methodology developed in our laboratory, lower blank values were obtained when enzymes were treated with anion exchange resin Dowex 1-X8-400.

Figure 3.1: Standard assay curve for folate using *L. casei*. Enzymes were treated with no dowex (top), dowex treated enzymes (bottom).

3.3.2 Standard solutions

1. Folic acid standard solution of 100 µg/ml

A folic acid stock standard solution was prepared according to AOAC guidelines (1999). A 50 mg sample of USP folic acid (Petroylglutamic acid, Vitamin M, C₁₉H₁₉N₇O₆, Molecular weight: 441.41, Sigma Chemical Co., St Louis, MO, USA; No. F-8798) was dissolved in 0.1 M pH 7.0 potassium phosphate buffer in a 500 ml volumetric flask and diluted to volume with 0.1 M phosphate buffer. This stock solution was stored, refrigerated, in the dark by covering it with aluminum foil. The purity of the standard and its concentration were determined by diluting 10 ml of standard with 90 ml of 0.1 M pH 7.0 phosphate buffer. The absorbance was measured at two wavelengths, 282nm and 346nm. The concentration of the diluted standard was calculated based on Beer's law.

$$\text{Folic acid concentration in stock solution (FA } \mu\text{g/ml)} = \frac{\text{absorbance}}{\epsilon} \times \text{dilution factor} \times 1000 \times M_w \times 1\text{cm}^{-1}$$

Where $\epsilon = 27,600 \text{ M}^{-1}\text{cm}^{-1}$ at 282nm and $7200 \text{ M}^{-1}\text{cm}^{-1}$ at 346 nm, Molecular weight (M_w) = 441.4 g/mole

2. Intermediate standard solution of 1µg/ml

An intermediate standard solution of 1µg/ml was freshly prepared by diluting 1 ml of stock solution with approximately 90ml water and the pH was adjusted to 7.5 with HCl. The volume was then brought to 100ml with water.

3. Working standard solution of concentration 3ng/ml

A working standard solution of concentration 3ng/ml was freshly prepared by placing 150 µl of the intermediate solution in 50 ml. The standard was then treated

identically to the samples. The working standard solution was used to construct a seven point standard curve using the following concentrations 0.088, 0.132, 0.197, 0.296, 0.444, 0.666, 1.000 ng/ml which are obtained as follows:

The concentration of folate in the stock standard was 100µg/ml. A 1:100 dilution of the stock solution was prepared (1 µg/ml). From this standard solution, 0.15 ml was added to 50 ml in a deconjugation tube. At this point the standard concentration was 3ng/ml.

A 150 µl aliquot of the above concentration (3ng/ml) was added to wells G1-G2 of a 12x8, 96-well microplate (**Figure 3.3**), to which 75 µl of plate buffer pH 6.8 had already been added. The standard concentration was therefore, further diluted by 2/3rds, and the standard concentration was 2ng/ml.

A 150 µl aliquot of the 2 ng/ml concentration was removed for further serial dilutions (leave 75 µl of 2 ng/ml in first standard well). Then another 75 µl of the plate buffer was added to wells after the dilutions have been made and the standard concentration would be 1ng/ml.

Therefore the range for our standard curve was 1.0 ng/ml to 0.0877 ng/ml, and the concentration of folate standard in each well of the microplate is as follows:

G1-G2	1.0000 ng/ml
F1-F2	0.6660 ng/ml
E1-E2	0.4443 ng/ml
D1-D2	0.2962 ng/ml
C1-C2	0.1974 ng/ml
B1-B2	0.1316 ng/ml
A1-A2	0.0877 ng/ml

3.3.3 Inoculum Preparation

In order to prepare inoculum, lyophilized *Lactobacillus casei rhamnosis class I* *L.casei* (ATCC No.7469 American Type Culture Collection, Rockville, MD, U.S.A.) was suspended in 1.0 ml of Difco folic acid casei medium (4.7g Difco Folic Acid Casei Medium in 50 ml of double distilled water, boiled, and cooled on an ice bath). A 50 ml aliquot of distilled water, 25 mg of sodium ascorbate, and 0.5 ml of diluted folic acid stock solution (100ng/ml) was added to the solution and it was sterile filtered.

A 0.15 ml aliquot of the bacterial solution was added to the culture medium aseptically and incubated at 37 °C for 18 hours. The bacterial culture was then cooled on an ice bath and 100 ml of cold 80% glycerol was added to the solution. The glycerol was not more than one year old, as it may become toxic after prolonged storage (Freshney, 1994). The solution was mixed very well and gently rotated by hand after addition of glycerol. Aliquots of 2.0 ml were placed in disposable sterile cryogenic round bottom, polypropylene tubes and stored at -80 °C until use.

3.3.4 Control Sample

Powdered infant formula (NIST Standard Reference Material # 1846, from The National Institute of Standards and Technology, Gaithersburg, MD, 20899) which is a spray-dried, milk-based infant formula was used as an internal check sample. The mass fraction value for folic acid in the formula is $1.29 \pm 0.28 \mu\text{g/g}$ folate.

3.3.5 Sample preparation

1. Liver samples

Approximately 2.5 g samples of liver were homogenized in 22.5 mL buffer (phosphate buffer pH 7.8, 10 mg/mL ascorbic acid) using a Kinematica CH-6010 Kriens-LU homogenizer. The homogenate was divided into four test tubes (5 mL polypropylene round-bottom falcon tube, Becton Dickinson and company, Franklin Lakes, NJ. U.S.A) of 4 mL each and kept frozen at -20°C until use. Prior to analysis, liver homogenate samples were thawed at room temperature. About 2 mL of the samples were pipetted into 50 mL autoclaveable centrifuge tubes. To each sample was added 10 mL buffer, pH 7.8, 15 mL ddH₂O, 0.15 mL octanol. Samples were autoclaved for 15 minutes at 121°C . Then they were cooled, and 5 mL additional buffer was added to each tube. To each sample tube was added 0.5 mL of protease enzyme and the samples incubated at 37°C for 3 hours. The tubes were boiled for 5 minutes to inactivate the protease enzyme and cooled on ice. A 0.5 mL aliquot of amylase was added to each tube and incubated at 37°C for 2 hours. A 2 mL aliquot of chicken pancrease (CP) was added and the samples were incubated for 16 hours at 37°C . The following morning, the samples were boiled for 10 minutes and their pH was adjusted to 4.5. The volume of the tubes was adjusted to 50 mL in a volumetric flask by adding ddH₂O. Samples were then filtered through a 0.22 μm syringe filter.

2. Fecal samples

Fecal samples were collected by placing fresh cardboard mats under the rat cages. Fecal pellets were collected from under each cage 24 hours later and kept in pre-weighted plastic containers.

Fecal samples were freeze-dried and the percentage moisture of each sample was determined. Samples were ground to a constant size of 2 μm by using a 20-mesh screen in a Wiley mill (Arthur Thomas Co. Scientific Apparatus, Philadelphia, P.A, U.S.A). The total folate concentration of the feces was determined in about 0.4-0.5 g of the dried feces. The methodology was identical to that used for the liver samples except dry feces were added to the same volume of ddH₂O instead of phosphate buffer pH 7.8.

3. Diet samples

A 10 g representative sample of each of the diets was stored in a lab fridge (4 $^{\circ}\text{C}$) in the dark. The folate concentration in each sample was measured as described for the fecal samples.

3.3.6 Folic Acid Casei Media

The assay medium was prepared by dissolving 9.40 g of Folic Acid Casei dehydrated medium (Difco Laboratories, Detroit MI 48232-7058 U.S.A) and 50 mg of L-ascorbic acid (Vitamin C, Sigma Chemical Co. P.O Box 14508 St. Louis, MO, 63178, U.S.A) into approximately 90 ml of dd H₂O. The media was boiled for 90 seconds (from the time the solution started to boil) and cooled on an ice bath. The pH was adjusted to 6.70 using 1N HCl. The total volume of the solution was brought up to 100 ml using ddH₂O. The media was filter sterilized through a 150 mL presterilized Millipore express 0.22 μm membrane (Millipore Corporation, Bedford, Massachusetts, U.S.A 01730).

On the day of the experiment, 1 mL of the thawed inoculum was mixed with 2.5 mL of normal saline. A 25 μL aliquot of the above solution was added to 25 mL of the medium. 14.4 mL of the inoculated media was used for each 96-well microplate.

Before choosing a plate buffer, three different concentrations (1X, 2X, 3X) of phosphate buffer pH 6.8 were tested in order to obtain maximum growth of *L. casei* (Figure 3.2). Standard curves of folate concentration using different plate buffer concentrations indicated that *L. casei* bacteria have a better growth in one fold concentration of phosphate buffer as compared with other concentrations. It seems that higher concentrations of phosphate buffer have an inhibitory effect on *L. casei* growth. Therefore all microplate folate assays used 1X phosphate buffer.

Figure 3.2: Effect of different concentrations of phosphate buffer on *L. casei* growth.

Figure 3.3: Schematic diagram of microplate used for folate assay.

3.3.7 Microplate assays

Each microplate was incubated in a sealed container with water at 37 °C for 22 hours. After the incubation time it was allowed to equilibrate at room temperature for 30 minutes. Wells were mixed using a 12-channel pipettor from lowest to highest concentrations and read at 595 nm using a Dynatech MR 5000 (Dynatech laboratories Inc. 14340 Sullyfield Circle, Chantilly, VA 22021, U.S.A) and using Biolinx Version 2.2 software to analyse results. A standard curve with a range of 0.0877 ng/ml to 1.0 ng/ml was plotted for each plate to allow calculation of the unknown samples.

A standard concentration response curve was prepared for each microplate assay by plotting a sigmoidal fit of the average optical density reading for each level of standard solution against the amount of folate contained in each well. From this standard curve, folate concentration for each sample was determined.

The extreme upper and lower portions of the standard curve do not give a reliable result. The linear portion of the curve (mostly between the 0.666 ng/ml and 0.1316 ng/ml) was used for the calculation of assay values only. Samples with high concentrations of folate, were measured several times with different dilutions. This ensured that their concentration fell within the linear portion of the standard curve (Figure 3.4).

Data from the microplate reader was copied to a predesigned spreadsheet to calculate the concentration of folate in the analyzed sample.

Figure 3.4: Standard assay curve for folate using *L. casei*.

3.4 Short Chain Fatty Acid Analysis

Short chain fatty acids (SCFAs) were analyzed by gas chromatography.

3.4.1 Standard mix

A volatile acid standard mix (Supelco, Supelco park, Bellefonte, PA, U.S.A, Lot # LA94808, cat # 46975), which included acetic acid, propionic acid, butyric acid, isobutyric acid, valeric acid, isocaproic acid, isovaleric acid, hexanoic acid, and heptanoic acid was used for the identification of the elution time of the SCFAs.

3.4.2 Sample preparation

About 1g of the cecal material was added to 3.5 mL distilled water plus 40 μ L of 50 % (v/v) sulphuric acid. A 0.5 mL aliquot of 10 mM 2-Ethylbutyric acid (Aldrich # 10.995-9, Lot # 24016) was added as an internal standard (Aldrich # 10.995-9, Lot # 24016, M_w = 116.16 g/mole). The sample was homogenized for 15 sec using a Kinematica CH-6010 Kriens-LU homogenizer and centrifuged at 15000 rpm for 30 minutes. After centrifugation, the supernatant was filtered through a 0.45 μ m filter (Acrodisc 25 mm filter, Gelman Sciences, 600 S. Wagner Rd, Ann Arbor, MI, U.S.A, 48103). One microlitre of the sample was used for chromatographic analysis with a Hewlett Packard 5880A Series Gas chromatograph with a fused silica capillary column 60m x 0.25mm x 0.25 μ m film thickness coated with nitroterephthalic acid modified polyethylene glycol polymer (Supelco, Inc., Supelco Park, Bellefonte, PA 16823-0048). The oven temperature was increased from 100 $^{\circ}$ C to 140 $^{\circ}$ C at a rate of 5 $^{\circ}$ C per minute, held at this temperature for 1 minute, increased to 200 $^{\circ}$ C in 10 minutes, and held at this final temperature for 15 minutes. The detector and injector port temperatures were 325 $^{\circ}$ C

and 225°C respectively. Helium was used as the carrier gas with a flow rate of 0.6 ml/min. Detector air flow was at a rate of 300 ml/min, detector hydrogen flow was at a rate of 30 ml/min and post column He flow was 24 ml/min. The data were plotted and integrated using an integrator (Hewlett Packard series II). SCFA compositions and amount of samples were calculated using standard response factors, which were determined from the standard mixture run at the beginning of samples run. The response factors (R_i) for each fatty acid were calculated according to the Ali *et al*, (1997).

$$R_i = \frac{\text{Peak area of the fatty acid in Standard} \times \text{Molarity of the internal standard}}{\text{Molarity of the fatty acid in Standard} \times \text{Peak area of the internal standard}}$$

Where R_i is response factor.

The concentration of each fatty acid in the test samples was calculated according to the following equation.

$$\text{Conc'n of fatty acid (mM)} = \left(\frac{\text{Peak area of the sample}}{\text{Peak area of the internal standard}} \right) \times \left(\frac{\text{Molarity of internal standard}}{\text{Response factor}} \right)$$

3.5 Fibre Analysis

Total dietary fibre in diets and fecal samples was determined by a combination of enzymatic and gravimetric methods, using the rapid Health Protection Branch (HPB) method. This method (Mongeau and Brassard, 1986) consists of parallel measurements of insoluble fibre and soluble fibre from two separate duplicate samples of about 0.4-0.5 g of freeze dried feces and diets which were ground through a 20-mesh screen.

Soluble fibre (the fibre material which dissolves in water at 100°C and precipitates in alcohol) was determined using a Fibretec-E (Tecator). The preweighed

duplicate samples were placed in 50 mL, screw cap glass tubes. To each tube, 10 mL distilled water, 2 mL of 2 M acetate buffer pH 4.5 and another 10 mL distilled water to wash down sides of tube, were added, and autoclaved at 120°C, 15 psi for 60 minutes. The tubes were removed from the autoclave and 100 µL of heat stable α -amylase (A-3306, Sigma Chemical Co, P.O Box 14508 St. Louis, MO, 63178, U.S.A) was added. The tubes were mixed and incubated in a boiling water-bath for 30 minutes, with mixing by hand at 10-minute intervals.

Soluble fibre was separated by filtration hot into flasks on a fibretec-E filtration apparatus using P₂ crucibles containing 2g of celite. The tubes were rinsed twice with 5 mL hot water and added to the crucibles. After filtration, the crucibles were removed from the instrument and saved for insoluble fibre determination. Rinsing of the Fibretec-E tubing with 5-ml hot water prevented contamination between samples. Four mL aliquot of 2 M sodium acetate and 300 µL of amyloglucosidase (A-9913 from *Aspergillus niger*, Sigma Chemical Co, P.O Box 14508 St. Louis, MO, 63178, U.S.A) was added to the filtrate flasks mixed and incubated in a water-bath at 60°C for 30 minutes. Without removing the flasks from the water-bath, 100 µL protease solution, 50 mg/ml (P-3910, Sigma Chemical Co, P.O Box 14508 St. Louis, MO, 63178, U.S.A) was added to each flask shaken in the water-bath for 5 minutes, covered and incubated for a further 25 minutes at 60 °C. The enzymes were added to remove residual starch and most of the protein. Anhydrous ethanol (166 mL) was added to each of the flasks, mixed, covered and left at room temperature for one hour to allow precipitation. The contents were filtered through a 50ml medium Gooch crucible, with a porosity of 10-15 µm containing a mat of celite prepared by adding celite to crucible, adding water, filtering and then

drying on bench top. The crucibles were rinsed twice with two 20 mL portions of 80% v/v ethanol and then rinsed with 20 mL of acetone. The crucibles were dried overnight in an oven at 105°C and weighed hot by a hot weighing technique. Crucibles and contents were placed in a muffle furnace and ashed at 525 °C for 4 hours. They were then placed in an oven at 105°C for 2 to 3 hours, and weighed hot every 30 seconds by placing a hot crucible on the analytical balance plate for 1 minute to heat up the balance prior to zeroing the balance. At time zero, the crucible was removed from the oven and placed on the balance plate. At time 20 sec, the weight of the crucible was recorded and the crucible was removed from the balance. Time 30 sec, the zero of the balance was recorded and another crucible was placed on the balance plate. The zero deflection due to the temperature change of the balance was subtracted for each weight.

$$\% \text{ soluble fibre} = \{[(\text{cruc} + \text{solfibre}) - (\text{cruc} + \text{ash})] - \text{wt of blank}\} \times 100 / \text{wt of dried sample}$$

Insoluble fibre was determined by refluxing precipitate in P2 crucible set aside when determining soluble fibre with neutral detergent using a Fibretec 2010 extractor (Tecator, Sweden) for 60 minutes from the time of boiling, and then remove neutral detergent by vacuum filtration followed by a 65 minute treatment with α -amylase (A-3176 EC.3.2.1.1 Type VI-B from Porcine Pancreas, Sigma Chemical Co, P.O Box 14508 St. Louis, MO, 63178, U.S.A) to remove residual starch and most protein. The α -Amylase solution was prepared daily by mixing 7.5g α -amylase powder for 15 minutes with 150 mL buffer solution, pH 7 (61 mL 0.1 M Na_2HPO_4 + 39 mL 0.1M NaH_2PO_4), centrifuging for 10 minutes at 3000 rpm and filtering through a coarse Gooch crucible

containing glass wool. After refluxing with neutral detergent, the crucibles were filled with 10 mL cold α -amylase solution and about 15 mL hot distilled water and held on the fibretec heat extractor system for five minutes. Filter crucibles were washed with 25 mL hot distilled water twice. A number 7- rubber stopper was used to seal the bottom of the crucibles. The crucibles were filled with 10 mL cold alpha-amylase solution and 15 mL hot distilled water and were placed in an oven at 55 °C for 60 minutes. After incubation, stoppers were removed and the crucibles were placed on the Fibretec E and samples were filtered. The residues were washed four times with 25 mL hot distilled water and twice with 20 mL of acetone. After the acetone was evaporated, the crucibles were dried overnight at 105 °C and weighed hot. The crucibles and contents were placed in a muffle furnace and ashed at 525 °C for four hours, placed in an oven at 105°C for 2 to 3 hours and weighed hot. The insoluble fibre content of each sample was calculated as follows:

$$\% \text{ insoluble fibre} = \{[(\text{wt cruc} + \text{wt residue}) - (\text{wt cruc after ashing})]\} \times 100 / \text{wt of dried sample}$$

Total dietary fibre value was obtained by adding the soluble fibre value to that of insoluble fibre value.

$$\% \text{ Total Fibre} = \% \text{ Soluble Fibre} + \% \text{ Insoluble Fibre}$$

A blank was run in duplicate through the entire procedure to control possible contributions from reagents to final results. These results were used to determine the total dietary fibre and excreted fibre.

