

**Influence of the gut microbiota and probiotics  
on selenium metabolism in the rat: *In vitro*  
and *in vivo* studies**

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*To my grandparents*

## ***“Knowledge”***

*Knowledge never dies*

*You can learn and gain from it*

*Knowledge never dies*

*You can earn, burn or forget it*

*Knowledge never dies*

*With a reminder, you will get it*

*Knowledge never dies*

*It will follow you wherever you go*

*Knowledge never dies*

*Even when your remains become dust*

*Knowledge never dies*

*People will remember and talk about it*

*Knowledge never dies*

*Your full name remains forever and ever*

## ABSTRACT

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Selenium plays a major role in the immune system and in decreasing the risk of cancer. Plasma selenium levels are low in patients with certain gastrointestinal disorders suggesting a role for the gut microbiota in selenium metabolism and disposition. Probiotic treatment can modulate the gut microbiota but the effect of such treatment on the metabolism of selenium supplements is unknown. The present study investigated the metabolism of L-selenomethionine (L-SeMet) and selenite, commonly used as selenium supplements, by probiotic bacteria *in vitro* and by rat gut contents *ex vivo*. The effect of probiotic treatment on the disposition of selenium after oral dosing with L-SeMet and selenite in rats was also investigated.

After anaerobic incubation of L-SeMet (0.51 mM) with 10% w/w suspensions of the contents of jejunum, ileum, caecum and colon from male Wistar rats at 37°C for 3 h, L-SeMet metabolism (30%) was greatest in caecum contents followed by colon, ileum and jejunum. Dimethyldiselenide (DMDSe) was produced to the extent of 8.7% of the L-SeMet added and 28.9% of the L-SeMet lost. A similar result was obtained after incubation of selenite (0.58 mM) with metabolism being complete in caecum contents and almost complete in colon. Dimethylselenide (DMSe) (5.7% of the selenite added) was produced accompanied by a red precipitate of elemental selenium.

When L-SeMet (0.51 mM) was incubated anaerobically with individual antibiotic-resistant probiotic strains (*Streptococcus salivarius* K12, *Lactobacillus rhamnosus* 67B, *Lactobacillus acidophilus* L10 and *Bifidobacterium lactis* LAFTI® B94) ( $1 - 5 \times 10^{10}$  cfu/mL) and with a mixture of the four probiotic strains (ca.  $3 \times 10^{10}$  cfu/mL) at 37°C for 24 h, 10 - 18% was metabolised with 36-80% of L-SeMet being converted to DMDSe and DMSe. In similar incubations with selenite (0.58 mM), metabolism was more extensive (26 - 100%) particularly by the lactobacilli



with 0-4.8% of selenite being converted to DMS<sub>2</sub>Se and DMDSe accompanied by the formation of elemental selenium. Metabolism of L-SeMet or selenite in incubations with a combination of gut contents and the four probiotic strains indicated some suppression of L-SeMet metabolism and enhancement of selenite metabolism. These results suggest probiotics and gut microorganisms interact in relation to selenium metabolism in the gut.

In the *in vivo* study, three groups of rats ( $n = 3/\text{group}$ ) were given saline or a single oral dose of 2 mg selenium/kg as L-SeMet or selenite by gavage (untreated rats). Another four groups of rats ( $n = 6/\text{group}$ ) were given the same dose of either L-SeMet or selenite (2 mg selenium/kg) at the time of the last dose of treatment with 3 mL of a mixture containing equal numbers of the four antibiotic-resistant probiotic strains (total cell count ca.  $1 \times 10^{10}$  cfu/mL) or vehicle (a mixture of the lyoprotectants trehalose, maltodextrin and lactitol) every 12 h for three days (treated rats). Blood was collected from five rats in each treatment group over 24 h and serum analysed for selenium along with samples of liver and kidney obtained at 24 h. The sixth rat in each treatment group was used to determine the counts of total bacteria and of each of the antibiotic-resistant probiotic strains in the four segments of the gut at 24 h.

Serum selenium concentrations over 24 h were not significantly different between probiotic and vehicle treated rats but were more sustained after L-SeMet or significantly higher after selenite than in untreated rats. In the liver and kidney of probiotic treated rats at 24 h, L-SeMet produced a significantly higher selenium level in the liver and lower selenium level in the kidney than in untreated rats. A similar trend was observed in rats given selenite but the differences were not significant. Total bacterial counts in corresponding segments of probiotic and vehicle treated rats were similar to each other and to corresponding counts in normal rats except in jejunum which were  $10^3 - 10^4$  cfu/g higher. In rats treated with probiotics, only *L. rhamnosus* 67B and *L. acidophilus* L10 were detected in

all gut segments in approximately equal numbers which were  $10^2$  -  $10^4$  cfu/g less than the corresponding total bacterial counts.

The present study indicates that: L-SeMet and selenite are metabolised in rat gut contents *ex vivo* to form volatile methylated selenium compounds and, in the case of selenite, elemental selenium; probiotic bacteria can metabolise L-SeMet and selenite in a manner similar to the gut microbiota; oral treatment with lyoprotectants stimulates bacterial growth in the gut leading to changes in the metabolism of L-SeMet and selenite; and treatment with probiotics and lyoprotectants exerts an effect on selenium disposition over and above that produced by lyoprotectants alone. Overall these results suggest that the gut microbiota play an important role in metabolising selenium supplements and that treatment with probiotics and lyoprotectants can influence selenium disposition after oral doses of L-SeMet and selenite. Whether these changes indicate a beneficial role for combination treatment with probiotics and selenium supplements requires further research.

# PUBLICATIONS

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## Journal articles

**Woravimol Krittaphol**, Philip A Wescombe, Christine D Thomson, Arlene McDowell, John R Tagg, J Paul Fawcett. (2011). Metabolism of L-selenomethionine and selenite by probiotic bacteria: *In vitro* and *in vivo* studies. *Biological Trace Element Research* DOI 10.1007/s12011-011-9057-2 (Epub ahead of print).

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**Woravimol Krittaphol**, Christine D Thomson, Arlene McDowell, Philip A Wescombe, John R Tagg, John P Fawcett. Do probiotics influence L-selenomethionine and selenite metabolism by indigenous microbes in different segments of the rat intestinal tract? Proceedings of the 19<sup>th</sup> International Congress of Nutrition, October 2009, Bangkok, Thailand. *Annals of Nutrition and Metabolism* **55** (suppl 1): 127 (**A distinguished poster presentation**). Internet: <http://www.karger.com/anm ISSN1421-9697>.

**Woravimol Krittaphol**, Christine D Thomson, Arlene McDowell, John R Tagg, J Paul Fawcett. Can supplemental probiotics influence L-selenomethionine metabolism in the rat intestinal tract? Proceedings of the 11<sup>th</sup> Conference on Formulation and Delivery of Bioactives, February 2009, Dunedin, New Zealand. (**Poster presentation**).

**Woravimol Krittaphol**, Philip A Wescombe, Arlene McDowell, John R Tagg, Christine D Thomson, J Paul Fawcett. Probiotics and selenium metabolism: Does it matter whether the bacteria are dead or alive? Proceedings of the 13<sup>th</sup> International Meeting on Trace Elements in Man and Animals, November 2008, Pucón, Chile. (**Oral presentation with a full-fellowship award**). Internet: <http://www.rowett.ac.uk/tema/Symposia/TEMA-13/archivos/oral.html>.

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## ABBREVIATIONS

AAS	Atomic Absorption Spectrometry
AFS	Atomic Fluorescence Spectrometry
AUC	Area Under the Curve
BLIS <sup>®</sup>	Bacteriocin-Like Inhibitory Substance
C <sub>max</sub>	Maximum concentration
CRM	Certified Reference Material
Cys	Cysteine
DMS <sub>e</sub>	Dimethylselenide
DMDS <sub>e</sub>	Dimethyldiselenide
GC-MS	Gas Chromatography Mass Spectrometry
GIT	Gastrointestinal Tract
GPx	Glutathione Peroxidase
GSH	Glutathione
HGAAS	Hydride Generation Atomic Absorption Spectrometry
HPLC	High Performance Liquid Chromatography
HR MS-TOF	High Resolution Time of Flight Mass Spectrometry
h	Hour
ICP-MS	Inductively Coupled Plasma Mass Spectrometry
ID	Iodothyronine deiodinase
LD <sub>50</sub>	Median of Lethal Dose

Met	Methionine
MRS	de Man Rogosa and Sharpe
MS	Mass Spectrometry
NAC	<i>N</i> -acetyl-L-cysteine
OPA	<i>o</i> -Phthaldialdehyde
PBS	Phosphate Buffered Saline
RDA	Recommended Dietary Allowance
RDI	Recommended Dietary Intake
RNI	Reference Nutrient Intake
s	Second
SeCys	Selenocysteine
Sel	Selenoprotein
SeMet	Selenomethionine
L-SeMet, DL-SeMet	L-Selenomethionine, DL-Selenomethionine
SRM	Standard Reference Material
T3, T4	5-Triiodothyronine, Thyroxine
TPN	Total Parenteral Nutrition
TrxR	Thioredoxin reductase
TMSe <sup>+</sup>	Trimethylselenonium
UV	Ultraviolet
WHO	World Health Organization

# CHAPTER 1

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## General introduction

### 1.1. Role of selenium in health and disease

Selenium, an essential trace element, plays several important roles in mammals including antioxidant defence, thyroid hormone metabolism, redox control of enzymes and proteins, and the optimal function of the immune system (Behne and Kyriakopoulos, 2001; Gromer *et al.*, 2005; Papp *et al.*, 2007). Selenium deficiency has also been linked to a variety of diseases in animals such as white muscle disease in cattle and sheep, hepaticosis dietetica in swine, liver necrosis in rats and exudative diathesis in poultry (Schwarz, 1951; Wolf *et al.*, 1963; Andrews *et al.*, 1968; Underwood, 1977; Jenkins *et al.*, 1993). In 1973, the antioxidant enzyme glutathione peroxidase (GPx) was found to be a selenoenzyme which, along with vitamin E (a lipid-soluble antioxidant), prevents the development of these animal diseases (Levander *et al.*, 1995). This ability helped to explain the interrelationship between selenium and vitamin E (Levander *et al.*, 1995) and subsequently led to the recognition of selenium deficiency in two human diseases (Thomson, 2007) namely Keshan disease (cardiomyopathy) and Kashin-Beck disease (endemic osteoarticular disorder). Keshan disease results in cardiac muscle failure whereas Kashin-Beck disease causes problems in the skeletal structure, especially in young children and women of child-bearing age (Yang *et al.*, 1984; Kolsteren, 1992).



Selenium deficiency can lead to lower 5-triiodothyronine (T3) and thyroxine (T4) levels and may contribute to the aetiology of myxoedematous cretinism and hypothyroidism (Schomburg and Köhrle, 2008). Selenium is also important in the immune system through its interactions with lysozymes, phagocytes, macrophages, neutrophils and B and T lymphocytes (Arthur *et al.*, 2003; Hoffmann and Berry, 2008). The precise mechanism by which selenium maintains optimal immune function is unknown but is thought to be related to its antioxidant function mediated through GPx (Arthur *et al.*, 2003). Selenium deficiency has been associated with a high risk of HIV-related mortality and faster disease progression in children (Campa *et al.*, 1999; Rayman, 2000). In addition, evidence for a role of selenium in viral infections has emerged from a study of the aetiology of Keshan disease where selenium deficiency leads to a viral genome change which is associated with increased virulence of the virus (Beck *et al.*, 1994; Beck *et al.*, 1998). Several studies in China suggest that an infectious cofactor is required along with a deficiency in selenium for Keshan disease to develop. This cofactor was later identified as a coxsackievirus (Su *et al.*, 1979; Bai *et al.*, 1980; Beck *et al.*, 2003).