Total fibre intake (g) =

$$[\text{Food consumption during the repletion phase (g)}] \times [\text{Dietary fibre (g/g)}]$$

Total fibre excreted (g) = [Fecal output (g)] $\times$ [fecal fibre (g/g)]

3.6 Measurement of Blood parameters

A 2 mL aliquot of the rat blood was collected in trace element-free evacuated tubes containing EDTA (Becton Dickinson vacutainer systems, Rutherford, NJ). Complete blood counts (red and white blood cell counts, platelet count, hematocrit, hemoglobin, mean cell volume, mean cell hemoglobin, and mean cell hemoglobin concentration) were determined by the hematology laboratory, Animal Resource Division, Health Canada, using a Coulter S Plus IV (Coulter Electronics, Hialeah, Fla.) instrument. The leukocyte differential determinations were done by using Wright Stain smears.

3.7 Phase 2: Animals

A total of 133 male weanling rats (45.29 ± 2.81 g, mean $\pm$ SD; 30 day old, Charles River Breeding Laboratories, Wilmington, MA) were individually housed in mesh-bottomed stainless steel cages in a room with a controlled temperature (23 ± 2 °C) and a twelve hour light dark cycle. In the depletion phase, rats were fed a low folate AIN 93G basal diet with free access to tap water for 28 days. At the end of the depletion phase, 7 rats were anesthetized with Isoflurane[®] and their blood, livers, cecal contents, and feces were removed. The remaining 126 folacin-depleted animals were divided into 18 groups of 7 animals randomly assigned to cages. These animals were fed repletion

diets containing variable amounts of folic acid (Sigma Chemical Co., St. Louis, MO 63178) as well as different sources of dietary fibre (ground corn, inulin, wheat germ, and polydextrose). Succinylsulfathiazole was also added to one half of the groups to inhibit intestinal bacteria growth (the composition of the phase 2 diets and the level of the folate and fibres sources are shown in **Table 3.2**). After 27-29 days of repletion, rats were randomly selected from each diet group, sacrificed with blood, liver, cecal contents, feces collected for analysis.

3.8 Phase 2: Formulation of Diets

All diets were formulated according to the American Institute of Nutrition (AIN 93G) recommendations (American Institute of Nutrition, 1993) as described in **Table 3.1**. Folate, fibre, and short chain fatty acid analysis were determined as described in Phase 1.

3.9 Phase 2: 2-6-Diaminopimelic acid analysis in rats feces

The concentration of 2-6-Diaminopimelic acid (DAPA) in rat feces was determined by Mr. Brian Lampi, of the Nutrition Research Division Of Health Canada, based on a modification of methods described by Czerkawski, (1973), El -Shazly, and Hungate (1966).

1. Ninhydrin Reagent

Ninhydrin, 25.0 g, (Sigma N-4876) was dissolved in mixture of 600 mL glacial acetic acid and 400 mL 6M phosphoric acid.

2. Chromosep SPE SCX column

A 500 mg, 2.8 mL column was purchased from Chromatographic Specialist (Cat #C612099). The column was washed with 5 mL, 6M HCl.

3. Carbograph SPE column

A 300 mg, 6 mL column was purchased from Chromatographic Specialist (Cat #C61210101).

4. Standard preparation

Standard DAPA (D-1377, Lot # 116H1265, MW=190.2, Sigma Chemical Co., St. Louis, MO 63178) was dissolved in 0.1 M citrate buffer, pH 4.2 and standard concentrations of 60, 30, 15, 7.5, 3.75, 1.875, 0.9375, and 0 $\mu\text{g/mL}$ were prepared using serial dilutions from a stock standard of 120 $\mu\text{g/mL}$.

5. Sample preparation

The procedure involved hydrolyzing 50 mg of dry rat feces with 3 mL of 6M HCl by incubating at 105 $^{\circ}\text{C}$ overnight in sealed tubes. The hydrolysate and 2mL 6M HCl (washing solution) were transferred to carbograph columns, which had been already washed with 3 mL 6M HCl. The columns were allowed to void.

A 3 mL 20% ethanol solution was added to the columns in order to remove any residual DAPA. All the eluents were then collected in 100 x 16 RTU glass tubes and dried at 70 $^{\circ}\text{C}$ under nitrogen gas. Dried samples were quantitatively transferred with 2x 5mL, 0.05M citric acid to 500 mg Chromosep SCX Columns which had already been washed with 2 mL 0.05 M citric acid and allowed to swell for 30 minutes. Columns were washed by adding 40 mL 0.1 M citric buffer, pH 3.0, to the column and the eluents were discarded. DAPA was extracted with 10 ml 0.1M citrate buffer, pH 4.2 and collected in preweighed 15 mL polypropylene centrifuge tubes. The tubes and eluent were reweighed to determine the volume of eluent for analysis.

6. Ninhydrin reaction:

A 1 mL aliquot of unknown or standard was added to 16 x 100 RTU glass tubes followed by 2 mL ninhydrin reagent. The top of each tube was covered by a marble and heated at 100⁰ C for exactly 5 minutes. After cooling to room temperature, a 250 µL aliquot of each sample was transferred to a microplate and the absorbance was read at 425 nm. The concentration of DAPA for each sample was calculated from a linear standard curve (**Figure 3.5**).

Figure 3.5: DAPA standard curve.

3.10 Muramic acid analysis in rat feces

Muramic acid was determined by Mr. Brian Lampi, of the Nutrition Research Division of Health Canada based on the Hadzija (1974) method.

1. Standard solution

Standards containing 0 to 50 µg/mL of lactic acid from a muramic acid stock solution were prepared by dissolving muramic acid (2- Amino-3-O- [1-carboxyethyl]-2deoxy-D-glucose, $C_6H_{17}NO_7$, MW=251.2, M-2503, Sigma Chemical Co, P.O Box 14508 St. Louis, MO, 63178, U.S.A) in water to give a 1 mg/mL solution. Muramic acid contains 30% by weight of O-ether-linked lactic acid.

2. Copper sulfate solution

$CuSO_4$, 4 g, (MW=159.6, C-1297, Sigma Chemical Co, P.O Box 14508 St. Louis, MO, 63178, U.S.A) was dissolved in 100 ml of ddH₂O.

3. *p*-Hydroxydiphenyl reagent

p-Hydroxydiphenyl, 1.5 g, (*p*-Phenylphenol,) was dissolved in 100 ml of 95% (vol/vol) ethanol.

Method:

A 3 mL aliquot of 6M HCl was added to about 70 mg of freeze dried feces and hydrolysed in reaction vials with a Teflon-lined screw cap overnight at 105°C to remove D-lactate from the 3-position of muramic acid. The samples were then cooled and a 0.2 mL aliquot was added to 15 mL glass tubes with a Teflon-lined screw cap. Hydrochloric acid was evaporated at 70°C under nitrogen, since it interferes with the reaction forming a green precipitate, probably cupric chloride. A 1 mL aliquot of dH₂O was added to the

samples. Then 0.5 mL of 1.0 N NaOH was added and the samples incubated at 37°C for 30 minutes. After incubation, 10 mL of concentrated sulphuric acid was added to the tubes. The tubes were then sealed and heated in a boiling water-bath for 10 minutes. Samples were cooled, and 0.1 mL of the copper sulfate reagent (4 g of CuSO₄ was dissolved in 100 mL ddH₂O, and 0.2 mL of the *p*-Hydroxydiphenyl reagent (1.5 g of *p*-Hydroxydiphenyl was dissolved in 100 mL of 95% ethanol) were added, mixed and incubated at 30°C for 30 minutes in a water bath.

A 200 µL aliquot of each sample was transferred to a microplate and the absorbance was measured at 560nm. A reagent blank was used to adjust the spectrophotometer to zero absorbance. The concentration of muramic acid was determined from a standard curve of known concentration of muramic acid (Figure 3.6).

Figure 3.6: Muramic acid standard Curve.

3.11 Phase 2: Kjeldahl Nitrogen Analysis

Total nitrogen in rat feces was determined by Mr. Brian Lampi of the Nutrition Research Division, Health Canada. Dried rat feces (0.4-0.5 g) were weighed into digestion tubes and digested at 350°C for at least 1.5 hour in 7 mL concentrated sulphuric acid with a Kjel tab pellet (potassium sulfate, 95.24%, and mercuric oxide 4.76%) in a Tecator 1016 Digestion system 40. Nitrogen was liberated and retained as ammonium sulfate. About 10 minutes after the digestion, 25 mL of the dH₂O was added to the tubes having a clear solution at this stage. Samples were then placed in the Kjeltec Auto 1030 Analyzer (TECATOR) and steam distilled. The resulting ammonia was collected and titrated with 0.200 N HCl until the methylred end point color indicated all ammonia had been titrated.

$$\% N = 14.01 \times 0.200 \times f \times 100 / \text{mg sample} \times (\text{mL titrant} - \text{mL blank})$$

Where atomic weight of nitrogen is 14.01 g/mole

Molarity of acid is 0.200 mole/L

F; Standard Kjeldahl factor (f=1 if determining %N)

3.12 Statistical Data analysis

All data were checked for a potential correlation between means and standard deviations prior to analysis by ANOVA. When a correlation was observed (**Figure 3.7**) the data was transformed using the formula $[(\text{value}^\lambda - 1)/\lambda]$. Arbitrary values of λ were chosen to reduce the correlation to almost zero (**Figure 3.8**).

All data was analysed by a 2 factor ANOVA (diet $\times$ drug), unless otherwise indicated, values are reported as means $\pm$ standard deviation. Post Hoc analysis, when

warranted by a significant F values were performed by Tukey's HSD test (Sigma Stat version 5, 97 edition, Jandel Scientific, San Rafael CA). The comparison of the groups are reported as the a, b, c superscript. A value of $P < 0.05$ was taken as the criterion of significance.

Figure 3.7: Relationship between standard deviation and means.

Figure 3.8: Relationship between standard deviation and means.

CHAPTER IV

Results

Phase 1

Folate repletion kinetics were determined by feeding rats a depletion diet for 28 days and then sacrificing eight rats at the beginning of repletion phase (time zero) and seven rats from each diet group every week after, for a total of four weeks (120 rats).

Liver and fecal folate were analyzed by a bacterial growth assay preceded by a three-enzyme extraction procedure. Short chain fatty acids in cecal contents were analyzed by gas chromatography as an indirect indication of fermentation.

Food Consumption and Body weight

No physical signs of folate deficiency (tendency for the eyelids to stick together, thinning of the hair on the head, loss of weight) were observed in the rats after 28 days of depletion. The mean food consumption and mean body weights during this period were 26.23 ± 2.38 g/d and 191.38 ± 13.14 g respectively.

Food consumption and body weights were not statistically different between diet groups during the repletion phase. However, differences in food consumption and body weight were noted over time (**Table 4.1** and **Figure 4.1**).

Hematology parameters

Leukocytes, erythrocytes, platelets, hemoglobin, hematocrites, mean cell volume, mean cell hemoglobin, and mean cell hemoglobin concentration were determined in the 28d animals of the repletion phase (**Figure 4.2**). Analysis of variance indicated no significant effect of diet or day on different parameters. The mean cell hemoglobin

concentration (MCHC), showed a significant (diet×day) interaction (**Figure 4.3**) but further analysis showed that this was not relevant.

Liver Folate

Feeding rats a depletion diet for 28 days reduced their liver folate content from approximately 7 µg/gw to a value of 3µg/gw. Repletion with folate-containing diets showed a variable response (**Figure 4.4**) with diets higher in folate giving higher liver folate concentrations. The values began to plateau around 28 days.

The experiment was designed to test the contribution of fermented material to physiologically available folate. In order to assess the ability of various fibre sources to contribute to liver folate, secondary graphs of liver folate versus total dietary folate were constructed for each time point (**Figure 4.5** and **Figure 4.6**). Liver folate on day 7 and 14 of the repletion phase linearly correlated with the folate content of the diets (**Figure 4.5**). On day 21 and 28 liver folate in rats fed with oat bran was higher than that of the wheat bran diets, even though the measured folate content of the oat bran diet was lower compared with the wheat bran diet (**Figure 4.6**).

Cecal Short chain fatty acids (SCFAs)

Short chain fatty acids in rat cecum were monitored to assess fermentation indirectly. The cecal short chain fatty acid concentrations were low in the depletion phase and increased in the repletion phase in both wheat bran and oat bran diets. This suggests that these diets promoted increased bacterial fermentation (**Figure 4.7** and **Figure 4.8**).

Table 4.1: Food consumption during the repletion period in different diet groups¹

Diet	Food Consumption (g/d)		
	Day 7 ^a	Day 14 ^{b,c}	Day 28 ^c
Low Standard	31.24±2.09	25.15±2.44	23.44±2.07
High Standard	31.07±2.96	25.33±2.73	24.54±2.74
Wheat Bran	31.93±3.79	26.89±2.36	24.16±0.78
Oat Bran	30.76±3.60	26.65±2.68	24.51±1.67

¹Results are expressed as means ± stdev on a sample size of 7 rats/group. Within a column, means are not significantly different (P>0.05). ANOVA analysis revealed a significant effect of diet (P=0.0000) on weekly food intake. The comparison of the groups are reported as the superscript a, b. Groups with the same superscript are not statistically different.

Pooled means for each day are: Day 7= 31.00 ± 0.45 g/d; Day 14= 25.58 ± 0.50 g/d; Day 21= 26.18 ± 0.35 g/d; Day 28 = 24.08 ± 0.36 g/d.

Figure 4.1: Rat body weight in different weeks of the repletion phase¹.

¹Results are expressed as means $\pm$ stdev on a sample size of 7 rats/group.

ANOVA analysis revealed a significant effect of diet ($P=0.0000$).

Groups with the same superscript are not statistically different.

Figure 4.2: Hematology parameters as a function of diets in the depletion, and repletion periods of phase one¹.

¹Depletion phase (depl), low standard (LS), high standard (HS), wheat bran (WB), oat bran (OB). Leukocytes (LKCS); erythrocytes (ERCS); hemoglobin (HB); hematocrite (Hct); mean cell volume (MCV); mean cell hemoglobin (MCH); mean cell hemoglobin concentration (MCHC); red cell distribution width (RDW); platelets (PLT); mean platelet volum (MPV).

Groups with identical letters are not significantly different as determined by ANOVA analysis followed by Tukey's LSD analysis.

MCHC

Figure 4.3: Mean cell hemoglobin concentration (g/Litre of red blood cells) as a function of days of the depletion and repletion period in phase one¹.

¹Depletion diet (open circles), low standard (closed circles), high standard (open squares), wheat bran (closed triangles), and oat bran (inverted triangles).

Figure 4.4: Liver folate concentration as a function of time.

Rats fed diets containing, 0.50 mg added folacin /kg of diet (low standard, closed circles), 1.32 mg added folacin /kg of diet (high standard, open squares), 0.86 mg added folacin /kg (wheat bran fibre, closed triangles), or 0.52 mg added folacin /kg (oat bran fibre, inverted triangles).

Figure 4.5: Liver folate concentration as a function of dietary Folate in day 7 (Top) and 14 (Bottom), of the repletion phase. Low standard (closed circle), high standard (open square), wheat bran (closed triangle), oat bran (inverted triangle).