In 1979, the first case of clinical selenium deficiency was reported in New Zealand in a patient receiving total parenteral nutrition (TPN) (Van Rij *et al.*, 1979). After giving the patient TPN containing L-selenomethionine (L-SeMet) for two weeks, the symptoms of selenium deficiency disappeared. However, not all patients with low selenium status develop clinical symptoms of selenium deficiency. Therefore, other factors appear to be involved, as noted for Keshan disease (Jochum *et al.*, 1999).

There is also controversy surrounding the possible inverse correlation between selenium intake and mortality from cancer of the prostate, colon, rectum and other tissues (Whanger, 2004; Combs Jr, 2005; Rayman, 2005; El-Bayoumy, 2009; Lippman *et al.*, 2009). In a Nurse's Health Study conducted in the USA, three years of data on selenium status based on analysis of toenail clippings showed the

benefit of high selenium status in reducing the incidence of certain forms of cancer (Garland *et al.*, 1995). In fact, a variety of cell culture and animal experimental models have shown that most selenium compounds serve to prevent the formation of carcinogens (anticarcinogenesis) (Combs Jr and Gray, 1998) although potency in this regard is dependent on the form of selenium. Recently, several studies have shown that a high dose of selenium is associated with a decreased risk of prostate and colorectal cancers (Clark *et al.*, 1998; Nakamuro *et al.*, 2000; Trumbo, 2005; Boosalis, 2008) but other studies have not supported this finding (Peters *et al.*, 2007; Ravn-Haren *et al.*, 2008; Lippman *et al.*, 2009).

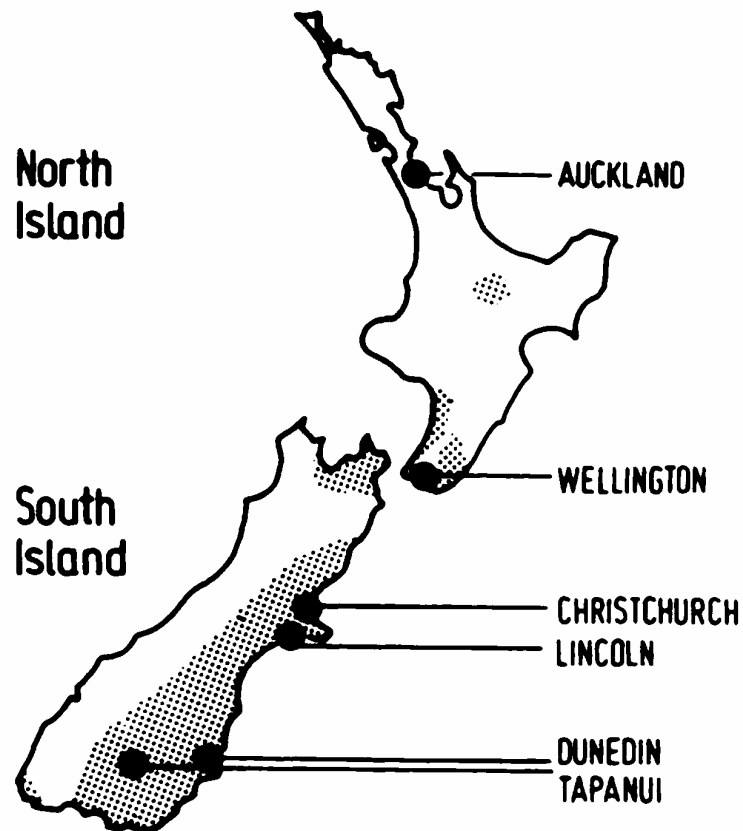
Much selenium research has been driven by early reports that selenium intake beyond Dietary Reference Intakes (DRI) reduced the risk of cancer, especially prostate cancer. These reports were the impetus for the largest prostate cancer trial ever conducted, the SELECT trial. Designed to run for 12 years, the interim results, determined in 2008, showed that selenium supplementation had no effect on cancer but caused an arithmetic increase in diabetes. The results of this study effectively ended most funding for research into selenium and cancer. However, the increase in diabetes was not statistically significant and there was insufficient evidence to suggest a causal effect of selenium intake above the DRI. There are several factors which need to be considered such as the different baseline selenium status of populations in different countries and regions, the function of selenium as it relates to particular disease states, the selenium species given and the health condition of the population including their exposure to stress, and whether their intake of selenium and other nutrients was adequate (Bleys *et al.*, 2007; Stranges *et al.*, 2007; Rayman, 2008).

In fact, the relationship between selenium and anticarcinogenesis may be due to the production of selenium metabolites (e.g. methylselenol) which enhance immune surveillance, promote apoptosis or inhibit angiogenesis (Ip, 1998; Ganther, 1999; Ip *et al.*, 2000; Combs Jr and Lü, 2006; Reilly, 2006). For other health conditions such as Crohn's disease and ulcerative colitis, the effects of

selenium supplementation are also contradictory (Rayman, 2000; Kuroki *et al.*, 2003; Lippman *et al.*, 2009).

Dietary intake of selenium depends on the level of selenium in the soil where food is grown and on food consumption patterns (Nowak *et al.*, 2004). The bioavailability of selenium in soil and plants depends on several factors such as the concentration and form of selenium, type and pH of the soil, and climate (Nowak *et al.*, 2004). The country of Finland has one of the lowest selenium intakes in the world such that, in the early 1980s, the government decided to address this problem by adding selenium to fertilizer. In a few years the blood selenium level of Finns rose to levels similar to those seen in the US. However, extensive epidemiologic reports failed to note any benefits from the increased level. Even though no epidemiological benefits have been observed, blood concentrations of selenium have increased to healthy levels and, because of this, the Finland study led the UK and other European countries to increase selenium levels in food crops in order to improve selenium status in their populations (Arthur, 2003; Hartikainen, 2005; Broadley *et al.*, 2006).

In New Zealand, the soil selenium level is low leading some food crops to have low levels of selenium. As a result, the New Zealand population has low selenium status. New Zealand children (5-14 years) and young women have a low concentration of selenium in serum, particularly those resident in the South Island (Thomson, 2004b; Thomson *et al.*, 2007). Animals raised in areas where soil is low in selenium generally have low selenium concentrations in their edible tissues (Hoffman *et al.*, 1972). As a result, New Zealand livestock and poultry are supplemented with selenium to prevent selenium-deficiency diseases (Thomson *et al.*, 2008). Figure 1.1 shows the regions where selenium-deficiency animal diseases may occur. Selenium appears to be present in proteinaceous tissue in animals primarily as L-SeMet and selenocysteine (SeCys), the ratio of which depends on the food consumed (Beilstein and Whanger, 1992).



**Figure 1.1.** Map of New Zealand showing shaded regions where selenium-deficiency animal diseases occur (Thomson and Robinson, 1980).

Food sources of selenium are red meat, eggs, poultry, fish and seafood. Cereals, vegetables and fruit have a lower selenium content than meat products (McNaughton and Marks, 2002). Seafood, chicken, eggs, breads and grain products are the main sources of selenium in the diet of the New Zealand population (Thomson, 2004b; Thomson *et al.*, 2008). Since selenium intake in the New Zealand population is slightly lower than the recommended dietary intake (Thomson, 2004b; Thomson *et al.*, 2008), selenium supplementation is common. Organic forms of selenium are the most common supplements since they provide higher bioavailability and utilisation of selenium than inorganic forms (Schrauzer, 2000; Whanger, 2002; Finley, 2006).

The terms “Recommended Dietary Allowance (Reddy *et al.*)”, “Recommended Dietary Intake (RDI)” and “Reference Nutrient Intake (RNI)” are country specific and based on each country’s Estimated Average Requirement (EAR) for selenium. The RDA of selenium in the USA/Canada is 55 µg/day, a value lower than in the UK (RNI of 75 µg/day for males and 60 µg/day for females) and Australia/New Zealand (RDI of 70 µg/day for males and 60 µg/day for females) (Department of Health, 1991; Food and Nutrition Board, 2000; NHMRC, 2006). The maximum safe dietary intake is considered to be 400 µg/day (Food and Nutrition Board, 2000; NHMRC, 2006).

## **1.2. Functions of selenium**

Selenium is present in a number of selenoproteins as part of the amino acid SeCys. In humans there are 25 selenoproteins (Reeves and Hoffman, 2009), the functions of which are summarised in Table 1.1. The functions can be classified into three main groups: as antioxidants in GPx enzymes; as regulators of thyroid hormone metabolism in iodothyronine deiodinase (ID) enzymes; and as participants in the redox control of enzymes and proteins in thioredoxin reductase (TrxR) enzymes and selenoproteins (Sels) (Gromer *et al.*, 2005; Behne *et al.*, 2009; Bellinger *et al.*, 2009; Reeves and Hoffman, 2009). These roles are described in the following sections.

**Table 1.1.** Human selenoproteins, their functions and locations.

<b>Selenoprotein</b>	<b>Main function</b>	<b>Location</b>	<b>Reference</b>
<b>GPx1</b>	Cytosolic glutathione peroxidase	Antioxidant; over-expression may increase risk of diabetes	Cytoplasm Lei <i>et al.</i> (2007)
<b>GPx2</b>	Gastrointestinal glutathione peroxidase	Protection from intestinal cancer	Gastrointestinal tract Florian <i>et al.</i> (2001)
<b>GPx3</b>	Plasma glutathione peroxidase	Modulation of nitrous oxide for cardiovascular protection and as an antioxidant in thyroid gland	Secreted into plasma Kenet <i>et al.</i> (1999) Schmutzler <i>et al.</i> (2007)
<b>GPx4</b>	Phospholipid hydroperoxidase glutathione peroxidase	Antioxidant, structural protein in sperm and sensor of stress and pro-apoptotic signals	Cytoplasm Seiler <i>et al.</i> (2008) Foresta <i>et al.</i> (2002) Borchert <i>et al.</i> (2003) Kryukov <i>et al.</i> (2003)
<b>GPx6</b>	Olfactory glutathione peroxidase	Possibly involved in developing embryo and olfactory epithelium in adults	
<b>TrxR1</b>	Thioredoxin reductase Type I	Cofactor in dithiol-disulfide exchange reactions and involved in developing embryo	Cytoplasm, nucleus Jakupoglu <i>et al.</i> (2005)
<b>TrxR2</b>	Thioredoxin reductase Type II	Cofactor in dithiol-disulfide exchange reactions and involved in developing embryo	Mitochondria Conrad <i>et al.</i> (2004)
<b>TrxR3</b>	Thioredoxin reductase Type III, Testes-specific thioredoxin-glutathione reductase	Involved in testes-specific expression	Osborne and Tonissen (2001)

Table 1.1. continued.

Selenoprotein		Main function	Location	Reference
<b>ID1</b>	Deiodinase Type I	Systemic action to increase thyroid hormone levels	Membrane-associated	Bianco <i>et al.</i> (2002)
<b>ID2</b>	Deiodinase Type II	Local action to increase thyroid hormone levels	Membrane-associated	Steinsapir <i>et al.</i> (2000)
<b>ID3</b>	Deiodinase Type III	Inactivates thyroid hormone	Membrane-associated	Bianco <i>et al.</i> (2002)
<b>Sel H</b>	Selenoprotein H	Involved in transcription and as an antioxidant	Nucleus	Panee <i>et al.</i> (2007)
<b>Sel I</b>	Selenoprotein I	Possibly involved in phospholipid biosynthesis	Transmembrane	Horibata and Hirabayashi (2007)
<b>Sel K</b>	Selenoprotein K	Transmembrane protein	Endoplasmic reticulum (ER), plasma membrane	Kryukov <i>et al.</i> (2003) Lu <i>et al.</i> (2006)
<b>Sel M and Sel 15</b>	Selenoprotein M and selenoprotein 15	Thiol-disulfide oxidoreductases	ER	Korotkov <i>et al.</i> (2001) Hwang <i>et al.</i> (2008)
<b>Sel N</b>	Selenoprotein N	Involved in ryanodine receptor-related calcium mobilisation and the movement of skeletal muscles	ER	Juryneć <i>et al.</i> (2008)
<b>Sel O</b>	Selenoprotein O	Possibly involved in redox function		Kryukov <i>et al.</i> (2003)
<b>Sel P</b>	Selenoprotein P	Extracellular antioxidant in brain and testis, intracellular antioxidant in phagocytes	Secreted into cytoplasm	Hill <i>et al.</i> (2003) Burk and Hill (2005) Schomburg <i>et al.</i> (2003)

Table 1.1. continued.

Selenoprotein		Main function	Location	Reference
<b>Sel R</b>	Selenoprotein R	Antioxidant in brain	Cytoplasm	Fomenko <i>et al.</i> (2008)
<b>Sel S</b>	Selenoprotein S	Transmembrane protein; possibly involved in protection from ER stress	ER, plasma membrane	Gao <i>et al.</i> (2004; 2007)
<b>Sel T</b>	Selenoprotein T	Possibly involved in calcium mobilisation	ER	Grumolato <i>et al.</i> (2008)
<b>Sel V</b>	Selenoprotein V	Involved in testes-specific expression		Dikiy <i>et al.</i> (2007); Kryukov <i>et al.</i> (2003); Hoffmann <i>et al.</i> (2007)
<b>Sel W</b>	Selenoprotein W	Antioxidant and possibly important in muscle growth	Cytoplasm	Beilstein <i>et al.</i> (1996) Loflin <i>et al.</i> (2006)
<b>SPS2</b>	Selenophosphate synthetase 2	Possibly involved in synthesis of all selenoproteins	Cytoplasm	Low <i>et al.</i> (1995) Guimaraes <i>et al.</i> (1996)



### 1.2.1. Antioxidant defence

GPx is the best characterised selenoenzyme with five subclasses identified based on their site of action. Cytosolic or classical GPx (GPx1) was the first to be discovered and is present in all cells. The remaining GPxs occur specifically in different body tissues; GPx2 in the gastrointestinal tract (GIT), GPx3 in plasma, GPx4 in plasma membranes and GPx6 in the olfactory region. These GPxs catalyse the reduction of hydrogen peroxide and organic hydroperoxides and protect the cell from oxidative damage (Behne *et al.*, 2009; Bellinger *et al.*, 2009; Reeves and Hoffman, 2009).

### 1.2.2. Thyroid hormone metabolism

IDs play an important role in the activation and metabolism of thyroid hormones. Three IDs (ID1, ID2 and ID3) have been identified (Table 1.1) (Gromer *et al.*, 2005; Behne *et al.*, 2009; Bellinger *et al.*, 2009; Reeves and Hoffman, 2009). ID1 is located in thyroid, liver and kidney where it is responsible for converting T4 to T3 (Bianco *et al.*, 2002). ID2 is found in the brain, brown adipose tissue, pituitary gland and placenta (Gereben *et al.*, 2000; Steinsapir *et al.*, 2000). Its main function is to produce T3 from circulating plasma T4 in intracellular fluids (Bianco *et al.*, 2002). ID3 is located in the central nervous system (CNS), placenta and skin (Salvatore *et al.*, 1995; Bianco *et al.*, 2002). It contributes to the local regulation of T3 levels in the human CNS (Santini *et al.*, 2001). Thus, all three IDs play an important role in thyroid hormone homeostasis (Steinsapir *et al.*, 1998; Gereben *et al.*, 2000; Steinsapir *et al.*, 2000).

### 1.2.3. Redox control of enzymes and proteins

TrxR catalyses the NADPH-dependent reduction of thioredoxin and regenerates ascorbic acid from its oxidised metabolite (Arnér and Holmgren, 2000; Lillig and Arnér, 2009). There are three mammalian TrxRs; TrxR1 found in the cytoplasmic and nuclear regions of cells, TrxR2 found in mitochondria, and TrxR3 a testes-

specific thioredoxin-glutathione reductase (Osborne and Tonissen, 2001; Sun *et al.*, 2001; Rundlof *et al.*, 2004; Turanov *et al.*, 2006). TrxR1 and TrxR2 are found in a variety of tissues and several spliced forms have been described in animals and humans which may reflect the complex regulation of expression of TrxR1 and TrxR2 in organelle and cell type-specific locations (Rundlof *et al.*, 2004; Turanov *et al.*, 2006). TrxR1 appears to be involved in HIV-1 infection since a low TrxR1 level has been found to accompany HIV infection (Gladyshev *et al.*, 1999; Kalantari *et al.*, 2008). TrxR3 can reduce glutathione disulfide but its specific function remains unknown (Sun *et al.*, 2001).

#### **1.2.4. Other functions**

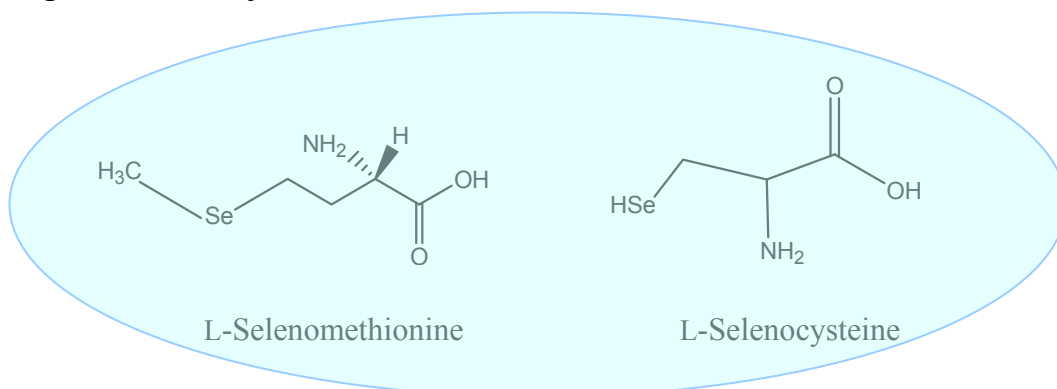
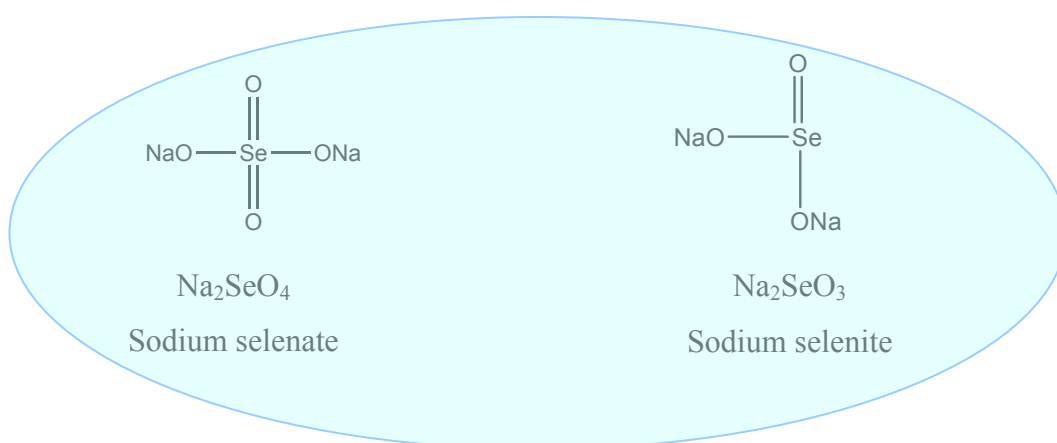
A wide variety of Sels has been identified in mammals (Table 1.1) (Gromer *et al.*, 2005; Behne *et al.*, 2009; Bellinger *et al.*, 2009; Reeves and Hoffman, 2009), most of which are probably involved in antioxidant defence. Sel P which contains ten selenium atoms per molecule in the form of SeCys (Burk and Hill, 2009) provides selenium to the brain and testes (Saito and Takahashi, 2002; Burk and Hill, 2009). It functions as a selenium transport protein and antioxidant and may also be involved in heparin binding and heavy metal ion complexation (Arteel *et al.*, 1998; Saito *et al.*, 1999; Burk and Hill, 2009). Recently, a low level of Sel P has been associated with an increased risk of Alzheimer's disease, Crohn's disease, inflammation and prostate cancer (Hill *et al.*, 2003; Bellinger *et al.*, 2008; Cooper *et al.*, 2008). Low Sel P levels can also lead to neurological problems and male sterility (Cooper *et al.*, 2008; Renko *et al.*, 2008). The function of other Sels probably depends on the type of organ or tissue with which they are associated (Table 1.1) but, in many cases, their specific function remains to be investigated.

### 1.3. Chemical forms of selenium

Selenium and sulfur have similar chemical and physical properties but their biochemistry differs. In biological systems, selenium compounds tend to be metabolised to more reduced states while sulfur compounds tend to be converted to more oxidised states. Reduction plays a major role in selenium metabolism for all selenocompounds in the diet (Combs Jr and Combs, 1984).