Figure 4.6: Liver folate concentration as a function of dietary Folate in day 21 (Top) and 28 (Bottom), of the repletion phase. Low standard (closed circle), high standard (open square), wheat bran (closed triangle), oat bran (inverted triangle).

Figure 4.7: Short chain fatty acid concentration as a function of time in rats fed with different diets.

Low basal diet in depletion phase (open circles), low standard (closed circles), high standard (open squares), wheat bran (closed triangles), oat bran (inverted triangles).

Figure 4.8: Short Chain Fatty acid concentration as a function of time in rats fed with different diets.

Low basal diet in depletion phase (open circles), low standard (closed circles), high standard (open squares), wheat bran (closed triangles), oat bran (inverted triangles).

Phase 2

Status of Rats after Depletion and Repletion Period

Phase 1 indicated that a 28 day repletion period was optimal for measuring folate liver repletion. The second phase of the experiment, therefore, measured the effect of several different dietary fibre and fermentable substrates after 28 days of repletion.

Rat liver and fecal folate were analyzed by a bacterial growth assay preceded by a three-enzyme extraction procedure. Cecal short chain fatty acids content and fecal nitrogen were analyzed by gas chromatography and Kjeldahl analysis, respectively, as an indirect indication of fermentation. Diamonopimelic acid (DAPA), and muramic acid (MA) in feces were analyzed as an indirect measure of bacterial numbers in the large intestine.

Food Consumption and Body weight

Similar to phase 1, no physical signs of folate deficiency (like the tendency for the eyelids to stick together, thinning of the hair on the head, loss of weight) were observed after the 28 day depletion period of phase 2. Mean food consumption and mean body weights during this period were 17.00 ± 3.81 g/d and 248.12 ± 7.83 g respectively.

During the repletion phase, rats were fed one of nine different diets containing dietary fibre and fermentable substrates with or without the sulfa drug, Succinylsulfathiazole (0.5%) (Table 3.2a, Table 3.2b). ANOVA analysis revealed no significant effect of diet or drug on weekly food intake (Figure 4.9) or body weight (Figure 4.10). This was true, despite an apparent but non-significant increase in food intake of animals fed ground corn diets (Figure 4.9).

Hematology parameters

The leukocyte, erythrocyte, and platelet count as well as red cell hemoglobin, hematocrite, mean cell volume, mean cell hemoglobin, and mean cell hemoglobin concentration were determined in the depletion and the repletion phase.

The leukocyte count was higher in animals fed diets containing oat bran, ground corn, wheat germ and polydextrose in the presence of 0.5% SST (**Figure 4.11**). However, statistical analysis of variance indicated no significant effect of diet ($P=0.1123$) or drug ($P=0.0557$), and no diet $\times$ drug ($P=0.4085$) interaction.

Statistical analysis indicated a significant effect of diet ($P=0.000283$) on the percentage of white blood cells that are monocytes (**Figure 4.12**). There was no consistent pattern of diet or supplement on this effect. No significant effect of drug ($P=0.07599$), and no diet $\times$ drug ($P=0.254440$) interaction were observed.

No differences in erythrocyte and platelet counts or in hemoglobin, hematocrit, mean cell volume, mean cell hemoglobin and mean cell hemoglobin concentration were observed (**Table 4.3 and Table 4.4**).

Cecal contents and cecal weights

In the repletion period of phase 2, rats cecal contents and cecal weights were analyzed as a function of different dietary fibre and fermentable substrates with or without 0.5% SST. Rat cecal contents and cecal weights were higher in diets containing polydextrose (cecal content: $10.36 \pm 0.63\text{g}$, cecal weight: $1.88 \pm 0.25\text{g}$) and inulin (cecal content: $6.56 \pm 0.58\text{g}$, cecal weight: $1.52 \pm 0.08\text{g}$). These differences were even more significant when 0.5% SST was added to polydextrose (cecal content: $16.59 \pm 0.62\text{g}$,

cecal weight: $2.55 \pm 0.18\text{g}$), and inulin (cecal content: $13.94 \pm 2.12\text{g}$, cecal weight: $2.41 \pm 0.07\text{g}$) containing diets (**Figure 4.13** and **Figure 4.14**).

The effect of diet on the weight of the cecum itself is shown in (**Figure 4.14**). The addition of the sulfa drug significantly increased cecal weight ($P=0.0000$). Diet also had a significant effect. Rats fed polydextrose and inulin had significantly higher cecal weights than animals fed other diets. ANOVA analysis also indicated a significant diet $\times$ drug interaction ($P=0.0114$). Post hoc analyses show that the difference was only significant for animals fed inulin.

Fecal excretion and fecal composition

Fecal dry mass output (g/d) was lower in animals fed diets containing ground corn, inulin and polydextrose (**Figure 4.15a**). Several different factors can contribute to a change in fecal output including the presence of dietary fibre, changes in bacterial mass and overall digestability of dietary fibre. Figure (**4.15b**) shows that part of the difference was due to a lower fecal fibre content in animals fed these diets. However, the fibre content in the feces of animals fed wheat bran, oat bran and wheat germ diets was also low demonstrating that other effects may be responsible. Bacterial mass was indirectly measured by measuring nitrogen, muramic acid (MA) and diaminopimelic acid (DAPA). MA and DAPA are amino acids unique to bacteria. MA and DAPA are found principally (up to 50%) in the peptidoglycan layer of the gram positive and usually less than 10% of the wall, and sometimes as little as 1% in the gram negative bacteria. The data (**Figure 4.16**, **Figure 4.17**, **Figure 4.18**) suggest lower bacterial amounts in feces from animals fed ground corn but the results for inulin and polydextrose are less clear cut.

The reliability of these methods for measuring bacteria has not been demonstrated absolutely. SST had no consistent effect on fecal nitrogen, MA and DAPA. Addition of SST to rat diets increased fecal nitrogen output but did not significantly affect MA or DAPA output, except for specific cases; DAPA excretion was lower in SST-treated inulin and polydextrose fed rats and MA was lower in SST-treated polydextrose fed rats.

Liver and Fecal Folate

Rat liver folate content (**Tabel 4.5 and Table 4.6**) is an indicator of the total amount of folate that is bioavailable from dietary sources. This could potentially include folate produced by bacterial fermentation in the large intestine. If bacterially derived folate were bioavailable, liver folate values should exceed those predicted by the standard addition of folacin alone to the diet. Thus, these points should lie above the standard curve. As shown in figures (4.19) and (4.20), a linear relationship between dietary folacin intake ($\mu\text{g/d}$) and liver folate stores (measured as $\mu\text{g/g}$ liver) was apparent in the absence and presence of SST. Thus, Figure (4.19) shows that the addition of dietary fibre or fermentable substrates had no additional effect on liver folate concentration. This was tested statistically by comparing the liver folate/dietary folate ratios for all diets. No significant differences were observed. This observation also applies to the animals fed diets containing SST (**Figure 4.20**). However, the total liver folate of animals fed diets with SST was lower than that of the animals fed diets without drugs. This effect was statistically significant ($P < 0.01$).

Although the liver folate values were unaffected by addition of dietary fibre or FS, fecal folate values were significantly increased in rats fed diets containing these substances (**Figure 4.21 and Figure 4.22**). In the absence of SST, statistically increased

fecal folate was observed for animals fed wheat bran ($P = 0.000133$), oat bran ($P = 0.000133$), ground corn ($P = 0.000133$), wheat germ ($P = 0.000133$), and inulin ($P = 0.000133$). No increase was observed with polydextrose (PD) ($P = 1.0000$). Addition of the sulfa drug to the diets reduced the amount of folate in the feces ($P = 0.000103$). Individual diets, however, responded differently. Addition of SST decreased folate excretion in animals fed wheat bran, oat bran, ground corn, wheat germ, Inulin and PD to 59%, 51%, 28%, 60%, $< 0\%$, and $< 0\%$ respectively. In the presence of drug significantly higher fecal folate was observed for animals fed wheat bran ($P = 0.000133$), oat bran ($P = 0.0025$) and wheat germ ($P = 0.000140$). No change was observed for ground corn ($P = 1.000$) and decreased fecal folate was observed for animals fed Inulin ($P = 0.000139$) and PD ($P = 0.000140$).

The changes in fecal folate and liver folate as a function of diet suggest that there may be a relationship between these two variables. **Figure 4.23**, shows that, no simple relationship exists between these parameters.

Fibre digestability and Folate balance

Fermentation degrades dietary fibre and all sources of dietary fibre and fermentable substrates showed evidence of this (**Figure 4.24**). Apparent dietary fibre fermentation was calculated from the amount of alphacel in each diet and the dietary fibre excretion in the feces obtained from the standard diets. The degree of dietary fibre fermentation differed slightly depending on the type of fibre. Nonfermentable alphacel was very poorly digested as shown by the low fibre digestibility of the standard diets. Approximately 40% of the wheat bran fibre was digested while between 80 and 100% of oat bran, ground corn and wheat germ was fermented. Addition of SST decreased

digestion of oat bran, ground corn and wheat germ dietary fibre. The degree of dietary fibre digestibility in animals fed the standard diets was low.

Measurements of inulin and polydextrose digestion were performed directly by Mr. Brian Lampi, of the Nutrition Research Division of Health Canada, since inulin and polydextrose do not precipitate during the dietary fibre method. These values showed that $99.80 \pm 0.05\%$ and $99.40 \pm 0.17\%$ of inulin and $82.70 \pm 5.00\%$ and $81.50 \pm 3.30\%$ were digested in diets without or with SST, respectively.

Short chain fatty acids are a by product of fermentation. **Figure 4.25** shows that the major products of fermentation are acetic, propionic and butyric acids. **Figure 4.25** also shows a varied pattern of SCFA production that depends on the diet. Butyric acid was the major product of oat bran fermentation while acetic acid and propionic dominated in inulin and polydextrose fermentation. Other dietary fibre and fermentable substrates had a more even distribution of acetic, propionic and butyric acids. Addition of SST had a varied effect on SCFA production. In the presence of SST, acetic acid was the major product of wheat germ, inulin, and polydextrose fermentation (**Figure 4.25**). Propionic acid dominated in oat bran, and inulin fermentation, while butyric acid was the major product of oat bran, wheat germ and inulin. Dietary fibre and fermentable substrates had an even distribution of isobutyric and isovaleric acid, while inulin and polydextrose fermentation showed a significant increase in isobutyric and isovaleric acid (**Figure 4.25**).

Another product of bacterial fermentation in the colon is folate which can be measured in the feces (**Figure 4.26, Table 4.5 and Table 4.6**). Using the folate concentration in the rat diets and the fecal folate content, folate balance can also be

determined. **Figure 4.26** shows an increase in folate balance in rats fed the standard diet as dietary folate increases. As fecal folate increases, folate balance decreases making it appear that the animals are retaining less of the dietary folate when fed diets containing dietary fibre or fermentable substrates. This effect could be attributed to an inhibition of folate absorption or to an increased excretion. **Table 4.5** and **Table 4.6** show that the effect is due to increased folate excretion. Addition of SST drug had an inhibitory effect on folate production by bacterial microflora resulting in a decrease in folate excretion (**Table 4.5** and **Table 4.6**) and leading to an apparent increase in folate balance in all diets (**Figure 4.26**). This was true even though liver folate values were lower (**Table 4.5** and **Table 4.6**).

Fecal folate is a product of bacterial fermentation. As indicated above, fecal MA, DAPA and nitrogen are indirect measure of fecal fermentation. Thus, one may expect a proportional relationship between fecal nitrogen, MA, DAPA and fecal folate.

Figure 4.27 shows a very poor relationship ($r^2 = 0.02$) between fecal folate and fecal nitrogen excretion in the presence of sulfa drug. However, in the absence of SST, fecal folate was linearly dependant on the fecal nitrogen production ($r^2 = 0.9$). The relationship between fecal folate and DAPA in the absence of sulfa drug ($r^2 = 0.14$), and with the sulfa drug ($r^2 = 0.401$) is also poor (**Figure 4.28**), considering that a $r^2 = 0.401$ means that only 40% of the variance can be explained by the graph.

Figure 4.29, shows that no relationship exists between fecal folate and fecal muramic acid in the presence or absence of SST ($r^2 < 0.1$).

Figure 4.9: Relationship between food intake and diet: effect of drug on food intake. Open circle, low standard; open square, medium standard; open diamond, high standard; open triangle, wheat bran; closed circle, oat bran; closed square, ground corn; closed diamond, wheat germ; closed triangle, inulin; plus sign, polydextrose. ANOVA analysis revealed no significant effect of diet ($P=0.071$) or drug ($P=0.226$) in weekly food intake, and no diet $\times$ drug interaction ($P=0.053$).

Phase 2: Body Weight after 28 d repletion

Figure 4.10: Rat Body weight at the end of repletion phase, fed different diets with or without 0.5% SST.

ANOVA analysis revealed no significant effect of diet ($P=0.4568$) or drug ($P=0.2788$) and no diet x drug interaction ($P=0.3608$).

Phase 2: Leukocytes

Figure 4.11: Leukocytes count in rat fed different diets with or without 0.5% SST at the end of repletion phase.

ANOVA analysis revealed no significant effect of diet ($P=0.1123$) or drug ($P= 0.0557$), and no diet x drug interaction ($P= 0.4085$).

Phase 2: Monocytes

Figure 4.12: Percentage of monocytes in total rat white blood cells as a function of different diets.

ANOVA analysis revealed a significant effect of diet ($P=0.000283$) but no significant effect of drug ($P=0.075990$), and no diet x drug interaction ($P=0.254440$).

Table 4.3: Hematology parameters in rat fed different dietary fibres without succinylsulfathiazole at the end of repletion phase

Diet	Description	Drug	LKCS	ERCS	HB	Hct	MCV	MCH	MCHC
1	Low Std	No	9.50±1.79	7.12±0.28	143.57±5.29	0.40±0.02	55.71±2.09	20.17±0.77	362.00±9.35
2	Med Std	No	7.88±1.01	7.26±0.48	146.86±5.73	0.40±0.02	55.07±1.70	20.29±0.87	367.86±6.87
3	High Std	No	8.77±2.25	6.93±0.22	141.71±5.53	0.39±0.02	55.87±2.08	20.43±0.39	366.29±8.85
4	Wheat Bran	No	8.70±2.41	7.07±0.34	142.67±3.15	0.39±0.01	54.98±1.46	20.20±0.86	367.33±8.38
5	Oat Bran	No	8.97±3.30	6.99±0.45	142.43±6.92	0.39±0.02	55.86±1.41	20.41±0.76	365.29±13.45
6	Ground Corn	No	9.56±1.25	7.01±0.44	141.71±6.95	0.39±0.03	55.76±1.17	20.21±0.69	363.00±10.74
7	Wheat Germ	No	8.66±3.13	7.35±0.50	146.43±5.38	0.41±0.02	55.30±2.06	19.96±0.83	361.14±11.68
8	Inulin	No	8.61±3.08	7.21±0.38	144.29±4.39	0.40±0.02	55.70±1.55	20.04±0.92	360.14±14.75
9	PolyDextrose	No	6.27±2.47	7.15±0.33	141.57±5.09	0.39±0.02	54.41±1.68	19.83±1.09	364.29±11.00

* Units are as follows: Leukocytes (LKCS)×10⁹/L; Erythrocytes (ERCS)×10¹²/L; Hemoglobin (HB), g/L; Mean cell volume (MCV), fL; Mean cell hemoglobin (MCH), pg; Mean cell hemoglobin concentration (MCHC), g/L.

Table 4.3: Hematology parameters in rat fed different dietary fibres without succinylsulfathiazole at the end of repletion phase

Diet	Description	Drug	RDW	PLT	MPV	SEG	LYMPH	MONO	EOSIN
1	Low Std	No	12.10±0.59	908.71±107.10	6.41±0.46	9.93±4.62	81.93±4.25	2.00±0.91	1.80±0.84
2	Med Std	No	12.34±0.59	946.57±112.37	6.34±0.23	11.64±3.46	81.00±4.68	1.21±0.82	1.36±1.38
3	High Std	No	11.94±0.69	808.71±141.41	6.43±0.37	11.21±3.02	78.71±6.49	1.50±0.90	1.40±0.57
4	Wheat Bran	No	12.15±0.84	926.67±136.67	6.40±0.44	10.67±4.06	78.67±3.65	3.50±1.02	1.25±0.49
5	Oat Bran	No	12.03±0.73	965.14±110.24	6.26±0.20	12.86±4.38	78.93±3.42	2.08±1.07	1.17±0.68
6	Ground Corn	No	11.93±0.21	830.57±71.42	6.43±0.28	12.86±5.30	77.00±8.33	2.07±1.27	1.33±0.75
7	Wheat Germ	No	11.59±0.62	841.00±94.47	6.30±0.31	12.21±8.27	81.43±8.72	1.43±0.45	0.67±0.26
8	Inulin	No	11.67±0.52	812.57±50.40	6.24±0.40	9.07±2.19	81.14±4.50	2.14±1.03	1.14±0.56
9	PolyDextrose	No	11.93±0.43	872.29±112.53	6.30±0.23	15.00±9.22	76.43±8.31	1.43±0.73	1.21±0.86

*Units are as follows: Red cell distribution width (RDW, no unit); platelets (PLT)×10⁹/L; Mean platelet volum (MPV) fL; segment (SEG) %; lymphocytes (LYMPH)%; monocytes (MONO)%; eosinophils (EOSIN)%.

Table 4.4: Hematology parameters in rat fed different dietary fibres with succinylsulfathiazole at the end of repletion phase

Diet	Description	Drug	LKCS	ERCS	HB	Hct	MCV	MCH	MCHC
1	Low Std	Yes	7.87±1.51	7.33±0.36	144.14±4.78	0.40±0.02	54.50±1.85	19.71±1.00	361.29±12.78
2	Med Std	Yes	8.51±1.75	7.28±0.45	146.29±2.87	0.41±0.02	56.11±1.81	20.14±0.94	359.14±10.53
3	High Std	Yes	9.39±2.51	7.10±0.37	145.86±6.49	0.40±0.03	56.59±0.97	20.56±0.54	363.71±10.73
4	Wheat Bran	Yes	8.76±2.46	7.37±0.28	144.00±5.51	0.40±0.02	53.67±1.36	19.56±0.80	364.43±11.75
5	Oat Bran	Yes	11.54±3.57	7.28±0.21	144.57±4.58	0.40±0.01	55.64±1.25	19.89±0.69	357.43±14.77
6	Ground Corn	Yes	10.82±4.69	7.06±0.31	141.33±6.28	0.39±0.01	55.78±1.61	20.00±0.42	358.83±13.53
7	Wheat Germ	Yes	10.99±2.94	7.31±0.21	145.86±2.19	0.41±0.01	56.24±1.87	19.96±0.54	354.71±7.09
8	Inulin	Yes	8.09±1.63	7.35±0.73	144.00±8.50	0.41±0.03	55.57±2.73	19.66±1.07	353.86±8.99
9	PolyDextrose	Yes	8.99±2.91	7.16±0.17	144.00±3.79	0.40±0.02	56.24±1.66	20.10±0.35	357.71±8.99

* Units are as follows: Leukocytes (LKCS)×10⁹/L; Erythrocytes (ERCS)×10¹²/L; Hemoglobin (HB), g/L; Mean cell volume (MCV), fL; Mean cell hemoglobin (MCH), pg; Mean cell hemoglobin concentration (MCHC), g/L.

Table 4.4: Hematology parameters in rat fed different dietary fibres with succinylsulfathiazole at the end of repletion phase.

Diet	Description	Drug	RDW	PLT	MPV	SEG	LYMPH	MONO	EOSIN
1	Low Std	Yes	12.01±0.95	870.29±68.30	6.31±0.31	12.14±3.12	76.79±4.35	2.83±1.63	1.50±0.71
2	Med Std	Yes	11.54±0.22	806.86±63.97	6.23±0.33	11.00±6.17	79.86±4.48	1.00±0.29	1.00±0.41
3	High Std	Yes	11.93±0.44	876.43±106.15	6.29±0.28	16.86±10.68	72.93±12.92	1.43±0.73	1.50±0.58
4	Wheat Bran	Yes	11.73±0.88	900.71±121.70	6.43±0.44	12.93±11.88	73.64±10.73	2.43±1.67	0.70±0.45
5	Oat Bran	Yes	11.83±0.53	876.71±98.82	6.47±0.25	8.71±3.97	76.93±4.68	1.07±0.35	1.00±0.50
6	Ground Corn	Yes	12.13±0.69	963.33±112.26	6.33±0.44	7.58±3.06	77.67±3.06	1.75±1.08	0.90±0.65
7	Wheat Germ	Yes	11.49±0.71	906.14±109.93	6.27±0.29	6.79±3.44	81.50±3.69	1.21±0.39	0.92±0.58
8	Inulin	Yes	13.11±3.47	908.57±123.33	6.51±0.38	9.07±3.23	79.57±4.22	1.36±1.03	1.00±0.35
9	PolyDextrose	Yes	11.84±0.40	846.29±48.72	6.41±0.19	8.93±2.54	78.86±4.01	1.40±0.74	1.29±0.64

*Units are as follows: Red cell distribution width (RDW, no unit); platelets (PLT)×10⁹/L; Mean platelet volum (MPV) fL; segment (SEG) %; lymphocytes (LYMPH)%; monocytes (MONO)%; eosinophils (EOSIN)%.

Phase 2: Total Cecal Contents

Figure 4.13: Effect of diets and drug on cecal content.

The comparison of the groups are reported as the superscript a, b.

Groups with different superscripts are significantly different.

ANOVA analysis revealed significant effect of diet ($P=0.0000$) or drug ($P=0.0000$) and no diet $\times$ drug interaction ($P=0.0505$).

Figure 4.14: Effect of diets and drug on cecal weight.

The comparison of the groups are reported as the superscript a, b.

* Significant effect of drug on rat cecal weight

ANOVA analysis revealed significant effect of diet ($P=0.0000$) or drug ($P=0.0010$) and diet $\times$ drug interaction ($P=0.0114$).

Figure 4.15: Rat fecal dry weight (Top, Figure: 4.15a) and % fibre in the feces (bottom, Figure: 4.15b) at the end of repletion phase, fed different diets with or without SST.

Figure 4.16: Total fecal nitrogen excreted in rat fed different diets at the end of repletion phase.

* Significant effect of drug on total nitrogen excretion.

ANOVA analysis revealed significant effect of diet ($P < 0.0000$) and drug ($P < 0.0000$).

Diets with different letters are significantly different at the $P < 0.05$ level.

Figure 4.17: Total fecal 2-6 diaminopimelic acid excreted in rat fed different diets at the end of repletion phase.

*Significant effect of drug on total DAPA excretion

ANOVA analysis revealed significant effect of diet ($P < 0.0000$) and drug ($P = 0.0003$).

Diets with different letters are significantly different at the $P < 0.05$ level.

Figure 4.18: Total fecal muramic acid excreted in rats fed different diets at the end of repletion phase.

*Significant effect of drug on total muramic acid excretion.

ANOVA analysis revealed significant effect of diet ($P < 0.0000$) and drug ($P = 0.0008$). Diets with different letters are significantly different at the $P < 0.05$ level.

Figure 4.19: Liver folate concentration as a function of dietary folate in different diet samples without succinylsulfathiazole after 28 days repletion. Closed circle, standards; closed square, wheat bran; open square, oat bran; closed triangle, ground corn; open triangle, wheat germ; closed inverted triangle, Inulin; open inverted triangle, polydextrose.

Phase 2: Liver folate Day 28, Repletion phase

Figure 4.20: Liver folate concentration as a function of dietary folate in different diet samples with succinylsulfathiazole after 28 days repletion.

Closed circle, standards; closed square, wheat bran; open square, oat bran; closed triangle, ground corn; open triangle, wheat germ; closed inverted triangle, Inulin; open inverted triangle, polydextrose.

Phase 2: Fecal folate Day 28, Repletion phase

Figure 4.21: Fecal folate output as a function of dietary folate in different diet samples without succinylsulfathiazole after 28 days repletion.

Closed circle, standards; closed square, wheat bran; open square, oat bran; closed triangle, ground corn; open triangle, wheat germ; closed inverted triangle, Inulin; open inverted triangle, polydextrose.

ANOVA analysis revealed a significant effect of diet ($P < 0.0000$) or drug ($P < 0.0000$), and diet x drug interaction ($P < 0.0000$).

Figure 4.22: Fecal folate concentration as a function of dietary folate in different diet samples with succinylsulfathiazole after 28 days repletion.

Closed circle, standards; closed square, wheat bran; open square, oat bran; closed triangle, ground corn; open triangle, wheat germ; closed inverted triangle, Inulin; open inverted triangle, polydextrose.

**Phase 2: Liver folate
Day 28, Repletion phase**

Figure 4.23: Liver folate concentration as a function of fecal folate in different diet samples with or without succinylsulfathiazole after 28 days repletion.

Closed circle, low standard with drug; open circle, low standard without drug, closed circle with cross, medium standard with drug; open circle with cross, medium standard without drug; closed square, high standard with drug; open square, high standard without drug; closed square with cross, wheat bran with drug; open square with cross, wheat bran without drug; closed triangle oat bran with drug; open triangle oat bran without drug; closed diamond, wheat germ with drug; open diamond, wheat germ without drug; open hexagonal, inulin with drug; closed hexagonal, inulin without drug; closed hexagonal with cross, polydextrose with drug; open hexagonal with cross, polydextrose without drug.

Table 4.5: Food consumption, dietary folate and fibre intake and excretion, folate balance and liver folate in animals fed diets without SST.

Diet	Description	Total Food		Dietary		Folate		Folate		Dietary		Fibre	
		Consumption	(g/28 d)	Folate intake	(µg/d)	excreted	(µg/d)	balance	(µg/d)	Liver folate	(µg/g)	Fibre intake	excreted (g/d)
0	Depletion	448 ± 40		0.45 ± 0.1		--	--	--	--			1.7 ± 0.1	--
1	Low Std	695 ± 41		12.4 ± 0.7		3.1 ± 0.6		9.2 ± 0.8		4.9 ± 0.7		1.7 ± 0.1	1.47 ± 0.14
2	Medium Std	680 ± 68		14.1 ± 1.4		4.1 ± 1.0		10.0 ± 1.2		5.3 ± 1.2		1.7 ± 0.2	1.53 ± 0.11
3	High Std	617 ± 31		19.7 ± 1.0		3.4 ± 0.7		16.3 ± 1.6		6.2 ± 0.8		1.6 ± 0.1	1.34 ± 0.14
4	Wheat Bran	725 ± 60		12.6 ± 1.0		9.2 ± 1.3		3.4 ± 0.9		4.8 ± 0.9		1.7 ± 0.1	1.05 ± 0.19
5	Oat Bran	685 ± 72		11.9 ± 1.3		7.3 ± 0.8		4.5 ± 0.7		5.0 ± 0.3		2.4 ± 0.3	1.27 ± 0.15
6	Ground Corn	664 ± 78		8.8 ± 1.0		5.3 ± 1.2		3.5 ± 0.8		3.7 ± 0.3		2.8 ± 0.3	0.85 ± 0.15
7	Wheat Germ	683 ± 77		18.6 ± 2.1		9.2 ± 2.2		9.7 ± 1.9		5.7 ± 0.5		2.1 ± 0.2	0.92 ± 0.33
8	Inulin	649 ± 83		11.7 ± 1.5		6.9 ± 2.9		5.9 ± 3.1		5.9 ± 1.7		1.2 ± 0.2	0.89 ± 0.13
9	Polydextrose	615 ± 91		13.9 ± 2.0		3.4 ± 0.7		10.5 ± 2.0		4.7 ± 1.1		1.3 ± 0.2	0.78 ± 0.11

Table 4.6: Food consumption, dietary folate and fibre intake and excretion, folate balance and liver folate in animals fed diets with 0.5% SST.

Diet	Description	Total Food		Dietary		Folate		Folate		Dietary		Fibre	
		Consumpti on (g/28 d)		Folate intake ($\mu\text{g/d}$)		excreted ($\mu\text{g/d}$)		balance ($\mu\text{g/d}$)		Liver folate ($\mu\text{g/g}$)		Fibre intake (g/d)	excreted (g/d)
10	Low Std	659 $\pm$ 27		10.7 $\pm$ 1.0		1.0 $\pm$ 0.2		9.7 $\pm$ 0.9		2.3 $\pm$ 0.6		2.0 $\pm$ 0.2	1.34 $\pm$ 0.09
11	Medium Std	679 $\pm$ 46		13.4 $\pm$ 1.6		1.6 $\pm$ 0.3		11.8 $\pm$ 1.4		4.3 $\pm$ 0.4		1.5 $\pm$ 0.2	1.37 $\pm$ 0.17
12	High Std	684 $\pm$ 27		20.2 $\pm$ 2.1		2.3 $\pm$ 0.6		17.9 $\pm$ 2.1		5.2 $\pm$ 0.5		1.6 $\pm$ 0.2	1.45 $\pm$ 0.09
13	Wheat Bran	648 $\pm$ 34		10.3 $\pm$ 1.2		0.9 $\pm$ 1.2		6.5 $\pm$ 0.7		3.2 $\pm$ 0.4		1.8 $\pm$ 0.2	0.94 $\pm$ 0.24
14	Oat Bran	684 $\pm$ 19		9.7 $\pm$ 0.7		2.8 $\pm$ 0.7		7.0 $\pm$ 8.5		3.0 $\pm$ 0.4		2.1 $\pm$ 0.2	1.36 $\pm$ 0.12
15	Ground Corn	764 $\pm$ 45		12.4 $\pm$ 1.6		1.6 $\pm$ 0.3		10.8 $\pm$ 1.5		2.4 $\pm$ 0.4		1.6 $\pm$ 0.2	0.78 $\pm$ 0.12
16	Wheat Germ	701 $\pm$ 44		19.4 $\pm$ 1.6		4.0 $\pm$ 0.8		15.3 $\pm$ 1.5		4.0 $\pm$ 0.4		2.3 $\pm$ 0.2	1.07 $\pm$ 0.14
17	Inulin	631 $\pm$ 56		12.1 $\pm$ 2.0		0.7 $\pm$ 0.2		11.4 $\pm$ 1.9		3.1 $\pm$ 0.8		1.2 $\pm$ 0.2	0.71 $\pm$ 0.11
18	Polydextrose	677 $\pm$ 23		11.9 $\pm$ 0.8		0.6 $\pm$ 0.2		11.3 $\pm$ 0.8		2.5 $\pm$ 0.2		1.6 $\pm$ 0.1	0.51 $\pm$ 0.09

Estimated Fibre Digestion

Figure 4.24: Percent alphacellulose dietary fibre digested by rats fed different diets in the repletion phase.

Digestibility of dietary wheat bran, oat bran, ground corn and wheat germ were estimated by subtracting alphacel excretion (derived from digestibility values in standard diets).

Diet groups with different superscripts are significantly different at the $P < 0.05$ level.

ANOVA analysis revealed a significant effect of diet ($P < 0.000000$) or drug ($P = 0.04986$), and diet x drug interaction ($P < 0.000000$).

*Significant effect of drug on % dietary fibre digested.

SCFAs in rat cecum: total amount

Figure 4.25: Total amount of short chain fatty acids in rat cecum

*Significant effect of diet on total short chain fatty acid production.

Figure 4.26: Effect of diet and drug on folate balance.

Diet groups with different superscripts are significantly different at the $P < 0.05$ level.

Figure 4.27: The relationship between fecal folate and fecal nitrogen concentration in rats fed different dietary fibre with or/without 0.5% SST.

Closed circle, low standard with drug; open circle, low standard without drug; closed circle with cross, medium standard with drug; open circle with cross, medium standard without drug; closed square, high standard with drug; open square, high standard without drug; closed square with cross, wheat bran with drug; open square with cross, wheat bran without drug; closed triangle oat bran with drug; open triangle oat bran without drug; closed diamond, wheat germ with drug; open diamond, wheat germ without drug; open hexagonal, inulin with drug; closed hexagonal, inulin without drug; closed hexagonal with cross, polydextrose with drug; open hexagonal with cross, polydextrose without drug.

Figure 4.28: The relationship between fecal folate and fecal DAPA concentration from rats fed different dietary fibres with or/without 0.5% SST.

Closed circle, low standard with drug; open circle, low standard without drug, closed circle with cross, medium standard with drug; open circle with cross, medium standard without drug; closed square, high standard with drug; open square, high standard without drug; closed square with cross, wheat bran with drug; open square with cross, wheat bran without drug; closed triangle oat bran with drug; open triangle oat bran without drug; closed diamond, wheat germ with drug; open diamond, wheat germ without drug; open hexagonal, inulin with drug; closed hexagonal, inulin without drug; closed hexagonal with cross, polydextrose with drug; open hexagonal with cross, polydextrose without drug.

Figure 4.29: The relationship between fecal folate and fecal MA concentration in rats fed different dietary fibres with or/without 0.5% SST.

Closed circle, low standard with drug; open circle, low standard without drug; closed circle with cross, medium standard with drug; open circle with cross, medium standard without drug; closed square, high standard with drug; open square, high standard without drug; closed square with cross, wheat bran with drug; open square with cross, wheat bran without drug; closed triangle oat bran with drug; open triangle oat bran without drug; closed diamond, wheat germ with drug; open diamond, wheat germ without drug; open hexagonal, inulin with drug; closed hexagonal, inulin without drug; closed hexagonal with cross, polydextrose with drug; open hexagonal with cross, polydextrose without drug.

Figure 4.30: The relationship between DAPA and rat fecal nitrogen excreted (top), MA and rat fecal nitrogen excreted (middle), and MA and DAPA (bottom).