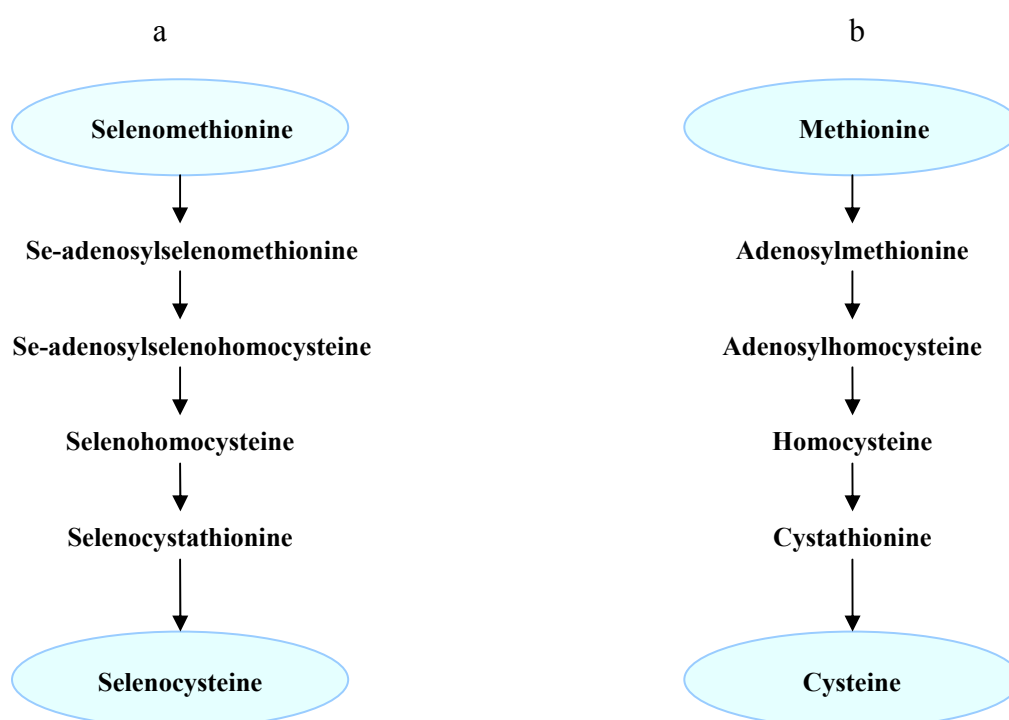
Selenium occurs in either an organic form such as the selenoamino acids, L-SeMet and SeCys, or an inorganic form such as selenate and selenite (Figure 1.2). Since 1974, inorganic selenium salts have been used in farm animal feed (Schrauzer, 2001; Finley, 2006) and, in 1984, synthetic L-SeMet became available. Some supplements containing L-SeMet are augmented with other organic or inorganic selenium compounds (Schrauzer, 2000).

Selenomethionine (SeMet) exists as the D- and L-forms and as the racemic mixture, DL-SeMet, which is obtained synthetically (Schrauzer, 2001; Kuehnelt *et al.*, 2005). L-SeMet is better utilised in the trans-selenation pathway in which it is converted to SeCys, and is used in preference to DL-SeMet in supplements (McAdam and Levander, 1987; Kuehnelt *et al.*, 2005). For rats, the two isomers are acceptable and at high levels are equally toxic to rat skeletal muscle, erythrocytes, liver and heart cells. Thus caution is needed when administering SeMet to rats (Nakamuro *et al.*, 2000; Schrauzer, 2000).

**Organic selenium forms****Inorganic selenium forms****Figure 1.2.** Organic and inorganic forms of selenium.**1.4. Metabolism of selenium****1.4.1. Selenium metabolic pathways in mammals**

The chemical structures of methionine (Met) and L-SeMet are similar and only differ in the heteroatom attached to the α-carbon of the amino acid. The molecules are virtually the same shape and size and hence most enzymes cannot discriminate between them. This allows L-SeMet to replace Met in the proteins of body tissues (McConnell and Hoffman, 1972) and to be metabolised by the same enzymes. For example, the trans-selenation pathway (Figure 1.3a) that converts L-SeMet to SeCys is similar to the trans-sulfuration pathway which converts Met to cysteine (Cys) (Figure 1.3b) (Finkelstein and Martin, 1984; Suzuki, 2005; Korendyaseva *et*

*al.*, 2008). Subsequently, Cys is converted to sulfide and, in a similar manner, SeCys is converted by  $\beta$ -lyase to selenide (Esaki *et al.*, 1982; Schomburg *et al.*, 2004; Suzuki, 2005). At this point the two pathways differ in that sulfide is very toxic and is immediately detoxified to sulfate by multiple reactions (Finkelstein and Martin, 1984) whereas selenide is converted to Sels that are utilised by the body or converted to methylated products (Suzuki, 2005).

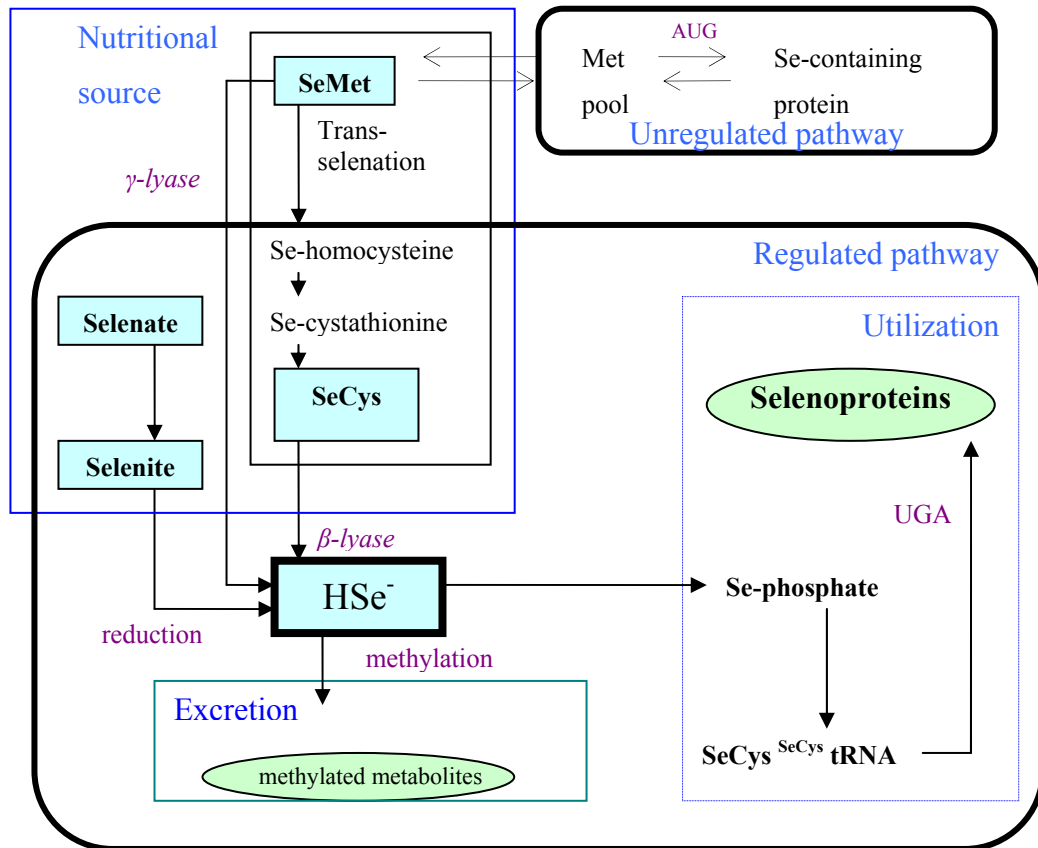


**Figure 1.3** Comparison of (a) the trans-selenation pathway and (b) the trans-sulfuration pathway in mammals (Finkelstein and Martin, 1984; Suzuki, 2005; Korendyaseva *et al.*, 2008).

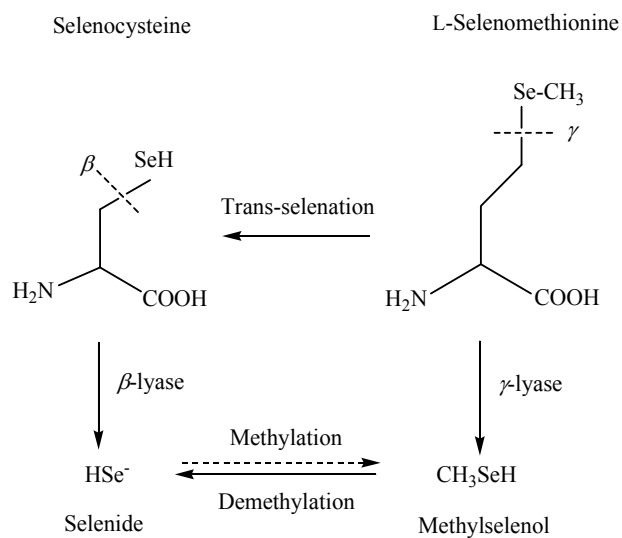
In mammalian systems, metabolism of organic and inorganic forms of selenium occurs mainly in the liver and kidney (Figure 1.4) (Suzuki *et al.*, 1995; Suzuki, 2005). L-SeMet is transformed to selenide ( $\text{HSe}^-$ ) via two pathways. The first direct route involves cleavage of the C-Se bond by  $\gamma$ -lyase to selenide (Suzuki, 2005). A subsequent study by Suzuki and colleagues refined this pathway and showed that  $\gamma$ -lyase first converts L-SeMet to methylselenol ( $\text{CH}_3\text{SeH}$ ) which is then demethylated to produce selenide (Figure 1.5) (Suzuki *et al.*, 2007). The second indirect route is via the trans-selenation pathway whereby L-SeMet is

converted to SeCys which is then converted to HSe<sup>-</sup> by  $\beta$ -lyase (Esaki *et al.*, 1982; Dungan and Frankenberger Jr, 1999; Schomburg *et al.*, 2004; Suzuki, 2005). The structures of the various intermediates in the route from L-SeMet to HSe<sup>-</sup> via trans-selenation and  $\beta$ -lyase are shown in Figure 1.6. The enzymes involved include L-methionine S-adenosyltransferase, methyltransferase, Se-adenosylselenohomocysteine hydrolase, L-serine hydrolyase (adding homocysteine), selenocystathionine cysteine-lyase (deaminating) and selenocysteine  $\beta$ -lyase (Esaki *et al.*, 1982; Schomburg *et al.*, 2004; Suzuki, 2005; GenomeNet Database Resources: Kyoto University Bioinformatics Centre, 2006). These enzymes are the same as those in the metabolic pathway of Met in mammalian tissues (Finkelstein and Martin, 1984).