Chapter V

5.1 Discussion

The animal-based folate assay has been validated by many labs (Krause *et al*, 1996; Rong *et al*, 1991; Keagy, 1990; Hoppner and Lampi, 1989; Keagy and Oace, 1989; Baily *et al*, 1988; Keagy *et al*, 1988) and has proven useful in assessing the bioavailability of the many different chemical forms of folate. In a 1967 review of rat growth bioassays, Herbert and Bertino indicated that tissue folate concentrations more accurately reflect animal folate status when compared with the rat growth assay. For this reason, liver folate was measured and used as an indicator of folate bioavailability. The assay method requires the depletion of liver folate stores prior to feeding a test diet. Liver is the tissue of choice because it is the major site of folate stores in the body (Henderson, 1990). During the refeeding phase, liver stores are repleted, and the rate of repletion is a function of the folate content in the diet and its bioavailability. In order to assess relative bioavailability, standard diets containing known amounts of highly available folic acid are fed to the rats. The amount of liver folate in rats fed the test diets is then compared against those fed control diets to obtain relative bioavailability.

It is believed that some nutrients, like dietary fibre and fermentable substrates have health-promoting benefits such as stimulating the reproduction of health promoting bacteria (Younes *et al*, 2001; Kruse *et al*, 1999; Gibson *et al*, 1995) and producing bioavailable vitamins like folate (Keagy, 1990; Keagy and Oace, 1984). This latter effect is thought to arise from their ability to synthesize folic acid *de novo* (Rong *et al*, 1991).

Liver Folate as a function of diet

Liver folate analysis from the first phase of the experiment provided data on the kinetics of folate repletion in our rat model. In the rat, low liver folate values measured at the end of the depletion period confirmed the efficacy of the depletion diet. Increases in liver folate, as a function of increased dietary folate, showed that animals that were depleted could be repleted again by adding different concentrations of folate in their diets (**Figure 4.4**). Liver folate and dietary folate were linearly correlated on day 7 and day 14 of the repletion phase whereas liver folate in rats fed the oat bran diet was higher on days 21 and 28. This was true even though the measured folate content of the oat bran diet was lower than the wheat bran diet. Since oat bran fibre is approximately 100% fermentable, and wheat bran fibre is only about 40% fermentable, these first results suggested that fermentation might have a role in folate bioavailability.

In the second phase of the experiment, as previously observed in phase 1, increasing amounts of folic acid in the diet produced an approximately linear increase in liver folate content. However, addition of dietary fibre or fermentable substrates to the diet failed to increase the liver folate content. This result demonstrates that any folate produced through bacterial fermentation in the cecum does not significantly contribute to the amount of folate that is bioavailable in the diet. Thus, the ratio of liver folate/ dietary folate at the end of 28-day repletion phase was not significantly different for the nine different diets.

The failure of fermentable substrates and dietary fibre to increase liver folate was not due to a lack of folate production in the cecum. Increased fermentation, as shown by increased SCFA production (**Figure 4.25**), increased fecal nitrogen (**Figure 4.16**),

increased muramic acid (**Figure 4.18**), and increased diaminopimelic acid (**Figure 4.17**) excretion as well as by increased digestion of dietary fibre (**Figure 4.24**) was associated with an increased excretion of folate (**Figure 4.22**). This folate was presumably derived from intestinal bacteria since virtually all dietary folacin was absorbed (**Figure 4.26**). It is possible to estimate the dietary equivalent of this folate by comparing the excretion rate with the rate of folacin intake (**Table 4.5**). In the standard diets with added folate, the excretion rate was constant at about 3.5 $\mu\text{g/d}$. Using this as the baseline, one can calculate approximately 5.7 $\mu\text{g/d}$ (wheat bran), 3.8 $\mu\text{g/d}$ (oat bran), 1.8 $\mu\text{g/d}$ (ground corn), 5.7 $\mu\text{g/d}$ (wheat germ), 3.4 $\mu\text{g/d}$ (inulin), and 0 $\mu\text{g/d}$ (polydextrose) folate were produced by intestinal microflora. This represented 45% (wheat bran), 32% (oat bran), 20% (wheat germ), 30% (ground corn), 29 % (inulin), and 0% (polydextrose) of the total ingested folacin. Based on these folate values, we expected to see a significantly greater liver folate /dietary folate ratio than was observed for the standard diets (which are not fermented to any greater extent). Because this did not happen, we concluded that folacin derived from bacterial fermentation was not bioavailable.

In contrast to our results, several investigators have reported that dietary fibre sources contributed additional folacin to the diet by stimulating intestinal microflora and folate synthesis. Keagy and Oace (1984) examined the effect of several sources of dietary fibre and folic acid bioavailability on rat bioassay and reported wheat bran and California small white beans stimulated synthesis of intestinal bacterial folacin and increased liver folate. Since those two sources of dietary fibre have significant amounts of endogenous folate, it is not clear how much of the liver folate came from the diet and what portion was contributed by bacterial folate production.

In another study, Rong *et al* (1991) injected [^3H] PABA directly into rat cecums to determine whether [^3H] folate synthesized by bacteria could appear in the liver and kidney of rats. Their results indicated that the large intestine is capable of absorbing the folate produced by the gut flora. However, they did not quantitatively measure the amount of folate contributed by the intestinal bacteria and the absorption mechanism of the bacterially-synthesized folate in the large intestine has not been identified. Keagy *et al* (1988) studied the effect of wheat bran and California small white beans on the absorption of two different forms of folic acid (monoglutamyl and heptaglutamyl folic acid) in human diets. Mean folate concentrations in serum and 24-hour urine samples showed that, in the presence of wheat bran, the absorption of monoglutamyl folic acid was higher as compared with heptaglutamyl folic acid, whereas in the diets containing beans, monoglutamyl absorption was lowered. Therefore, they suggested that differences in the chemical structure of wheat bran might interact differently on monoglutamyl folic acid and heptaglutamyl folic acid might cause the different effects of bran on each compound. The results obtained by Keagy *et al* (1988) were based on the analysis of folate concentrations in the serum. Since liver is the major storage site for folate in animals and humans, it is a better indicator of the total amount of folate obtained from dietary sources than serum folate values, which fluctuate during the day and are sensitive to food intake.

In the present study, the effect of fermentation on liver folate was also assessed by adding 0.5% SST to each diet. Succinylsulfathiazole (SST) belongs to a group of sulphonamides that all have the same structure as PABA. They inhibit the synthesis of folic acid and folic acid containing co-enzymes responsible for the synthesis of DNA and

nucleic acids (Lee, 1999). Their low toxicity to eukaryotes (Welch and Wright, 1943), their low absorption by the alimentary tract (Miller, 1944), and the ease of incorporation in highly purified diets (Welch, 1942) makes them a preferred antibiotic in this study.

Adding SST lowered both total fecal folate (**Figure 4.22**) and total liver folate (**Figure 4.20**) content when compared with rats not fed the drug. The reduction in fecal folate can be explained by an SST-associated inhibition of folate producing bacteria in the cecum. This interpretation is in agreement with previous studies by Miller (1944) and Skeggs and Wright (1946) who observed a reduction in intestinal facultative anaerobes after feeding rats SST-containing diets. Although not directly measured by the present experiments, a reduction in fermentation was suggested by lower SCFA production (**Figure 4.25**) as well as reduced fecal nitrogen (**Figure 4.16**), fecal DAPA (**Figure 4.17**) and fecal MA (**Figure 4.18**) in several of the diets.

The SST-associated reduction in fecal folate values was accompanied by a reduction in liver folate concentrations. One possible explanation is that the reduced liver folate values were the result of reduced folate production by colonic bacteria. This possibility is ruled out by a decrease in the liver folate content in animals fed the standard diets in the presence of SST. The fact that the observed linear relationship between dietary folate intake and liver folate content is similar to that observed in animals fed diets without SST also suggests that this postulate does not explain the data. The simple explanation is a SST-associated inhibition of folate absorption in the small intestine. Other drugs and many food substances have been shown to affect folate absorption by their inhibitory effect on intestinal folate conjugase activity (Bailey, 1988). Intestinal conjugase activity is an important factor in hydrolyzing dietary polyglutamates to their

monoglutamyl forms, which is an essential step in the absorption process. According to Blakley and Benkovic (1984) drugs like dilantin and an anticonvulsant may inhibit the activity of the brush border conjugase and cause signs of folate deficiency and megaloblastic anemia.

The interpretation offered above for the effect of SST on bacterial folate production is over simplistic when one considers the bacterial ecosystem of the large intestine or cecum. There are over 400 different species of bacteria that colonize the rat cecum. Not all these species will respond to the addition of a sulfa drug like SST. In addition, not all species produce folate in concentrations large enough to be measured in the feces. Other factors also complicate the interpretation. For example, Gant *et al* (1943) showed that adding 0.5% SST to rat diets reduced the number of *Escherichia coli* in rat feces after 3-4 days but *E. coli* numbers returned to normal after 20-25 days. This was interpreted as the growth of SST-resistant organisms. *E.coli* are interesting because they are thought to be the major folate-producing species in the rat cecum (Coates *et al*, 1968; Daft *et al*, 1963; Miller and Lucky, 1963). Our results show a reduced folate excretion in rats fed SST for 28 days (**Figure 4.22**). If Gant *et al* (1943) are correct, this is more than enough time for the recolonization of *E. coli* in the cecum. The results, therefore, may be explained either by a loss of folate-synthesizing capacity in SST-resistant *E. coli* or by the appearance of other bacterial species to occupy the niche left vacant by the initial destruction of the *E.coli* population brought about through feeding of SST. The growth of drug-resistant species is a more likely scenario since Miller (1944) observed no change in the total number of excreted bacteria after feeding SST-containing diets even though the number of intestinal facultative anaerobes was reduced.

Fermentation

Bacterial fermentation is thought to produce several different products: an increased bacterial mass, heat, fermentation gasses like H_2 and CH_4 , and SCFA. Increased bacterial mass was measured indirectly by following increases in nitrogen, DAPA and MA while SCFA production was measured directly in the feces and cecum. Measuring fibre disappearance directly demonstrated dietary fibre digestion but is only an indirect measure of fermentation. It is technically difficult to measure how much dietary fibre actually reaches the large intestine and so the absolute extent of fermentation is in question. For this reason, fermentation products were also measured to verify relative changes in fermentation.

Bacterial fermentation degrades dietary fibre and fermentable substrates. In phase 2 of the study, the degree of dietary fibre digestibility in the standard diets was low while it was higher in diets containing more easily fermented fibres. The extent of fermentation depends on the solubility, chemical structure of the fibre, accessibility to colonic microflora, and adaptation of intestinal microflora to the dietary fibre (Oakenfull, 2001; Nyman and Asp, 1985). Low fibre digestion in the standard diets was due to the presence of cellulose (alphacel), which is a major constituent of plants cell walls (McCleary and Prosky, 2001). Cellulose is a polymer of glucose with a linear β - 1, 4 linkage between glucose molecules and like the other structural fibres (lignins and some hemicelluloses) is water insoluble, poorly fermentable and non-viscous (Stipanuk, 2000). In the absence of readily fermentable substrates, bacteria continue to grow using each other, colonic epithelial cells and mucin as energy sources (McBurney *et al*, 1987). These are poor substrates and account for the reduced fecal bacterial output. Dietary fibres like oat bran

fibre were about 80% fermented in our experiments because this fibre is very accessible to microflora and its chemical structure is composed of long-chain β -glucans (mixed $\beta(1-4)$ and $\beta(1-3)$) linkages that are not highly branched (Malkki, 2001). This irregular pattern of binding prevents the formation of hydrogen-bonded ordered crystalline structures making it water soluble, and consequently, easily accessible to colonic bacteria. It is relatively rapidly and completely fermented (Malkki, 2001).

Wheat bran fibre comes from the outer layer of the wheat seed, as shown in **Figure 5.1** (Cho *et al*, 1999). It is mostly water insoluble fibre and composed of hemicellulose and cellulose rich fibres strengthened by lignin. Hemicellulose, cellulose, and lignin are resistant to intestinal bacterial flora. In our experiment, we observed that about 40% of the wheat bran fibre was digested which agrees with Stephen and Cummings (1980). These authors reported 36% fibre digestibility for wheat bran using a gravimetric method to measure fecal fibre (the neutral detergent fibre method).

Wheat germ is the embryo of the seed and represents only a small portion (2%) of the seed weight (<http://www.smallgrains.org/WHFACTS/Kernel.html>, 2001). Wheat germ fibre is more available to bacteria as compared with wheat bran fibre since wheat germ fibre does not contain the three outer seed layers (aleurone, fused pericarp and testa and crushed nucellus). Compared with wheat bran, wheat germ contains minimal quantities of protein, but higher concentrations of B-complex vitamins and trace minerals (<http://www.smallgrains.org/WHFACTS/Kernel.html>, 2001). Measurement of fibre digestion showed that about 90% of this fibre was digested by intestinal bacteria.

Ground corn is a form of corn bran produced by a dry milling process that separates the bran, germ and endosperm. It is composed of about 65% hemicellulose, and

20% cellulose (Cho *et al*, 1999). Our experiments showed that about 95% of this fibre was digested by the rat suggesting that the hemicellulose and cellulose structures are more accessible to bacteria in this fibre presentation.

Polydextrose is a synthetic polymer of glucose, sorbitol, and citric acid with the ratio of (90:10:1) (Yoshioka, *et al*, 1994). In previous studies, polydextrose digestion has been measured at between 30 and 50%. Inulin is a naturally occurring oligomer of up to 35 fructose units, with a terminal glucose residue (Cho and Prosky, 1999) that is found in highest amounts in vegetables (Wang and Gibson, 1993). The inulin sugar polymer is made up of D-fructose linked by $\beta(2-1)$ bonds with an $\alpha(1-2)$ linked D-glucose at the terminal end (Yazawa and Tamura, 1982). Based on previous studies, inulin is undigestible by the enzymes of the gastrointestinal tract and reaches the colon unchanged (Rumessen *et al*, 1990). Large intestinal microflora readily ferment inulin to produce short chain fatty acids.

In our study, polydextrose and inulin diets as well as fecal samples was measured by the method of Craig *et al* (2000). Polydextrose and similar carbohydrates like inulin do not precipitate in ethanol, which is used to isolate dietary fibre in the gravimetric procedure (Craig *et al*, 2000). It is, therefore, not quantified by the AOAC method for measuring total dietary fibre (TDF). Our results showed that 82.7% and 99.8% of polydextrose and inulin were digested, respectively. In the presence of SST, digestibility was not statistically different (81.5% and 99.4%, respectively).

Figure 5.1: Longitudinal section through the wheat grain¹.

¹Adapted from <http://www.smallgrains.org/WHFACTS/Kernel.html>, 2001.

Measurement of SCFA were performed with cecal contents in this study since regional differences in SCFA concentration have been noted in humans and animals, with the highest concentration in cecum (Norgaard and Mortensen, 1995). Various patterns of short chain fatty acids were observed in this study with the different diets but the major end products were always acetic, propionic and butyric acids. Feeding rats with an oat bran diet led to a high production of butyric acid and other SCFAs to a lesser extent, while acetic acid and propionic acid were the major products of inulin and polydextrose diets. It is apparent that different types of fibre may differ in their ability to produce short chain fatty acids during fermentation. Production of cecal SCFAs in the repletion period of Phase 1 indicated a quick adaptation of rat microflora to their new diet.

Fermentation of inulin has been shown to increase the number and proportion of fecal bifidobacteria and reduce the cecal pH as well as increase cecal weight and cecal content. Potential benefits of inulin include a sequestering of toxic NH_3 by reaction with H_3O^+ to produce NH_4^+ which is not diffusible. This lowers blood ammonia levels. This is brought about by a lowering of the cecal pH, caused by the production of SCFAs. Another benefit associated with low pH is a suppression of the growth of pathogenic bacteria (Wang and Gibson, 1993). Inulin was virtually completely fermented in these experiments. Based on the previous studies (Wang and Gibson, 1993) increased intake of some of specific dietary fibres and fructose-based polymers like inulin specially increase bifidobacteria. This is thought to be beneficial for the host for the reasons discussed above. Rat cecal content and cecal weight were increased in diets containing polydextrose and inulin. These differences were even more significant when 0.5% SST was added to their diets. Several scientists have shown a change in cecal size in rats by

using different sources of dietary fibre. Yoshioka *et al* (1994) reported an increase in rat cecal weight after addition of 5% polydextrose to their diet for the period of 52 days. They also showed an inverse relationship between cecal weight and the pH of the cecum. Fermentable fibres in the diet produce volatile fatty acids in the large intestine and lower the pH of the large intestine (Cumming, 1983). Lupton *et al* (1988) reported the same results on rats after feeding them different sources of dietary fibre for 8 months, and suggested these differences are due to increased hydrogen ion concentrations, which stimulate cell proliferation of the cecum. Inulin generates high amounts of acid because of its rapid and complete fermentation while other dietary fibres are more slowly fermented allowing the gradual absorption of SCFA to occur. Polydextrose may have other effects like roughening the shape of colonic mucosa and thinning the cecal muscular layer (Yoshioka *et al*, 1994).