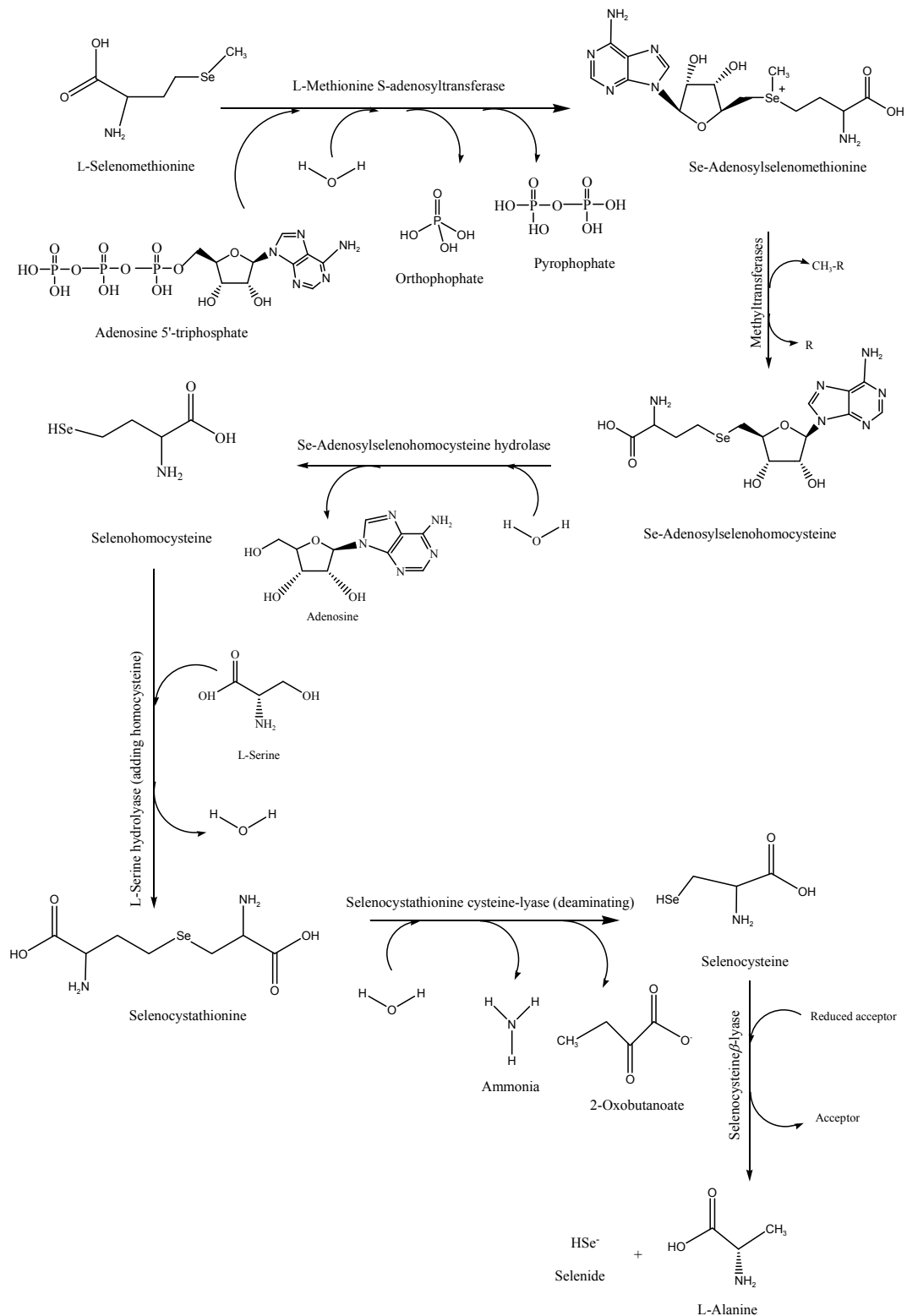
Both organic and inorganic selenium are converted via HSe<sup>-</sup> to selenophosphate and then incorporated into SeCys<sup>SeCys</sup>tRNA to meet a SeCys codon to produce Sels (Figure 1.4). HSe<sup>-</sup> is highly reactive and is readily incorporated into Sels and utilised in the body (Suzuki, 2005). Consequently free HSe<sup>-</sup> may not be present. However, excess HSe<sup>-</sup> is methylated and excreted out of the body via the lung and kidney in the form of dimethylselenide (DMSe) and trimethylselenonium (TMSe<sup>+</sup>), respectively (Suzuki, 2005). This is discussed in more detail in Chapter 3.



**Figure 1.4.** The metabolic pathway of selenomethionine (SeMet) and selenate/selenite in mammals. Abbreviation: Selenocysteine (SeCys), selenide (HSe<sup>-</sup>), selenophosphate (Se-phosphate), a selenocysteinyl transfer ribonucleic acid (SeCys<sup>SeCys</sup> tRNA), methionine (Met), a start codon encoding amino acids (AUG) and a stop codon encoding amino acids (UGA) (Suzuki, 2005).



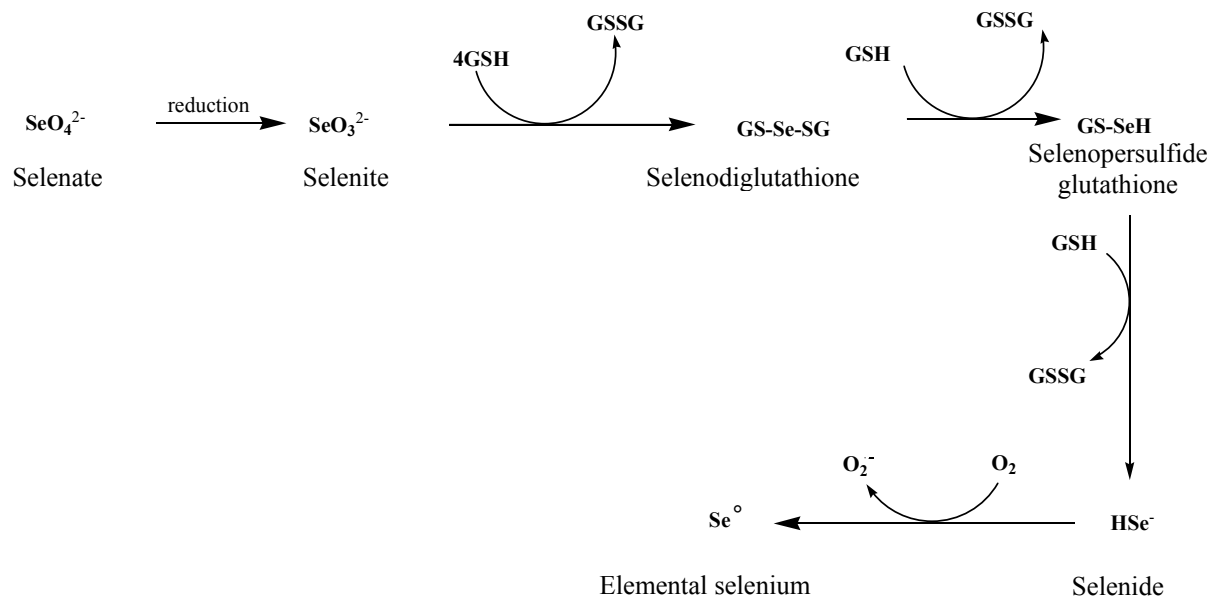
**Figure 1.5.** The metabolic pathways by which the selenoamino acids, L-selenomethionine and selenocysteine, form methylselenol and selenide (Suzuki *et al.*, 2007).



**Figure 1.6.** Metabolic pathway of L-selenomethionine to selenide showing the enzymes involved and the chemical structures of each intermediate (GenomeNet Database Resources: Kyoto University Bioinformatics Centre, 2006).

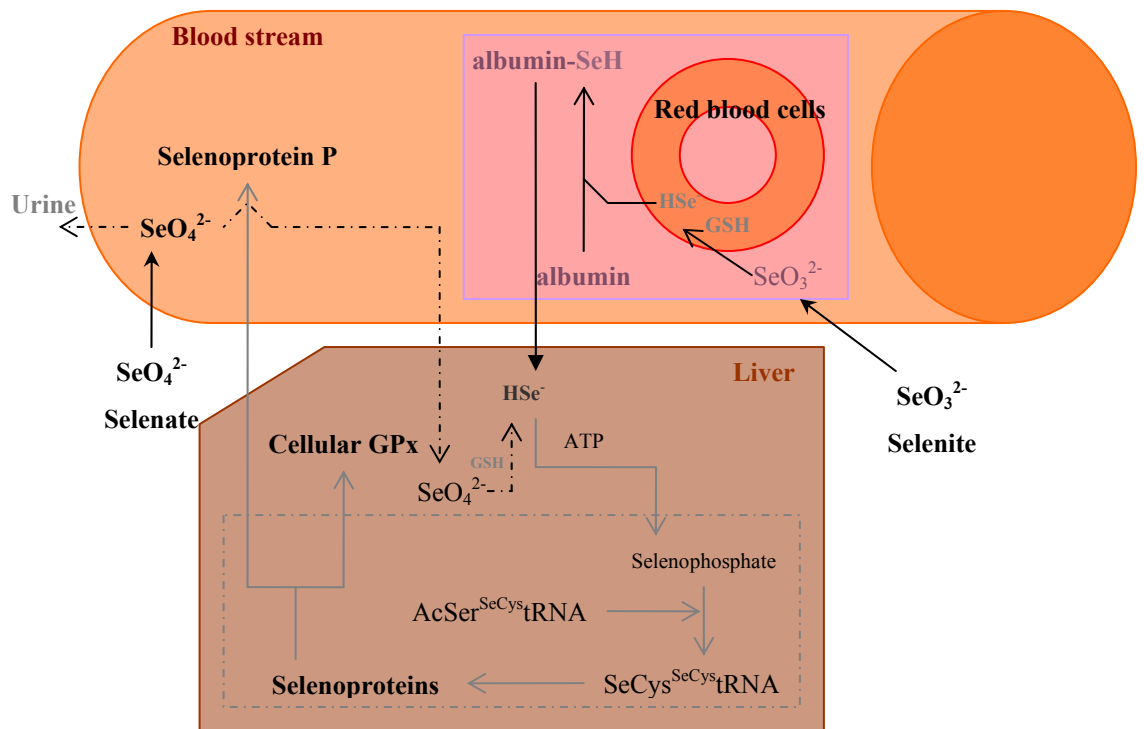


Prior to utilisation, selenate and selenite require bioreductive activation in the presence of glutathione (Li *et al.*) (Li *et al.*) (Li *et al.*) (Figure 1.7). GSH reduces selenate and selenite to selenide with selenite being more readily reduced than selenate. Elemental selenium is produced from selenide by oxidation (Zakharyan *et al.*, 2005).



**Figure 1.7.** The metabolic pathway for the conversion of inorganic forms of selenium (selenate and selenite) to selenide and elemental selenium (Zakharyan *et al.*, 2005).

In general, selenate and selenite are metabolised in the liver after oral administration and absorption from the intestinal tract (Swanson *et al.*, 1991). Following intravenous injection, selenate and selenite are transformed in the bloodstream or in the liver. Selenate is transferred unchanged from the blood to the liver where it is converted to selenide (Figure 1.8). In contrast, selenite is taken up by red blood cells, reduced to selenide and effluxed into the plasma where it binds to albumin. The albumin-SeH complex is then transferred to the liver where it is incorporated into Sels via selenophosphate (Figure 1.8) (Suzuki and Itoh, 1997; Suzuki, 2005).



**Figure 1.8.** Metabolic pathways of selenate and selenite in the blood and liver. Abbreviation: Glutathione peroxidase (GPx); Activated serine residue on selenocysteinyl residues (AcSer<sup>SeCys</sup>tRNA) in selenoprotein sequences SeCys<sup>SeCys</sup>tRNA; Selenide (HSe<sup>-</sup>) (Suzuki and Itoh, 1997; Suzuki, 2005).

## 1.5. Bioavailability of selenium

Selenium bioavailability is dependent on the form of selenium (organic and inorganic compounds) (Finley, 2006; 2007) and the influence of dietary factors such as protein intake and the presence of heavy metals (Navarro-Alarcon and Cabrera-Vique, 2008). Protein-rich foods containing high levels of selenoamino acids such as wheat products (bread and pasta), meats (beef, fish and eggs) and brazil nuts are highly absorbed into the body. However, the absorption of selenium varies with the selenium source and selenium status of the subject (Diaz-Alarcon *et al.*, 1996; Dumont *et al.*, 2006). It is also affected by interaction with

other trace elements, an interaction which can be additive, antagonistic or synergistic (Hamilton, 2006). In general, selenium toxicity can be reduced by arsenic, cadmium, copper, mercury, silver and tungsten but it appears to be unaffected by chromium, cobalt, fluorine, nickel and zinc (Davies, 1974; Diplock, 1976; Levander, 1977; Whanger, 1981). Mercury is an example of a metal that has both antagonistic and synergistic interactions with selenium (Rudd *et al.*, 1980; Turner and Rudd, 1983; Ohlendorf *et al.*, 1993) depending on the relative concentrations of the two elements in the environment. Low levels of selenium result in antagonistic effects whereas high levels result in a synergistic effect (Hill, 1975; Ewan, 1978; Ohlendorf *et al.*, 1993).

#### **1.5.1. Absorption, retention, transport and excretion**

In general, absorption of selenium is not under homeostatic regulation. Thus, selenium status did not regulate selenium absorption but it may regulate selenium excretion (Thomson, 2007). For example, in New Zealand where selenium status is low, excretion of selenium is also low to maintain concentrations in the body. On the other hand, in the USA where selenium status is high, excretion of selenium is also high (Bügel *et al.*, 2008).

After oral administration, L-SeMet is rapidly absorbed (> 90% of selenium) from all segments of the GIT (Vendeland *et al.*, 1992a) reaching a peak level in the blood after about 3 h (Spencer and Blau, 1962). A series of rat studies has shown that L-SeMet absorption is inhibited by high concentrations of Met suggesting absorption of L-SeMet and Met in the intestine uses the same active transport mechanism (Spencer and Blau, 1962; McConnell and Cho, 1967; Wolfram *et al.*, 1989).

Little information is available on the mechanism of SeCys absorption but it appears that the absorption of selenocystine is not inhibited by Cys (Wolfram *et*

*al.*, 1989). This suggests that Cys and selenocystine are absorbed by different mechanisms (Vendeland *et al.*, 1992b; Leblondel *et al.*, 2001).

Selenate and selenite are mainly absorbed in the ileum by passive transport (Ardüser *et al.*, 1985; Wolfram *et al.*, 1985; Wolfram *et al.*, 1986; Vendeland *et al.*, 1992a; Leblondel *et al.*, 2001; Thomson, 2007). For selenite, selenium absorption is less than 60% after oral administration (Swanson *et al.*, 1991; Thomson, 2007). The absorption can be enhanced by GSH but, at high concentrations, both thiosulphate and sulphate inhibit the absorption of selenate and selenite in rat (Anundi *et al.*, 1984; Ardüser *et al.*, 1985; Leblondel *et al.*, 2001). Following ingestion, selenate is absorbed from the ileum at a faster rate than selenite (Vendeland *et al.*, 1992a). About 17% of selenite is retained in the ileum whereas 92% of selenate is absorbed into the blood (Vendeland *et al.*, 1992b).

Retention of selenium in the body is dependent on the form of selenium administered. For example, L-SeMet persists in the body for longer and is more effective in raising blood selenium levels than either selenate or selenite (Schrauzer, 2000). The non-specific incorporation of L-SeMet into protein contributes to tissue selenium which is then unavailable for synthesis of new Sels until the old proteins are broken down (Thomson, 2007). Although selenium appears to be transported bound to plasma proteins, detailed information about selenium transport in the body is not available, although Sel P has been suggested to be a transport protein (Hill *et al.*, 2003).

Initially it was reported that  $\text{TMSe}^+$  is the major product of ingested selenium excreted in urine (Wrobel, 2003; Gammelgaard and Bendahl, 2004). Subsequently, it was shown that selenosugar 1 (methyl 2-acetamido-2-deoxy-1-seleno- $\beta$ -D-glucopyranoside), selenosugar 2 (methyl 2-acetamido-2-deoxy-1-seleno- $\beta$ -D-galactopyranoside) and selenosugar 3 (methyl 2-amino-2-deoxy-1-seleno- $\beta$ -D-galactopyranoside) (Ogra *et al.*, 2002; Bendahl and Gammelgaard,

2004; Suzuki *et al.*, 2010) are the major selenium metabolites in urine. Other selenium compounds including selenate, selenite, selenodiglutathione, methylselenol, selenocystine, SeCys, selenoethionine, L-SeMet, selenocystamine, methylselenomethionine and Se-adenosylselenomethionine have been reported in urine but there is insufficient evidence to accept these compounds as urinary selenium metabolites (Gammelgaard and Jons, 2000; Cao *et al.*, 2001; Wrobel *et al.*, 2002; Chatterjee *et al.*, 2003; Gammelgaard *et al.*, 2003). Unabsorbed selenium is excreted from the body by faecal elimination (Thomson and Stewart, 1973; 1974; Thomson and Robinson, 1986; Robinson *et al.*, 1997; Thomson, 2007) but the role of the gut microbiota in selenium excretion has not been studied.

### **1.5.2. Disposition of selenium in mammals**

Previous studies have used [<sup>74</sup>Se]selenite and [<sup>74</sup>Se]L-SeMet to study the disposition of these compounds in mammals. A kinetic model for selenite has been developed and later modified to apply to L-SeMet (Ben-Porath *et al.*, 1968; Graham *et al.*, 1971; Patterson *et al.*, 1989; Swanson *et al.*, 1991). In general, both selenium forms have similar dispositions except that the model for selenite has one tissue pool and the model for L-SeMet has two tissue pools. The difference between the tissue pools is that one represents short-term storage of selenium and the other represents long-term storage.

After absorption from the GIT, labelled selenium as selenite or L-SeMet leaves enterocytes by two pathways (Graham *et al.*, 1971; Patterson *et al.*, 1989). In the first, labelled selenium appears in the peripheral circulation after passing through the liver. In the second, labelled selenium leaves enterocytes via the hepatopancreatic subsystem or the lymphatic system and is then transported to the liver via plasma. Selenium undergoes enterohepatic recirculation which involves excretion of selenium into the gut via bile (46% of labelled selenium after L-SeMet administration) and then reabsorption from the GIT. From the liver and

pancreas, labelled selenium moves into tissues via plasma. Once in the tissues, labelled selenium is slowly turned over and exchanges with selenium in the tissue pools such as the kidneys, muscle and bone. The physiological advantage of L-SeMet over selenite as a supplement is that selenium can be reutilised by slow turnover of general body proteins (Ben-Porath *et al.*, 1968; Graham *et al.*, 1971; Patterson *et al.*, 1989; Swanson *et al.*, 1991).

## **1.6. Markers of selenium status and metabolism**

Levels of selenium in serum, whole blood, erythrocytes, urine, hair and toenails have been used as indicators of selenium status in human (Gibson, 2005) but serum/plasma selenium is most commonly used. In human studies, selenium concentration in serum has been shown to positively correlate with selenium concentration in liver and kidney (Oster *et al.*, 1988). Levels of selenoenzymes and Sels such as GPx, TrxR and/or Sel P are increasingly used to indicate selenium status and to diagnose selenium-related chronic diseases (Papp *et al.*, 2007). Faeces and urine levels have been used to investigate the metabolism of selenium (Thomson, 2007).

### **1.6.1. Selenium in serum**

An assessment of selenium concentration in human serum indicates that a concentration < 50 µg/L (0.63 µM) represents poor selenium status and > 120 µg/L (1.52 µM) represents high selenium status (Thomson, 2004a). The serum selenium level responds rapidly to an increase in selenium intake. In rat, several studies have used doses of 0.1 - 2 mg selenium/kg to investigate the dose vs serum-selenium level relationship. The serum selenium level in rat at baseline is higher than that in human (Behne *et al.*, 1996; Itoh and Suzuki, 1997; Shiobara *et al.*, 1998).

Dietary intake, age and sex are known to affect serum/plasma selenium concentrations in human. A positive correlation between dietary intake and serum selenium concentration has been found (Stead *et al.*, 1985; Wasowicz *et al.*, 2003) and the serum/plasma concentration appears to increase with age (Van Deal *et al.*, 1994; Micetic-Tuck *et al.*, 1996; Wang *et al.*, 1998; Diaz Romero *et al.*, 2001). However, several studies indicate that the selenium concentration is not dependent on gender in children and adults (Sesana *et al.*, 1992; Haldimann *et al.*, 1996; Diaz Romero *et al.*, 2001; Krittaphol *et al.*, 2006; Thomson *et al.*, 2007) although concentrations in boys tend to be lower than in girls (Viegas-Crespo *et al.*, 1994). There was no difference in plasma selenium concentration between young and older women, but a positive relationship between plasma selenium concentration and plasma estrogen concentration was found (Smith *et al.*, 2000).

Male rats have been used in several studies investigating selenium status (Levander *et al.*, 1983; Behne and Hofer-Bosse, 1984; Suzuki *et al.*, 1995), whereas only a few studies have used female rats (Suzuki *et al.*, 1995; Schomburg and Schweizer, 2009). In general, there is no difference in selenium status and tissue selenium level between male and female rats but a positive relationship between plasma selenium concentration and reproductive hormone levels has been found in tissues from female rats (Brown and Burk, 1973; Schomburg and Schweizer, 2009).

### **1.6.2. Selenium in urine**

Several studies in animals and humans have used 24 h urine samples to determine selenium status (Palmer *et al.*, 1969; Griffiths, 1976; Ogra *et al.*, 2002; Kuehnelt *et al.*, 2005). The dose of selenium may affect the type of selenium metabolites that are excreted in urine (Itoh and Suzuki, 1997; Thomson, 2007) but there is insufficient quantitative data to support this assertion (Kobayashi *et al.*, 2002; Gammelgaard and Bendahl, 2004). For example, in a study of five cancer patients taking a high dose (8 mg/Se/day) of L-SeMet, selenosugar was the major selenium

metabolite in urine in all patients and only one patient produced  $\text{TMSe}^+$  (Kuehnelt *et al.*, 2007). It seems that after a high dose of L-SeMet,  $\text{TMSe}^+$  is only a minor selenium metabolite in urine and therefore may not be a useful biomarker of excessive selenium intake in humans. In healthy mice, after injection of labelled selenium as selenite, selenium was excreted in the urine mainly as selenosugar with smaller amounts being excreted as selenite and  $\text{TMSe}^+$  (Suzuki *et al.*, 2010).

To investigate this inconsistency surrounding selenium metabolites excreted in urine at high selenium intakes, Suzuki and colleagues examined age-related changes in urinary selenium metabolites in rats given different concentrations of selenite (0.2 - 2.0  $\mu\text{g}$  selenium/mL) in their drinking water (Suzuki *et al.*, 2005). Although  $\text{TMSe}^+$  concentration in urine increased after selenosugar concentrations reached a plateau, it remained a minor urinary metabolite in adult rats. However, in young rats,  $\text{TMSe}^+$  was the main metabolite in urine at high selenium doses but only during short-term dosing. On chronic dosing, the  $\text{TMSe}^+$ /selenosugar ratio appeared to decrease (Suzuki *et al.*, 2005).

### **1.6.3. Selenium in faeces**

Faeces are commonly analysed to determine the cumulative loss of selenium. Assay of the 24 h faeces from animals has shown that, after oral doses of selenite or L-SeMet, 21% and 16% respectively of the selenium dose is excreted in the faeces (Thomson and Stewart, 1973). For humans given selenite, approximately 33 – 58% of the selenium was excreted in the faeces whereas, after giving a similar dose of L-SeMet, only 4 - 6% of selenium was excreted (Thomson and Stewart, 1974). These studies indicate that selenium is excreted in the faeces to a greater extent after ingestion of selenite than after ingestion of L-SeMet in animals and humans.