In our study, we estimated bacterial growth in the large intestine by measuring fecal nitrogen excretion based on Kjeldhal analysis. Total fecal nitrogen excreted in each diet group can be converted to crude protein by multiplying it by the factor of 6.25 representing 16% nitrogen in animal protein (Chen *et al*, 1998). Several other published correction factors are 4.3, representing the 23% nitrogen content of human fecal amino acid (Dintzis *et al*, 1979), and 5.7 representing 17.5% nitrogen in vegetable or animal protein (Marlett and Johnson, 1984). However, since there are many sources of nitrogen in the feces, those conversion factors are not very accurate (Chen *et al*, 1998). All these show that a single relationship does not exist between fecal nitrogen and fecal bacteria. Rats fed ground corn had lower amounts of nitrogen in their feces as compared with animals fed inulin and polydextrose. Diets containing oat bran fibre increased the total

nitrogen excretion. Nitrogen present in the large intestine may not be bacterial related and it may come from salivary, gastric, pancreatic, and biliary secretions as well as from intestinal cell secretions and cell desquamation (Mahe *et al*, 1994). In the large intestine, dietary protein that is not absorbed by the small intestine, epithelial cells, mucines, and urea are all available for bacterial fermentation (Nordgard and Mortensen, 1995). It has been estimated that this contributes 2-3 g/day of nitrogen or 10 % of the intake protein (Smith *et al*, 1979). Previous studies have shown that other factors such as malabsorption, intestinal inflammation and bleeding may increase the total fecal nitrogen excretion (Nordgard and Mortensen, 1995). In oat bran diets, these factors may be significant since an increase in leukocyte counts suggested intestinal inflammation in these diet groups. The presence of 0.5% SST increased the total fecal nitrogen excretion in rats fed different sources of dietary fibre and fermentable substrates.

A combination of the Czerkawski (1973) and El-Shazly methods (1966) was used to measure total DAPA excreted in the rat feces. DAPA is a di-amino acid that is found uniquely in bacterial cell walls. It has been previously shown that the DAPA/protein ratio is relatively constant in bacteria. This suggests that it can be used as an indirect marker of bacterial content in feces (Czerkawski, 1974). The DAPA content of bacterial feces was relatively low, representing approximately 0.004 % of the total protein weight. Total protein was estimated by multiplying the fecal nitrogen content by 6.25.

Muramic acid is another amino acid that is found uniquely in bacteria. It forms part of the peptidoglycan structure that surrounds most gram-positive bacteria (O'Leary, 1989). Determination was performed according to Hadzija (1974) which hydrolyses MA into its components and then measures the lactic acid released. The MA content of

bacterial feces was low, representing 0.01 % of the total protein. This is due, in part, to its unique location in the bacterial wall structure that forms only a small proportion of total bacterial protein.

Both DAPA and MA were followed in the feces as a function of the diet. During the fermentation process, bacterial biomass increases as a result of cell replication. These bacteria attach to the unfermented and partly fermented food products that are excreted in the feces (Puchala *et al*, 1992). The fecal bacteria should, therefore, represent a reliable sampling of the bacteria in the cecum and large intestine. According to the literature, one would predict a linear correlation between DAPA and MA since both amino acids are thought to adequately represent the bacterial population. Although a general trend was apparent, a close correlation between these two values was not obtained (**Figure 4.30**, $r^2=0.45$). Similarly, no good correlations between fecal nitrogen and DAPA or fecal nitrogen and MA (**Figure 4.30**) were observed suggesting that these parameters are, at best, estimates of the amount of fecal bacteria. This is not surprising given the large number of bacterial species present in the rat cecum; it has been estimated that greater than 400 species colonize this organ. In addition, not all these organisms are gram positive-the criterion for the presence of MA nor is the proportion of MA in the cell wall of gram positives constant. Thus, the presence of DAPA does not guarantee the presence of MA. These concerns are further compounded by the problem that dietary fibre and fermentable substrates may stimulate specifically bacterial genera. For example, bifidobacteria are known to increase during inulin feeding (Wang and Gibson, 1993). **Figure (4.18)** demonstrates that the same phenomenon may occur for polydextrose since the large increase in MA indicates a specific stimulation of gram positive bacteria (and perhaps

one with a high MA content in its bacterial wall). Correlations between fecal nitrogen and DAPA or MA may be confounded by differences in protein and nitrogen balance in the rats fed the different diets. These differences can arise as dietary fibre influences the secretion of mucin and the turnover of the gastrointestinal cells. These will provide a nitrogen source to the bacterial culture and may change the relative proportion of species present in the cecum.

Animal Health

The rats tolerated the deficiency and refeeding phases of the diet with no outward signs of distress or folate deficiency. Folate deficiency typically manifests as a tendency for the eyelids to stick together, a thinning of the hair on the head, and loss of weight. Rats also tolerated the addition of SST to the diets without showing any outward signs of folate deficiency. Addition of SST did not affect food consumption.

Addition of dietary fibre had no effect on food consumption; food intake was identical among all groups. Because the diets were designed to have identical energy densities, no differences in energy intake were observed. The greatest weight gains were observed in rats fed the oat bran diet while rats fed the polydextrose diet had the lowest weight gain. These differences were not statistically different. Diarrhea has been reported in Sprague-Dawley rats fed 0.5% SST during a five-week experimental time period (Gant *et al*, 1945) but our rats showed no signs of diarrhea. The addition of SST lowered weight gain and food intake in rats fed oat bran and wheat bran but this effect was not statistically significant. This change may have reflected a reduced preference for this diet.

For comparative purposes, male rats were chosen from the same supplier and from the same strain. In previous studies, researchers have found significant differences

in hematological and biochemical parameters between strains of rodents (Russel *et al*, 1951), rabbits (Laird *et al*, 1970) and monkeys (Altshuler *et al*, 1971). In addition, Lampe *et al* (1993) indicated that gender is an important factor in SCFA analysis. They have shown that digestibility of wheat bran fibre was 43% in women and 37% in men when they consumed the same amount of fibre per day. In women, fecal bulking was lower and mouth to anus transit time was longer than in men. To avoid any biological differences related to gender, male Sprague-Dawley rats were chosen for this project.

Hematological parameters were analyzed at the end of the depletion and repletion phases. The results revealed no sign of folic acid deficiency associated-anemia with different dietary fibres, fermentable substrates with or without sulphonamide drug in rat diets. This is true even though small changes in hemoglobin content, hematocrit and mean cell volume were observed. Increases in mean cell volume are indicative of anemia (Lee, 1999). Thus, the observed increase during the depletion phase suggests the onset of anemia, even though the values still fell within the normal range for rats. Anemia is also reflected in the hematocrit, a relative measure of packed cell volume. Lower hematocrit values and higher cell volume are also an indication of anemia.

In Phase 1, a significant diet $\times$ day interaction and slight decrease in mean cell hemoglobin concentration (MCHC) was also observed during the depletion phase. Since MCHC is the ratio of hemoglobin content to the volume of cells, any biological conditions that affect hemoglobin (Hb) or hematocrit (Hct) will effect the MCHC determination. The MCHC values alone, do not add any significant clinical information in contrast to mean cell volume (MCV), which is a very important factor in classification of anemia (Lee, 1999). Data from Wolford *et al* (1986) indicated that normal MCHC in

rats under the age of 6 months is 37.0 ± 0.95 g/dL. Our values were always in this range. In addition, further examination of the MCHC values over time revealed no clear pattern.

In Phase 2, blood parameters were determined at the end of the repletion phase. Values for the depletion phase were unreliable because of an insufficient concentration of anticoagulant (Ethylenediaminetetraacetic acid, EDTA) in the vacutainer tube used to collect blood. EDTA removes calcium, which is an important factor in blood coagulation (Lee, 1999). Blood parameters at the end of repletion phase are reported in Table: (4.3) and (4.4).

Unlike the situation observed in phase 1, no significant effect of diet or drug on rat leukocyte count was observed in the repletion period of phase 2. Based on Wolford *et al*, 1986, normal leukocyte counts in male rats under the age of six months, are $10.6 \pm 2.8 \times 10^9 / L$. In rats fed diets containing oat bran, ground corn, wheat germ and polydextrose SST increased leukocytes. This might be due to a drug-related side effect. We know of only one report of side effects related to sulfa drugs in animals fed purified diets with succinylsulfathiazole. Welch and Wright (1943) reported spontaneous blood loss, with high prothrombin times in their experimental rat model after 79 days of feeding a basal diet containing succinylsulfathiazole.

White cells were further analyzed to find the percentage of each leukocyte type (segments, lymphocytes, monocytes, eosinophils) by a differential leukocyte count. Analysis of variance revealed a significant effect of diet on monocytes with no consistent pattern of diet; no significant effect of diet and drug was observed on other blood parameters. Based on a Wolford *et al* (1986) study using 324 rats, normal monocyte values for male rats under the age of 6 months are $(4.0 \pm 2.0)\%$. Because the normal

values of circulating monocytes are not precisely defined in human and animals, the association of monocytopenia with a specific condition is not clear. Monocytosis in humans is associated with inflammatory bowel diseases, fevers of unknown origin, and viral or parasitic infections (Turgeon, 1999). In this study, certain dietary fibres, like wheat bran increased monocyte levels as compared with controls, although they were still in the normal range. Rat physiological parameters, such as body weight, food consumption and other blood parameters did not indicate any sign of infection, fever or bleeding. Therefore, those conditions were ruled out as the reason for the increase in monocytes.

The percentage of neutrophilic leukocytes in rats fed polydextrose without SST was higher as compared with other dietary fibre containing diets. This might reflect an inflammation of the large intestine associated with feeding a polydextrose diet. This has previously been observed by Yoshioka *et al* (1994), who noted rough intestinal mucosa and inflammation of the large intestine in rats fed diets containing 20% polydextrose for 79 days.

During the repletion period of phase 2, measured fecal dry weights were higher in rats fed wheat bran, oat bran, and wheat germ diet. Wheat bran is a highly lignified plant tissue (67% hemicellulose, 23% lignin, and 10% cellulose) and is mostly water insoluble. It resists fermentation in the colon and therefore increases dry fecal weight, decreases intestinal transit time, and increases bacterial cell mass in the feces (Lampe *et al*, 1993, Jenkins *et al*, 1987). Gravimetric fractionation of stools in human analysis by Stephan and Cummings (1980) indicated that most of the increase in stool weight with wheat bran diet was related to undigested fibre. Fecal dry weight in rats consuming oat bran was also

higher. In rats fed the oat bran diet, the increase in rat dry fecal weight is most likely due to increased bacterial excretion. This is because oat bran is very well fermented in the lower bowel so that very little of the ingested fibre is excreted. Our measurement show that only 20% of the ingested oat fibre is found in the feces. Data from other studies using the rat as an animal model examining the fecal bulking effect of wheat bran and oat bran support these conclusions (Ranhotra, *et al*, 1991; Mongeau *et al*, 1990). Dry fecal weight in animals fed the wheat germ diet was almost as high as wheat bran diet, while the estimated fibre digestion results showed that wheat germ fibre was about 95% digested and wheat bran fibre was only 40% digested in the rat large intestine. The percentage of fecal fibre in the wheat germ diet was lower than that of the wheat bran diet. So that other factors, like increased bacterial mass, might be involve in increasing the fecal output of animals fed wheat germ diets. Bacterial mass was measured indirectly by measuring nitrogen, muramic acid and diaminopimelic acid. Dry fecal weight in animals fed the ground corn, inulin and polydextrose were significantly lower to compare with standards. This is partly due to a lower fecal fibre content and lower bacterial amounts in feces from animals fed ground corn but the results for inulin and polydextrose are less clear cut.

Microbiological assay for folate

Total folate content in liver and feces was analyzed using a microbiological assay with a tri-enzyme extraction procedure. The tri-enzyme treatment was used to extract total folates that may be trapped in or bound to the matrices of protein and polysaccharides. This method also releases folates trapped during heating of foods. The freed folates are then extracted and diluted with Difco *L. casei* folic acid basal medium

containing all required growth nutrients except folate, and the turbidity of the *L.casei* (ATCC 7469) growth response is compared quantitatively to that of known standard folate solutions. The assay was carried out using a 96-well microplate with a microplate reader. The microbiological assay for folate analysis is not only very sensitive to small amounts of test material, but is very accurate as well.

L. casei (ATCC 7469) is a good choice of microorganism since its nutritional requirements (biotin, pantathenic acid, nicotinic acid, pyridoxine, riboflavin) are well defined and present in the media. As it grows, it acidifies the growth media. The rate of acid production is, therefore, proportional to its growth. This property has been used for other assays like riboflavin; *L. casei* is a familiar microorganism in laboratories (Landy and Dicken, 1942).

5.2 Conclusion

During the 28-day repletion phase, the increase in liver folate was proportional to the dietary folate content and did not vary with the inclusion of dietary folate and fermentable substrates. Fibre disappearance, SCFA production, an increase in fecal folate content, fecal muramic acid, diaminopimelic acid, and an increase in total N production provided evidence for an increased bacterial fermentation. Increased production of folate, accompanied the increase in fermentation. However, colonic folate produced by bacterial fermentation did not significantly contribute to folate bioavailability in the rat.

References

- Abad, A. R., and Gregory, J. F.III, Assessment of folate bioavailability in the rat using extrinsic dietary enrichment with radiolabels folates, *J. Agric. Food. Chem*, **1988**, 36: 97-104
- Abad, A. R., and Gregory, J. F.III, Determination of folate bioavailability with a rat bioassay, *J. Nutr*, **1987**, 117: 866-873
- Ali, L. H., Weaver, A. C. M., and Rader, J. I., Comparison of capillary column gas chromatographic and AOAC gravimetric procedure for total fat and distribution of fatty acids in foods, *Food chemistry*, **1997**, 58 (1), 149-160
- Altshuler, H. L., Stowell, R. E., and Lowe, R. T., Normal serum biochemical values of *Mocaca arctoides*, *Macaca fascicularis*, *Macaca radiata*, *Lab. Anim. Sci*, **1971**, 21, 916-926.
- Asp, Nils-Georg, Definition and analysis of dietary fibre, *Scand. J. Gastroent*, **1987**, 22 (Supplement 129), 16-20
- Basch Knudsen, K. E., Wisker, E., Daniel, M., Feldheim, and Eggum, B. O., Digestibility of energy, protein, fat and non-starch polysaccharides in mixed diets: comparative studies between man and the rat, *Br. J. Nutr*, **1994**, 71(4), 471-487.
- Bailey, L. B., Folate in health and disease, Marcel Dekker, Inc, New York, **1995**.
- Bailey, L. B., Barton, L. E., Hillier, S. E., and Creda, J. J., Bioavailability of mono and polyglutamyl folate in human subjects, *Nutr. Rep. Int*. **1988**, 38: 509-518
- Bailey, L. B., Cerda, J. J., Bloch, B. S., Busby, M. J., Varga, L., Chandler, C. J., and Halsted, C. H., Effect of age on poly- and monoglutamyl folacine absorption in human subjects, *J. Nutr*, **1984**, 114: 1770-1776
- Bahandari, S. D., and Gregory, J. F. III, Folic acid, 5-methyl-tetrahydrofolate and 5-formyl-tetrahydrofolate exhibit equivalent intestinal absorption, metabolism and in vivo kinetics in rats, *J. Nutr*; **1992**, 122: 1847-1854
- Bahandari, S. D., and Gregory, J. F. III, Inhibition by selected food components of human and porcine intestinal pteroylpolyglutamate hydrolase activity, *Am. J. Clin. Nutr*. **1990**, 51: 87-94
- Ball, G. F. M., Bioavailability and Analysis of Vitamins in foods, Chapman and Hall, New York, U. S. A, **1998**.
- Baxter, M. G., Millar, A. A., Webster, R.A., Some studies on the convulsant action of folic acid, *Br. J. Pharmacol*, **1973**, 48: 350-351

Bendich, A., and Deckelbaum, R. J., Preventive Nutrition, The comprehensive guide for health professionals, Human Press, New Jersey, U. S. A, 2001.

Bergman, E. N., Energy contributions of volatile fatty acids from the gastrointestinal tract in various species, *Physiol Rev*, 1990, 70(2): 567-90.

Bingham, S. A., Plant cell wall material and cancer protection in food and cancer prevention: chemical and biological aspects, (Waldron, K. W., Johnson I. T., and Fenwick, G. R. editors), Cambridge: The Royal Society of Chemistry, 1993, 339-347.

Bingham, S. A., Williams, D. R. R. and Cummings, J. H., Dietary fibre consumption in Britain: new estimates and their relation to large bowel cancer mortality, *Br. J. Cancer*, 1985, 52, 399-402.

Blakley, R., Benkovic, S., Folate and Pterins, John Wiley and Sons, New York, U. S. A 1984.

Brody, T., Nutritional Biochemistry, Academic Press, New York, 2nd Ed., U. S. A, 1999.