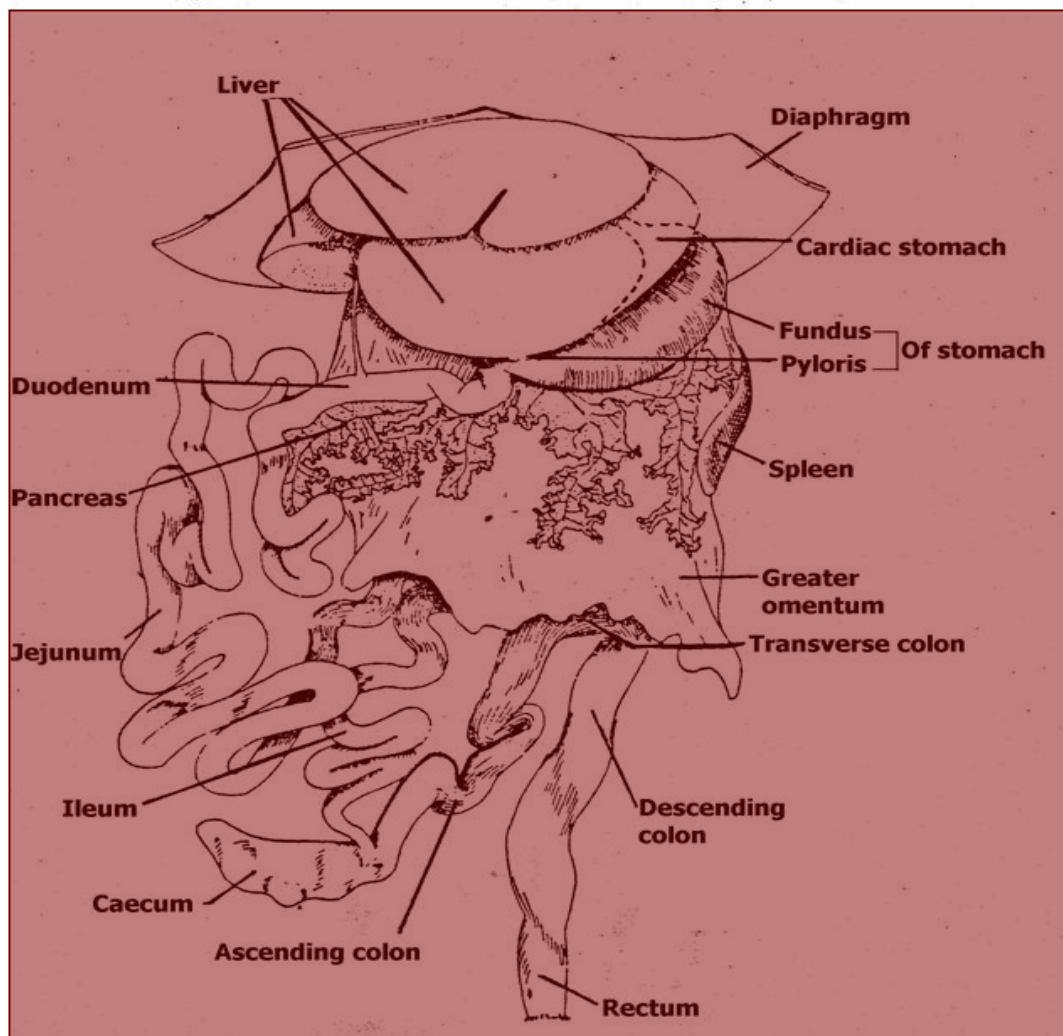
## 1.7. Role of the intestine

The disposition of selenium suggests that L-SeMet and selenite undergo enterohepatic recycling. Thus, it is possible that selenium metabolism occurs in the GIT. In order to understand the limitations of the rat as a model of the human GIT, an understanding of the anatomy and physiology of the rat GIT is required.

In the rat small intestine (Figure 1.9), most chemical digestion and absorption of nutrients occurs in three regions - the duodenum, jejunum and ileum (Levin, 1969). The pancreas and the intestine secrete digestive enzymes into the lumen of the small intestine to bring about the chemical breakdown of food into its constituent components. The ileum opens to the large intestine which consists of four compartments; the large caecum, the ascending colon, the transverse colon and the descending colon (Figure 1.9). The caecum is larger than the colon and contains a large number of microorganisms which breakdown fibre in the diet that cannot be digested by the enzymes of the small intestine. The major function of the large intestine is to reabsorb the large quantity of water secreted into the gut during digestion. Consequently, nutrients and water are removed from the undigested material in the colon and the faeces are then stored in the rectum until eliminated (University of Winnipeg, 2006).

In the present study, a rat model was used since it is possible to control the environment and diet during experiments. In addition, the collection of contents from different segments of the intestinal tract is virtually impossible in humans. It is also difficult to mimic intestinal conditions using a simulated system (Chen *et al.*, 2010) since it cannot support the large variety of microorganisms found in an actual gut. Thus, a rat experimental model is useful to elucidate the metabolism and utilisation of selenium in the intestinal tract.

The rat model can give results that apply to the human since, in both cases, metabolic activity in the gut is related to enzymes and the population of gut microorganisms in the different segments of the gut. However, it must be recognised that the human GIT is different from the rat in that the colon is longer and the caecum is smaller. This suggests that metabolism by the gut microbiota occurs predominantly in the caecum in rat and in the colon in human.



**Figure 1.9.** Anatomy of the rat intestinal tract (University of Winnipeg, 2006).

## 1.8. Gut microorganisms

The total counts of viable bacteria (cfu/mL or cfu/g contents) inhabiting the human jejunum, ileum and colon are approximately  $10^5$ ,  $10^4$ - $10^8$  and  $10^{10}$ - $10^{12}$ , respectively (Orrhage and Nord, 2000). In one study in rats, the total counts of viable bacteria in caecum and colon were approximately  $10^{10}$  cfu/g wet weight of contents (Wyatt *et al.*, 1988). In another study, the total viable bacterial counts in small and large intestine were  $10^8$  and  $10^{10}$  cfu/g wet weight of contents, respectively (Kararli, 1995). In mammals, microorganisms that make up the gut microbiota are important in digestion, recycling of nutrients and preventing colonisation by pathogenic organisms (Guarner and Malagelada, 2003). Gut microbiota are known to be involved in vitamin and drug metabolism (Boyle *et al.*, 2006; Jia *et al.*, 2008) but little is known regarding their role in selenium metabolism.

The gut microbiota is essential to the process of enterohepatic recirculation of compounds that enter the GIT via biliary excretion. Bile contains salts which are formed by conjugation of bile acids with the amino acids taurine or glycine in the liver (Martin *et al.*, 2007). It is possible that selenium containing compounds are also conjugated to bile acids in the liver and then undergo secretion into the GIT. Enzymes produced by the gut microbiota (i.e.  $\beta$ -glucuronidase, sulphatase and glycosidase) deconjugate such compounds allowing the parent compounds to be reabsorbed in the small intestine (Houten *et al.*, 2006). Based on the disposition of labelled selenium, selenium returns to the intestinal tract from the liver via the bile and is exposed to the gut microbiota.

A few studies of the enterohepatic recirculation of selenium have been carried out using [ $^{74}\text{Se}$ ]L-SeMet. In human, it was estimated that 46% of selenium from the liver is excreted into the GIT via the bile (Swanson *et al.*, 1991). The labelled selenium continued to appear in the faeces during a 12-day collection period

corresponding to the duration of enterohepatic recirculation (Swanson *et al.*, 1991).

### **1.8.1. Biotransformation of selenium by microorganisms**

Microorganisms play an important role in the mobility, speciation and toxicity of selenium (Oremland *et al.*, 1991). Two important processes brought about by microorganisms are methylation and demethylation. Based on selenium metabolism by bacteria found in soil and sewage sludge, methylation by microorganisms to form the volatile selenium metabolites, DMSe and dimethyldiselenide (DMDS<sub>2</sub>) is a means of reducing the toxicity of selenocompounds (Challenger, 1945; Chasteen, 1993). Two methyltransferase enzymes (a thiopurine methyltransferase and a homologue of calicheamicin methyltransferase) have been identified in *Rhodocyclus tenuis*, *Rhodospirillum rubrum* and *Pseudomonas syringae*. These bacteria can convert organic or inorganic selenium to DMDS<sub>2</sub> and DMSe, respectively (Ranjard *et al.*, 2002; Ranjard *et al.*, 2003; Stolz *et al.*, 2006). As regards demethylation, soil and water microorganisms remove a methyl group and use it as an energy source (Duran and Alexander, 1975). Selenite can be reduced by *Pseudomonas fluorescens* K27 to produce elemental selenium as a by-product (Hapuarachchi *et al.*, 2004). It has been suggested that the production of elemental selenium is a means of removing potentially toxic levels of selenium compounds (Wang *et al.*, 2007; Zhang *et al.*, 2008). However, information on the conditions under which elemental selenium is formed is limited. In regard to whether elemental selenium is bioavailable, it has been found that elemental selenium (nanoparticle size) is less toxic than selenite (Zhang *et al.*, 2004; Zhang *et al.*, 2005; Wang *et al.*, 2007) but its bioavailability has not been investigated.

## 1.9. Probiotic bacteria

Probiotic bacteria are defined as “live microorganisms which when administered in adequate amounts confer a health benefit on the host” (FAO/WHO, 2002). Several studies have shown that probiotic bacteria can enhance and maintain the composition of the healthy intestinal microbiota (Dunne *et al.*, 1999; Boyle *et al.*, 2006; Schultz and Lindström, 2008). There are several criteria for selecting probiotic bacteria: They should originally be human strains (i.e. species isolated from the intended host) and safe for human use; they should be stable to acid and bile and able to adhere to the intestinal mucosa; they should be able to produce antimicrobial components and have a beneficial effect on the host; they should be non-pathogenic and non-toxic, be capable of surviving and metabolising in the gut and remain viable during storage and use (Tannock, 1999; Dunne *et al.*, 2001; Schrezenmeir and de Vrese, 2001; Fooks and Gibson, 2002).

The means by which probiotics exert a therapeutic effect include modulating the immune system, production of antimicrobial substances and inhibition of pathogen adherence or translocation (Elina *et al.*, 1999; Fooks and Gibson, 2002; Yang *et al.*, 2009). Thus, probiotics appear to improve inflammatory bowel disease (Steidler, 2001) by regulating T-cell function (Shanahan, 2002) and interfering with tumour growth through regulating transcription factors of interferon- $\gamma$ , IL-1 $\beta$  and TNF- $\alpha$  (Geier *et al.*, 2006; Fotiadis *et al.*, 2008). They can also reduce the concentration of carcinogenic agents in the gut by reducing the activity of bacterial enzymes such as nitroreductase and  $\beta$ -glucuronidase which can produce carcinogenic agents as metabolic byproducts (Rowland and Grasso, 1975; Hayatsu and Hayatsu, 1993). The antitumorigenic activity occurs through decreasing the pH of the gut contents which helps to increase the proliferative activity of normal cells and aids the binding of mutagens in the diet (e.g. nitrosamines in cooked meat) (Hayatsu and Hayatsu, 1993). Generally, probiotics have been found to improve host health but some studies showed no effect after probiotic treatment. These studies may have suffered from use of the wrong strain

or use of insufficient cells to alter the metabolism of the indigenous microbiota (O'Sullivan and O'Morain, 2000; Thomas *et al.*, 2001; Marteau *et al.*, 2006).

Probiotic bacteria are commonly used as either single or mixed cultures of live microorganisms. *Lactobacillus* and *Bifidobacterium* species are commonly used because they increase intestinal lactic acid-producing bacteria which maintain gut homeostasis and modulate the immune response in the host (Sanders, 1994; Collins and Gibson, 1999; Isolauri, 1999).