Brouwer, I. A., Dusseldorp, M. V., Thomas, C. M. G., Duran, M., Hautvast, J. G. A. J., Eskes, T. K. A. B., and Steegers-Theunissen, R. P. M., Low-dose folic acid supplementation decreases plasma homocysteine concentrations: a randomized trial, *Am. J. Clin. Nutr*, 1999, 69:99-104.

Burkitt, D. P., Epidemiology of cancer of the colon and the rectum, *Cancer*, 1971, 28, 3-13

Butterworth, C. E., and Bendich, A., Folic acid and the prevention of birth defects, *Annu. Rev. Nutr*, 1996, 16: 73-97.

Butterworth, C. E., and Tamura, T., Folic acid safety and toxicity, A brief review, *Am. J. Clin. Nutr*, 1989, 50: 353-3558

Campbell, N. R., How safe are folic acid supplements? *Arch. Intern. Med*, 1996, 156: 1638-1644

Chen, T. S., and Copper, R. G., Thermal destruction of folacine: effect of ascorbic acid, oxygen and temperature, *J. Food. Sci*, 1979, 44: 713-716

Chen, H-L., Haack, V. S., Janecky, C. W., Vollendorf, N. W., and Marlett, J. A., Mechanisms by which wheat bran and oat bran increase stool weight in humans, *Am J Clin Nutr*, 1998; 68: 711-719

Chiasson, R. B., Laboratory anatomy of the white rat, Wm. C. Brown Publisher, U. S. A 1990.

Cho, S. S., Prosky, L., Dreher, M., Complex carbohydrates in foods, Marcel Dekker, Inc, New York, 1999.

Cho, S. S., Prosky, L., Complex carbohydrates: Definition and Analysis, In: Complex carbohydrates in foods, Marcel Dekker, Inc, New York, 1999.

Coates, M. E., Ford, J. E., and Harrison, J. F., Intestinal synthesis of vitamins of the B complex in chicks, *Br. J. Nutr.*, **1968**, 22:493-500

Craig, S. A. S., Holden, J. F., and Khaled, M. J., Determination of polydextrose in foods as dietary fibre, *J. AOAC Intern'l*, **2000**, 83, 1006-1012.

Cummings J. H., Fermentation in the human large intestine: evidence and implications for health, *Lancet*, **1983**, 28, 1(8335): 1206-9.

Cummings J. H., and Englyst, H. N., Fermentation in the human large intestine and the available substrates, *Am J Clin Nutr*, **1987**, 45, 1243-1255

Cummings J. H., and Macfarlane G. T., The control and consequences of bacterial fermentation in the human colon, *J. Appl. Bacteriol*, **1991**, 70, 443-459.

Czerkawski, J. W., and Faulds, C., Methods for determining 2-6-Diaminopimelic Acid and 2-Aminoethylphosphonic acid in gut contents, *J. Sci. Food Agric*, **1974**, 25, 45-55.

Daft, F. S., McDaniel, E. G., Herman, L. G., Romine, M. K., and Hegner, J. R., Role of coprophagy in utilization of B vitamins synthesized by intestinal bacteria, *Fed. Proc.* **1963**, 22:129-133

Darcy-Vrillon, B., Selhub, J., Rosenberg, I. H., Analysis of sequential events in intestinal absorption of folylpolyglutamate, *Am. J. Physiol*, **1988**, 255:G361-G366

Deguchi, Y., Morishita, T., and Mutai, M., Comparative studies on synthesis of water-soluble vitamins among human species of bifidobacteria, *Agric. Biol. Chem.*, **1985**, 49 (1), 13-19

Delzenne, N. M., and Roberfroid, M.R., Physiological effects of non-digestible oligosaccharides, *Lebensm.-Wiss.u-Technol*, **1994**, 27, 1-6

Devries, W. J., Keagy, P. M., Hudson, C. A., and Rader, J. I., AACC collaborative study of a method for determining total folate in cereal products microbiological assay using trienzyme extraction (AACC method 86-47), *Cereal. Food World*, **2001**, 46 (5): 216-219

Dintzis, F. R., McBrien, J. B., and Baker, F. L., Some effects of baking and human gastrointestinal action upon a hard red wheat bran In: Dietary fibres, chemistry and nutrition, Inglett, G. E., and Falkehag, S. I., eds, pp: 157-171, Academic press, New York, **1979**.

Divedi, C. M., Dormody, J., Chandler, C., Wang, T., and Halsted, C. H., Characteristics of human intestinal folate conjugases, *Fed. Proc.* **1983**, 42, 667 (abstract 2194)

Eichner, E. R., and Hillman, R. S., The evolution of anaemia in alcoholic patients, *Am. J. Med.*, **1971**, 50:218-232

El-Shazly, K., and Hungate, R. E., Method for measuring Diaminopimelic acid in total rumen contents and its application to the estimation of bacterial growth, *Applied Microbiology*, **1966**, 14 (1): 27-30.

Englyst, H. N., and Cummings, J. H., Digestion of the polysaccharides of potato in the small intestine of man, *Am. J. Clin. Nutr.*, **1987**, 45: 423-431.

Englyst, H. N., and Cummings, J. H., Digestion of the carbohydrates of banana in the human small intestine, *Am. J. Clin. Nutr.*, **1986**, 44: 42-50.

Englyst, H. N., and Cummings, J. H., Digestion of the polysaccharides of some cereal foods in the human small intestine, *Am. J. Clin. Nutr.*, **1985**, 42: 778-787.

Figdor, S. K., and Rennhard H. H., Caloric utilization and disposition of [¹⁴C] polydextrose in the rat, *J. Agric. Food. Chem.*, **1981**, 29:1181-1189

Fleming, A., The role of folate in the prevention of neural tube defects: Human and Animal studies, *Nutrition Reviews*, **2001**, 59(8): S13-S28.

Food and Drug Administration, Federal Register, Food Labeling, Health Claims and Label Statements: Folate and neural tube defects, Public Health Services, Tuesday March 5, **1996**.

Food and Nutrition Board Institute of Medicine, A report of the standing committee on the Scientific evaluation of dietary reference intakes and its Panel on folate, other B vitamind, and choline and Subcommittee on upper reference levels of nutrients, National Academy Press, Washington, D.C., U. S. A, **1998**.

Fox, A., Schwab J. H., and Cochran, T., Muramic acid detection in mammalian tissues by gas-liquid chromatography-mass spectroscopy, *Infect. Immun.*, **1980**, 29: 526-531

Friedrich, W., Folic acid and unconjugated pteridines In: Vitamins, Walter de Gruyler, New York, U. S. A, **1988**.

Gant, O. K., Ransone, B., McCoy, E., and Elvehjem, C. A., Intestinal flora of rats on purified diets containing sulfonamides, *Proc. Soc. Exp. Biol. and Med.*, **1943**, 52:276-279.

Garrett, R. H., and Grisham, C. M., Biochemistry, Saunders College Publishing, New York, **1995**.

Gestel, G., Besancon, P., and Rouanet, J. M., Comparative evaluation of the effects of two different forms of dietary fibre (Rice Bran vs. Wheat Bran) on rat colonic mucosa and faecal microflora, *Ann Nutr Metab*, **1994**, 38:249-256.

Gibson, G. R., Beatty, E. R., Wang, X., and Cummings, J. H., Selective stimulation of bifidobacteria in the human colon by oligofructose and inulin, *Gastroenterology*, **1995**, 108, 975-982

Gilbert, J., and Fox, A., Elimination of group A streptococcal cell walls from mammalian tissues, *Infect. Immun*, **1987**, 55: 1526-1528

Graham, D. C., Rose, D.A., and Ostertag, S.G., Radiometric determination and chick bioassay of folacine in fortified and unfortified frozen foods, *J. Food Sci*, **1980**, 45:47-51

Gregory, J. F., Case study: Folate Bioavailability, *J. Nutr*, **2001**, 131-1376S-1382S.

Gregory, J. F., Bahandari, S. D., Baily, L. B., Toth, J. P., Bbaumgartner, T. G., and Cerda, J. J., Relative bioavailability of deuterium-labeled monoglutamyl tetrahydrofolates and folic acid in human subjects, *Am. J. Clin. Nutr*, **1992**, 55: 1147-1153

Gregory, J. F., Chemical and nutritional aspects of folate research: analytical procedures, methods of folate synthesis, stability, and bioavailability of dietary folates, *Adv. Food Nutr. Res*, **1989**, 33:1-101

Guyton, A. C., and Hall, J. E., Human physiology and mechanisms of disease, W.B Saunders Company, U. S. A, **1996**.

Hadzija, O. A., Simple method for the quantitative determination of muramic acid, *Anal Biochem*, **1974**, 60(2): 512-7.

Halsted, C. H., Chandler, C. J., Wang, T., Beer, W. H., and Dwivedi, C.M., Brush border folate conjugase: a zinc activated enzyme, *Gastroenterology*, **1983**, 84:11791.

Halsted, C. H., The intestinal absorption of folates, *Am. J. Clin. Nutr*, **1979**, 32:846-855

Hawkes, J. G., and Villota, R., Folate in foods: reactivity, stability during processing, and nutritional implications, *Crit. Rev. Food Sci. Nutr*, **1989**, 28, 439-539

Health and Welfare Canada, Canada's food guide to healthy eating, Health Protection Branch, Health and Welfare Canada, **1992**.

Health and Welfare Canada, Report of the Expert Advisory Committee on Dietary Fibre, Health Protection Branch, Health and Welfare Canada, **1985**.

Health Protection Branch Laboratories, Sequential Method for rapid determination of total dietary fibre, In *laboratory procedure LPFC-162*; Bureau of Nutrition Sciences, Health and Welfare Canada: Ottawa, Canada, 1992.

Hebel, R., and Stromberg, M. W., Anatomy and Embryology of the laboratory rat, BioMed- Verlag, Germany, 1986.

Henderson, G. B., Folate Binding Proteins, *Annu. Rev. Nutr*, 1990, 10:319-335.

Hendrickson, B. A., Guo, J., Laughlin, R., Chen, Y., and Alverdy, J. C., Increased type I fimbrial expression among commensal Escherichia coli isolated in the murine cecum following catabolic stress, *Infect. Immun*, 1999, 67: 745-753

Herbert, V. D., Folate supplements should be appropriately labeled to protect consumers, *Pediatrics*, 1994, 93: 694-695.

Herbert V, Biochemical and hematologic lesions in folic acid deficiency, *Am J Clin Nutr*, 1967, 20(6): 562-72.

Herbert, V. D., and Colman, N., Folic acid and Vitamin B₁₂, In: Modern Nutrition in Health and Disease, Shils, M. E. and Young, V. R., eds, pp.388-416, Lea and Febiger, Philadelphia, 1988.

Herbert, V.D., and Das, K. C., Folic acid and vitaminB₁₂, In: Modern Nutrition in Health and disease, Shils, M. E., Olson, J. A. and Moshe, S., eds, Vol. 8, pp. 402-425, Lea and Febiger, Philadelphia, 1994.

Hipsley, E. H., Dietary Fibre and pregnancy toxemia, *Br. Med. J.* 1953, 2, 420-422.

Hoiyer M. A., Melief M. J., vanHelden-Meeuwssen, C. G., Eulderink, F., and Hazenberg, M. P., Detection of muramic acid in a carbohydrate fraction of human spleen, *Infect-Immun*, 1995, 63(5) 1652-7.

Hoppner, K., and Lampi, B., Antiacid ingestion and folate bioavailability in the rat, *Nutr. Rep. Int'l*, 1988, 38(3): 539-546.

Hoppner, K., and Lampi, B., Chemistry and Biology of Pteridines, Walter de Gruyter & Co., Berlin, New York-printed in Germany, 1986.

Hoppner, K., and Lampi, B., Liver folacin as a response parameter for a folacine bioassay in rats, *Nutr. Rep. Int'l*, 1985, 31, 203-211.

Hoppner, K., and Lampi, B., Folate levels in human livers from autopsies in Canada, *Am. J. Clin Nutr*, 1980, 33: 862-864.

Houghton, L.A., Green, T.J., Donovan, U.M., Gibson, R.S., Stephen, A.M., and O'Coner, D. L., Association between dietary fibre intake and the folate status of a group of female adolescents, *Am. J. Clin Nutr*, 1997, 66(6): 1414-1421.

Hume, I. D., Fermentation in the hindgut of mammals, In: Gastrointestinal microbiology. Gastrointestinal ecosystems and fermentations, Mackie, R. I. and white, B. A, eds, Chapman and Hall Microbiology Series, New York, 1996, v.1, 424-469.

Hurdle, A. D. F., Barton, D., and Searles, I. H., A method for measuring folate in food and its application to a hospital diet, *Am. J. Clin Nutr*, 1968, 21, 1202-1207.

Hutchings, B. L., Bohonos, N., and Peterson, W.H., Growth factors for bacteria, XIII. Purification and properties of an eluate factor required by certain lactic acid bacteria, *J. Biol Chem*, 1941, 141: 521-528.

Jenkins, D. J., Peterson, R. D., Thorne, M. J., Ferguson, P. W., Wheat fiber and laxation: dose response and equilibration time, *Am J Gastroenterol*, 1987, 82(12): 1259-63.

Jones, J., Update on defining dietary fibre, *Cereal food world*, 2000, 45 (5): 219-220.

Keagy, P. M., Folic acid metabolism in health and disease, Wiley-liss, Inc., U. S. A, 1990.

Keagy, P. M., and Oace, S. M., Rat bioassay of wheat bran folate and effects of intestinal bacteria, *J. Nutr*, 1989, 119: 1932-1939

Keagy, P. M., Shane, B., and Oace, S. M., Folate Bioavialability in humans: effects of wheat bran and beans, *Am. J. Clin Nutr*, 1988, 47: 80-88

Keagy, P. M., Rat bioassay of folate monoglutamate, heptaglutamate, and wheat bran, *Fed. Proc. Fed. Am. Soc. Exp. Biol*, 1985, 44, 777

Keagy, P.M., and Oace, S.M., Folic acid utilization from high fibre diets in rats, *J. Nutr*, 1984, 114, 1252-1259.

Keagy, P.M., and Oace, S.M., Development of folacin bioassay in rats, *J. Nutr*, 1982, 112: 77-81

Kehl, S. J., McLennan, H., and Collingridge, G.L., Effects of folic and Kainic acids on synaptic responses of hippocampal neurons, *Neuroscience*, 1984, 11: 111-124.

Kim, Y-I., Pogribny, I. P., Basnakian, A. G., Miller, J.W., Selhub, J., James, S.J., and Mason J. B., Folate deficiency in rats induces DNA strand breaks and hypomethylation with the p53 tumor suppressor gene, *Am. J. Clin Nutr*, 1997, 65:46-52.

Kobayashi, H., and Fleming, S. E., The Source of Dietary Fibre Influences-Short chain fatty acid production and concentrations in the large bowel, In: CRC hand book of dietary fibre, Susan S. C., and Dreher M. L., Ed, Marcel Dekker Inc, New York, 2001.

Kozar, M. P., Krahmer, M. T., Fox, A., and Gray, B. M., Failur to detect muramic acid in normal rat tissues but detection in cerebrospinal fluids from patients with pneumococcal meningitis, *Infect-Immun*, 2000, 68(8): 4688-4698

Krause, L. J., Forsberg, C. W., and O'Conner, D. L., Feeding human milk to rats increases bifidobacterium in the cecum and colon which correlates with enhanced folate status, *J. Nutr*, 1996, 126 (5): 1505-1511.

Krebs, N. F., Bioavialability of dietary supplements and impact of physiologic state: Infants, Children and adolescents, *J. Nutr*. 2001, 131: 1351S-1354S

Kruse, H. P., Klessen, B., and Blaut, M., Effects of inulin on fecal bifidobacteria in human subjects, *Br. J. Nutr*, 1999, 82, 375-382.

Landy, M., and Dicken, D. M., A microbiological assay method for six B vitamins using lactobacillus casei and a medium of essintially known composition, *J. Lab.Clin. Med.*, 1942, 27: 1086-1092.

Laird, C. W., Fox, R. R., Mitchell, B. P., Blau, E. M., and Schultz, H. S., Effect of strain and age on some hematological parameters in the rabbit, *Am. J. Physiol*, 1970, 218, 1613-1616

Lampe, J. W., Wetsch, R. F., Thompson, W. O., and Slavin, J. L., Gastrointestinal effects of sugarbeet fiber and wheat bran in healthy men, *Eur J Clin Nutr*, 1993, 47(8): 543-8.

LaRusso, N. F., Proteins in bile: How they get there and what they do, *Am. J. Physiol*, 1984, 247: G199-G205.

Lee, G. R., Wintrobe's clinical hematology, Williams and Wilkins, Baltimore, 1999.

Leichter, J., Switzer, V. P., and Landymore, A. F., Effect of cooking on folate content of vegetables, *Nutr. Rep. Int'l*, 1978, 18, 475-479

Lentz, S.R., and Sadler, J. E., Inhibition of thrombomodulin surface expression and protein C activation by the thrombogenic agent homocystein, *J. Clin Invest*, 1991, 88: 1906-1914.

Lin, K. C., Luh, B. S., and Schweigert, B.S., Folic acid content of canned garbanzo beans, *J. Food Sci.*, 1975,40, 562-565.

Lindenbaum, J. Rosenberg, I. H., Wilson, P. W., Stabler, S. P., and Allen, R. H., Prevalence of cobalamin deficiency in the Framingham elderly population, *Am. J. Clin Nutr*, **1994**, 60: 2-11(1996).

Lindenbaum, J., Heaton, E. B., Savage, D. G., Brust, J. C., Garrett, T. J., Podell, E. R., Marcell, P. D., Stabler, S. P., and Allen, R. H., Neuropsychiatric disorder caused by cobalamin deficiency in the absence of anemia or macrocytosis, *N. Engl. J. Med*, **1988**, 318, 1720-1728

Loots, J. M., Kramer, S., and Brennan, M. J. W., The effect of folates on the reflex activity in the isolated hemisected frog spinal cord, *J. Neural. Transm*, **1982**, 54: 239-249.

Low, A. G., Nutritional requirement of gastric secretion digestion and emptying, *Nutr. Res. Rev*, **1990**, 3, 229-252.

Lupton, J. R., Coder, D. M., Jacobs, L. R., Long-term effects of fermentable fibers on rat colonic pH and epithelial cell cycle, *J Nutr*, **1988**, 118(7): 840-5.

Macfarlane, G. T., and Cummings, J. H., Probiotic and prebiotics: can regulating the activities of intestinal bacteria benefit health?, *B. M. J*, **1999**, 318: 999-1003.

Mahe, S., Roos, N., Benamouzig, R., Sick, H., Baglieri, A., Huneau, J. F., and Tome, D., True exogenous and endogenous nitrogen fractions in the human jejunum after ingestion of small amounts of ^{15}N -labeled casein, *J. Nutr*, **1994**, 124: 548-555.

Malkki, Y., Oat fibre production, composition physiochemical properties, physiological effects, safety, and food applications, In: CRC hand book of dietary fibre, Susan S. C., and Dreher M. L., Marcel Dekker Inc, New York, **2001**.

Marlett, J. A., and Johnson, E. J., Composition of fecal fibre from human subjects, *J. Nutr*, **1984**; 115: 650-660

Mason, J. B., and Levesque, T., Folate: Effects on carcinogenesis and the potential for cancer chemo-prevention, *Oncology*, **1996**, 10: 1727-1736.

McBurney, M. I., Van Soest, P.J., and Jeraci, J. L., Colonic carcinogenesis: the microbial feast or famine mechanism, *Nutrition and cancer*, **1987**, 10: 23-28

McCleary, B. V., Enzyme purity and activity in fibre determinations, *Cereal Food World*, **1999**, 44, 590-596.

McCleary, B. V., and Prosky, L., Advanced Dietary Fibre Technology, Blackwell Science Ltd, U. S. A., **2001**.

Metges C. C., Contribution of microbial amino acids to amino acid homeostasis of the host, *J. Nutr*, **2000**, 130 (7): 1857S-1864S.

Miller K. A., The effect of Succinylsulfathiazole and phthylsulfathiazole on the bacterial flora of rat feces, *J. Nutr*, **1944**, 29: 143-154.

Miller H. T., and Lucky T. D., Intestinal synthesis of folic acid in monoflora chicks, *J. Nutr*, **1963**, 80: 237-242

Mongeau, R., Brassard, R., Deeks, J. R., Laffey, P. J., Nguyen, L., and Brooks, S. P. J., Comparision of dietary fibre contents of selected baby foods from two major brands in canada using three methods, *J. Agric. Food Chem*, **2001**, Vol 49 (8): 3782-3786.

Mongeau, R., Siddiqui, I. R., Emery, J., and Brassard, R., Effect of dietary fibre concentrated from celery, parsnip, and rutabaga on intestinal function, serum cholestrol, and blood glucose, *J Agri Food Chem*, **1990**, 38: 195-200.

Mongeau, R., and Brassard, R., Enzymatic-gravimetric determination in foods of dietary fibre as sum of insoluble and soluble fibre fractions: Summary of collabrative study, *J.AOAC Int*, **1993**, 76, 923-925.

Mongeau, R., and Brassard, R., A rapid method for the determination of soluble and insoluble dietary fibre: comparison with AOAC total dietary fibre procedure and Englyst's method, *J. Food Sci*, **1986**, 51: 1333-1336.

Mongeau, R., Scott. F. W., and Brassard, R., Definition and analysis of dietary fibre: Complex carbohydrates in foods, Edited by Sungsoo Cho, Prosky, L, Dreher, M, Marcel Dekker INC. New York, **1999**, 305-315.

Nordgaard, I., and Mortensen P. B., Digestive process in the human colon, *Nutrition*, **1995**, 11(1): 37-45.

Nyman, M., and Asp, N. G., Dietary fibre fermentation in the rat intestinal tract: effect of adaptation period, protein and fibre levels, and particle size, *Br. J. Nutr*, **1985**, 54 (3), 635-643.

Oakenfull D., Physiochemical property of dietary fibre overview, In: CRC hand book of dietary fibre, Susan S. C., and Dreher M. L., Ed, Marcel Dekker Inc, New York, **2001**.

Organozation for economic co-operation and development (OECD) guidelines for testing of chemicals, OECD Publications, France, **1993**.

O'Keefe, C. A., Bailey, L. B., Thomas, E. A., Hofler, S. A., Davis, A. B., Cerda, J. J., and Gregory J. F. III, Controlled Dietary Folate Affects Folate Status in Nonpregnant Women, *J. Nutr*, **1995**, 125:2717-2725.

O'Leary, W. M., Practical handbook of microbiology, CRC Press, Inc, Boca Raton, Florida, 1989.

Popesko, P., Rajtova, V., and Horak, J., A color atlas of anatomy of small laboratory animals, Wolfe publishing Ltd. England, 1992.

Prosky, L., What is dietary fibre, *J. AOAC Inter'l*, 2000, 83, (4), 985-987.

Prosky, L., and Devries, J., Controlling Dietary fibre in food products, Van Nostrand Reinhold, New York, U.S.A, 1992.

Puchala, R., Pior, H., and Kulasek, G.W., Determination of diaminopimelic acid in biological materials using high-performance liquid chromatography, *J. Chromatogr*, 1992; 623:63-67

Ranhotra, G. S., Gelroth, J. A., Glaser, B. K., and Rao C. S., Fecal bulking effect of whole grain flour from selected grains, *Cereal Chem*, 1991, 68, 556-558

Reddy, B., Engle, A., Katsifis, S., Simi, B., Bartram, H.P., Perrino, P. and Mahan, C., Biochemical epidemiology of colon cancer: effect of types of dietary fibre on fecal mutagens, acid, and neutral sterols in healthy subjects, *Cancer research*, 1982, 49, 4629-4635

Reddy, B. S., Hedges, A. R., Laakso, K. and Wynder, E. L., Metabolic epidemiology of large bowel cancer Fecal bulk and constituents of high risk North America and low risk Finnish population, *Cancer*, 1978, 42, 2832-2838.

Reisenauer, A. M., and Halsted C. H., Human folate Requirements, *J. Nutr*, 1987, 117, 600-602

Reisenauer, A. M., Halsted C. H., Human jejunal brush border folate conjugase, *Biochim. et Biophys. Acta*, 1981, 659: 62-69.

Reisenauer, A. M., Krumdieck, C. L., and Halsted, C. H., Folate conjugase: Two separate activities in human jejunum, *Science*, 1977, 98:196-197.

Report of the Committee on Medical Aspects of Food and Nutrition Policy, 50 Folic acid and the prevention of disease, Department of Health, Norwich, 2001.

Rijke, R.P., Plaisier, H. M., and Langendoen, N. J., Epithelial cell kinetics in the descending colon of the rat, *Virchows Arch. Cell. Pathol*, 1979, 30: 85-94.

Ristow, K.A., Gregory, J.F. III., and Damron, B.L., Effect of dietary fibre on the bioavailability of folic acid monoglutamate, *J. Nutr*, 1982, 112, 750-758.

Rong, N. I., Selhub, J., Goldin, B. R., and Rosenberg, I. H., Bacterially synthesized folate in rat large intestine is incorporated into host tissue folyl polyglutamates, *J. Nutr*, **1991**, 121: 1955-1959.

Royall, D, Wolever, T. M., and Jeejeebhoy, K. N., Clinical significance of colonic fermentation, *Am J Gastroenterol*, **1990**, 85(10): 1307-12.

Rozman, K., and Hanninen, O., Gastrointestinal toxicology, Elsevier, New York, **1986**.

Rumessen, J. J., Bode, S., Hamberg, O., and Gudmand-Hoyer E., Fructans of Jerusalem artichokes: intestinal transport, absorption, fermentation, and influence on blood glucose, insulin, and C-peptide responses in healthy subjects, *Am J Clin Nutr*, **1990**, 52(4): 675-81.

Russell, R. M., Dhar, G. J., Dutta, S. K., and Rosenberg, I. H., Influence of interluminal pH on folate absorption: Studies in control subjects and in patients with pancreatic insufficiency, *J Lab Clin Med*, **1979**, 93: 428-436.

Russell, R. M., Golner, B. B., Krasinski, S. D., Sadowski, J.A., Suter, P. M., and Braun, C. L., Effect of anti acid and H2 receptor antagonists on the intestinal absorption of folic acid, *J Lab Clin Med*, **1988**, 112:458-463.

Russell, R. M., Krasinski, S. D., Samloff, I. M., Jacob, R. A., Hartz, S. C., and Brovender, S. R., Folic acid malabsorption in atrophic gastritis, *Gastroenterology*, **1986**, 91:1476-1482.

Russell, R. M., Ismail-Beigi, F., and Reinhold, J.G., Folate content of Iranian breads and the effect of their fibre content on the intestinal absorption of folic acid, *Am. J. Clin. Nutr*, **1976**, 29, 799-802.

Russell, E. S., Neufield, E. F., and Higgins, C. T., Comparison of normal blood picture of young adults from 18 inbred strains of mice, *Proc. Soc. Exp. Biol. Med*, **1951**, 78: 761-766

Said, H. M., Horne, D. W. and Wagner, C. Effect of human milk folate binding protein on folate intestinal transport, *Archive. Biochem. Biophys*, **1986**, 251:114-120.

Schweigert, B.S., Pollard, A. E., and Elvehjem, C. A., The folic acid content of meats and the retention of this vitamine during cooking, *Arch. Biochem*, **1946**, 10, 107-111.

Selby, J. V., Friedman, G. D., and Fireman, B. H., Screening prescription drugs for possible carcinogenicity: Eleven to fifteen years of follow up, *Cancer Res*, **1989**, 49: 5736-5747.

- Seyoum, E., and Selhub, J., Properties of food folate determined by stability and susceptibility to intestinal pteroylpolyglutamate hydrolase action, *J. Nutr*, **1998**, 128: 1956-1960.
- Shane, B., Biochemical and physiological aspects of human Nutrition: Folic acid, Vitamin B12, and Vitamin B6, W.B. Saunders Company. U.S.A, **2000**.
- Shetty, P. S., and Kurpad, A. V., Increasing starch intake in the human diet increases fecal bulking, *Am J Clin Nutr*, **1986**, 43(2): 210-2.
- Shiota, T., Biosynthesis of folate from pterin precursors in Folate and Pterins, In: Chemistry and biochemistry of folates, Blakley, R. L., and Benkovic, S., John Wiley and Sons, New York, U.S.A, **1984**, 1, 121-134.
- Shoda, R., Mason, J. B., Selhub, J., and Rosenberg I. H., Folate binding in intestinal brush border membranes: evidence for the presence of two binding activities, *J. Nutr. Biochem*, **1990**, Vol (1): 257-261.
- Skeggs, H. R., and Wright, L. D., Vitamin B complex studies with diets differing in the carbohydrate component, *J. Nutr*, **1946**, 32: 375-386.
- Slavine, J., Dietary Fibre: Classification, Chemical analyses, and food sources, *J. Am. Diet. Assoc*, **1987**, vol: pp1164-1171.
- Soughgate, D. A. T., Polysaccharide food additives that contribute to dietary fibre, In: CRC hand book of dietary fibre, Susan S. C., and Dreher M. L., Marcel Dekker Inc, New York, **2001**.
- Steinberg, S. E., Mechanisms of folate homeostasis, *Am. J. Physiol*, **1984**, 246:G319-G324.
- Stephen, A. M., Cummings, J. H., Mechanism of action of dietary fibre in the human colon, *Nature*, **1980**, 284, 283-284.
- Stipanuk, M. H., Biochemical and physiological aspects of human nutrition, W.B. Saunders Company. U.S.A, **2000**.
- Tortora, G. J., Funke, B. R., and Case, L. C., Microbiology: an introduction. 3rd edition, The Benjamin/ Cummings Publishing Company, Inc, U.S.A, **1989**.
- Trowell, H., Crude fibre, dietary fibre and atherosclerosis, *Atherosclerosis*, **1972**, 16, 138-140.
- Trowell, H., Definition of dietary fibre and hypothesis that it is a protective factor in certain disease, *Am. J. Clin. Nutr*, **1976**, 29, 417-427

- Tortora, G. J., Funke, B. R., and Case, L. C., Microbiology: an introduction. 3rd edition, The Benjamin/ Cummings Publishing Company, Inc, U.S.A, 1989.
- Trowell, H., Crude fibre, dietary fibre and atherosclerosis, *Atherosclerosis*, 1972, 16, 138-140.
- Trowell, H., Definition of dietary fibre and hypothesis that it is a protective factor in certain disease, *Am. J. Clin. Nutr*, 1976, 29, 417-427
- Turgeon, M. L., Clinical Hematology, theory and procedures, 3rd edition, Lippincott Williams and Wilkins, New York, U.S.A, 1999.
- Ueland, P M., Refsum, H., Stabler, S. P., Malinow, M. R., Andersson, A., and Allen, R. H., Total homocystein plasma or serum: Methods and clinical applications, *Clin Chem*; 1993, 39 (9): 1764-1779.
- U.S. Food and Drug Administration, In Proceedings of the Food and Drug Administration Initiated Conference on Dietary Fibre and Cancer and Coronary Heart Disease, Fibre and Cancer, Arlington, MD, U.S. GPO: Washington, DC, 1994, pp74.
- Wang, X., and Gibson, G. R., Effect of the invitro fermentation of oligofructose and inulin by bacteria growing in the human large intestine, *J. Appl. Bacteriol*, 1993, 75, 373-380
- Wang, T. T. Y., Reisenauer, A. M., and Halsted, C. H., Comparison of folate conjugase activities in human, pig, rat and monkey intestine, *J. Nutr*, 1985, 115: 814-819.
- Wei, M. M., Bailey, L. B., Toth, J.P., and Gregory, J. F. III, Bioavailability for humans of deuterium-labled monoglutamyl and polyglutamyl folates is affected by selected foods, *J. Nutr*, 1996, 126:3100-3108.
- Welch, A. D., Succinylsulfathiazole as an inhibitor of bacterial synthesis in nutrition experiments, *Federation Proc*, 1942, 1: 171-173
- Welch, A. D., and Wright, L. D., The role of folic acid and biotin in the nutrition of the rat, *J. Nutr*, 1943, 25: 555-558
- Weller, M., Marini, A. M., Martin, B., and Paul, S. M., The reduced unsubstituted pteroate moiety is required for folate toxicity of cultured cerebellar granule neurons, *J Pharmacol Exp Ther*, 1994, 269: 393-401.
- Whitney, E. N., and Rolfes, S. R., Understanding Nutrition 8th edition, An international Thomson Publication Company. U.S.A, 1999.

Wolford, S.T., Schroer, R. A., Gohs, F. X., Gallo, P. P., Brodeck, M., Falk, H. B., Ruhren, R., Reference range data base for serum chemistry and hematology values in laboratory animals, *J. Toxicol. Environ. Health*, **1986**, 18 (2): 161-188.

Woods, M. N., and Gorbach, S. L., Influences of fibre on the ecology of the intestinal flora, In: *CRC handbook of dietary fibre in human nutrition*, GA Spiller, Ed. Boca Raton, FL: CRC Press Inc. **1993**, 361-370.

Yazawa, K., and Tamura, Z., Search for suger sources for selective increase of bifidobacteria, *Bifidobacteria Microflora*, **1982**, 1, 39-44

Yoshioka, M., Shimomura, Y., and Suzuki, M., Dietary polydextrose affects the large intestine in rats, *J. Nutr*, **1994**, 124, 539-547.

Younes, H., Coudray, C., Bellanger, J., Demigne, C., Rayssiguier, Y., and Remesy, C., Effect of two fermentable carbohydrates (inulin and resistannt starch) and their combination on calcium and magnesium balance in rats, *Br. J. Nutr*, **2001**, 86, 479-485.

Zimmerman, J., Gilula, Z., Selhub, J., Rosenberg I. H., Kinetic analysis of the effect of luminal pH on transport of folic acid in the small intestine, *Int J Vit Nutr R Res*, **1989**, 59:151-156.