### **1.9.1. Probiotic bacteria and selenium**

Probiotic bacteria are a subgroup of microorganisms and as such may have the capacity to metabolise selenium. It is possible that probiotics can transform selenium to Sels via selenide which can then be utilised by the host. Probiotics may be used to deliver selenium since they can accumulate selenium in their cells as Sels. In addition, probiotics may assist the endogenous microbiota by methylating selenium as a means of selenium detoxification. Despite these potential benefits, information about the effect of probiotics on selenium metabolism and disposition is limited and requires investigation in order to illuminate the possible consequences of probiotic treatment on selenium status.

Dias and Weimer (1998) showed that bacterial demethiolation enzymes (i.e. cystathionine  $\gamma$ - or  $\beta$ -lyase) can breakdown Met to methanethiol and that addition of Met in the growth medium of lactic acid bacteria can induce the level of methionine  $\gamma$ -lyase (Dias and Weimer, 1998). It is therefore possible that cystathionine  $\gamma$ - or  $\beta$ -lyase in probiotic lactobacilli or lactococci can produce methylselenol from L-SeMet as shown in Figure 1.1.

A few studies have revealed that probiotic bacteria can affect selenium metabolism and disposition. *Lactobacillus* and *Bifidobacterium* strains cultivated with selenite were able to absorb and accumulate selenium and were used as

selenium-enriched probiotics (Chen *et al.*, 2005; Mazo *et al.*, 2007; Zhang *et al.*, 2009). Selenite was also reduced in *Lactobacilli* to elemental selenium and selenite was transformed via selenide to SeCys or L-SeMet, which could be stored in the cells (Calomme *et al.*, 1995a; Calomme *et al.*, 1995b; Andreoni *et al.*, 2000). Selenium has been shown to enhance the action of certain probiotic bacteria. For example, the antimutagenic activity of the probiotic bacterium *Enterococcus faecium* M-74 was enhanced by selenium (Križková *et al.*, 2002).

A study of the biotransformation of inorganic selenium in two types of microorganisms, bacteria (*Lactobacillus*) and yeast (*Saccharomyces*), in the lactic fermentation process of yoghurt and kefir (a creamy drink made of fermented cow's milk) showed that selenocystine and Se-methylselenocysteine were produced. In kefir, the two selenium compounds were found together with L-SeMet (Alzate *et al.*, 2008). Se-Methylselenocysteine production was greater during the fermentation of kefir than during the fermentation of yoghurt. This may be important because there is some evidence that Se-methylselenocysteine is a better *in vivo* source of the putative anticarcinogen, methylselenol, compared to SeMet and selenite (Alzate *et al.*, 2008). For stimulation of selenium status, drinking yoghurt containing different antioxidants and *Lactobacillus plantatum* 299v increased the levels of serum selenium, total antioxidant capacity in plasma and Sel P (Önning *et al.*, 2003). There was also a significant increase in the number of *Lactobacillus plantatum* 299v in faeces (Önning *et al.*, 2003).

## **1.10. Conclusion**

Selenium research in the present study focused on the possible role of the gut microbiota and its interaction with probiotics in mediating the health benefits of selenium supplements. Selenium supplements are important to maintain selenium status when selenium intake from normal diets is inadequate. Organic (L-SeMet) and inorganic selenium (selenite) forms are commonly used as human and animal supplements (Garbisu *et al.*, 1996; Shiobara *et al.*, 1999; Zhang *et al.*, 2005;

Suzuki *et al.*, 2006; Abdulah *et al.*, 2007; Forceville, 2007; Ogra *et al.*, 2008; Hamad *et al.*, 2009; Jiang *et al.*, 2009). Metabolism of selenium is known to take place in the liver and kidney. As selenium undergoes enterohepatic recirculation, it is likely that the microbiota also play a role in selenium metabolism (Hrdina *et al.*, 2009). Microorganisms in the environment and in culture can metabolise selenium compounds and produce selenium metabolites by methylation and demethylation. Thus, understanding selenium metabolism by the indigenous gut microbiota may contribute to improved utilisation of selenium from dietary sources and selenium supplements. Probiotic treatment improves the immune response, modulates the gut microbiota and reduces carcinogenic agents in the diet. Selenium has a similar function in improving the immune system and reducing the risk of cancer. The use of a combination of probiotics and selenium supplements may have a synergistic effect which requires further investigation. Since radiolabelled L-SeMet or selenite were unavailable and argon interferes with the main isotope of selenium when analysed by inductively coupled plasma mass spectrometry, high doses (2 mg selenium/kg) of L-SeMet and selenite were used to investigate of metabolism of these compounds in this thesis.

### **1.11. Aims**

The first aim of this thesis was to examine whether the gut microbiota metabolise selenium supplements *ex vivo*. Gut contents from rat jejunum, ileum, caecum and colon and two forms of selenium supplements L-SeMet and selenite were used. The second aim was to assess the metabolic activity of a selection of probiotic bacteria towards these selenium supplements *in vitro* and to investigate their metabolic activity when combined with gut contents. The third aim was to determine whether probiotic treatment can modulate the disposition of selenium after oral administration of L-SeMet and selenite in rat.



These aims address two hypotheses under investigation in this study; first that L-SeMet and selenite are metabolised by the gut microbiota and by probiotic bacteria and second that oral treatment with probiotic bacteria increases the metabolism of orally administered L-SeMet and selenite and changes the disposition of selenium.

## CHAPTER 2

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### Analytical methods

Prior to an investigation of the metabolism of L-SeMet and selenite by gut microbiota and probiotic bacteria, analytical methods for the determination of L-SeMet, volatile selenium metabolites (DMSe and DMDS<sub>e</sub>), selenite and selenium concentration in gut contents, serum and tissues (liver and kidney) were required. The analytical methods described in this Chapter were developed based on literature methods. A derivatisation assay involving high performance liquid chromatography (HPLC) with fluorescence detection for the determination of L-SeMet in the complex matrix of rat gut contents is followed by a method using gas chromatography-mass spectrometry (GC-MS) to analyse the volatile selenium metabolites, DMSe and DMDS<sub>e</sub>. Finally, assays using atomic absorption spectrometry (AAS) for the determination of selenite and selenium are described.

#### 2.1. Assay for L-SeMet\*

Several techniques have been applied to the determination of L-SeMet. The majority of techniques used GC-MS (Reamer and Veillon, 1983) or HPLC (Encinar *et al.*, 2004) coupled to detectors based on hydride generation atomic absorption spectrometry (HFAAS), hydride generation atomic fluorescence spectrometry (HGAFS) or inductively coupled plasma mass spectrometry (ICP-MS) (Liang *et al.*, 2006).

\*Krittaphol *et al.* (2009) *Biomedical Chromatography* 23: 1169-1174

For GC analysis, L-SeMet has been derivatized with cyanogen bromide (CNBr) to form volatile methylselenocyanide which is then detected by ICP/MS (Ouyang and Wu, 1988; Zheng *et al.*, 1989; Yang *et al.*, 2004). In another GC method, L-SeMet was esterified with propan-2-ol and acylated with heptafluorobutyric anhydride to form a volatile species that was detected by MS (De la Calle-Guntinas *et al.*, 1997). GC-MS can discriminate Met and L-SeMet since they have a mass difference equal to the difference in the atomic weights of selenium and sulfur. The determination of L-SeMet in a protein matrix has been carried out by hydrochloric acid digestion under a nitrogen atmosphere followed by silylation and GC-MS. The derivatives were identified by their retention times and characteristic mass patterns (Lobinski *et al.*, 2000).

Chatterjee *et al.* (2001) used HPLC followed by HGAAS to determine L-SeMet in urine. The authors demonstrated that L-SeMet could be separated from other selenium compounds and, under acidic conditions, is reduced with NaBH<sub>4</sub> to produce methylselenol and subsequently DMDS<sub>e</sub>. This DMDS<sub>e</sub> is then carried to a quartz tube by the carrier gas where it decomposes to gaseous elemental selenium to be detected by AAS. Subsequently, Liang *et al.* (2006) showed that HPLC-HGAFS was more sensitive than HPLC-HGAAS. However, HPLC with HGAAS or HGAFS detection is a tedious and complex method for determination of L-SeMet.

Several studies have used HPLC coupled with other detectors to determine L-SeMet (Bird *et al.*, 1997; Li *et al.*, 1999; Encinar *et al.*, 2004). Bird *et al.* (1997) used HPLC/ICP-MS for the analysis of selenoamino acids. Reversed phase HPLC with pre-column derivatisation gave good separation and ICP-MS allowed the identification of selenium compounds with terminal amine functionality. HPLC/ICP-MS has also been used for the identification of selenium species (Bird *et al.*, 1997; Cao *et al.*, 2001).

The isocratic HPLC method for the determination of L-SeMet used in the present study is based on a method to determine amino acids using fluorescence derivatisation with *o*-phthaldialdehyde (OPA) (Seiler, 1993). The method was described by Hammel *et al.* (1997) but required extensive modifications for use in the present study. In particular, OPA together with *N*-acetyl-L-cysteine (NAC) was needed to produce a stable fluorescent derivative of L-SeMet. This strategy is based on previous work showing that OPA/NAC derivatisation is appropriate to separate and quantify amino acids (Seiler, 1993).

Determination of selenoamino acids in complex matrices such as gut contents has not been reported although complex mixtures of amino acids from protein hydrolysis have been analysed (Medina Hernandez *et al.*, 1991; Hammel *et al.*, 1997; van Eijk *et al.*, 1999). In the present method, acid was used to precipitate protein in gut content suspensions prior to derivatisation. The acid precipitation method was based on previous assays of amino acids and selenium yeast supplements in urine and blood (Graser *et al.*, 1985; Liang *et al.*, 2006). The preparation of the OPA/NAC derivatising agent was based on work by Molnar-Perl and Vasanits (1999) and Kutlan and Molnar-Perl (2003). The composition of the reaction mixture was adapted from that used by Graser *et al.* (1985).

### **2.1.1. Materials**

L-SeMet ( $\geq 98\%$  purity) and DL-SeMet ( $\geq 99\%$  purity) were purchased from Calbiochem (USA and Canada) and Sigma (St. Louis, USA), respectively. Analytical grade reagents (suppliers) were as follows: OPA (Fluka, Austria); NAC (Merck, Germany); anhydrous sodium acetate, sulphosalicylic acid, sodium hydroxide (BDH, England); potassium chloride (Ajax Finechem: UNILAB, Australia); sodium borate (Hopkin & Williams Ltd, England); HPLC grade methanol, acetonitrile and formic acid (Merck, Germany). In order to avoid adventitious selenium contamination, all glass and plastic ware were soaked overnight in 10% ultrapure nitric acid (BDH ARISTAR<sup>TM</sup>), rinsed with distilled

and deionised water three times and then oven dried before use.

Phosphate buffered saline (PBS; 0.01 M made up of 8 g sodium chloride, 0.2 g potassium chloride, 1.15 g disodium hydrogen phosphate, 0.2 g potassium dihydrogen phosphate in 1 L distilled deionised water) adjusted to pH 7.4 was chosen for the preparation of 10% gut content suspensions since most bacteria remain viable at this pH. The concentration of PBS (0.01 M) is expected to be sufficient to buffer any acidic or basic constituents in gut contents during the incubations.

### **2.1.2. Derivatisation**

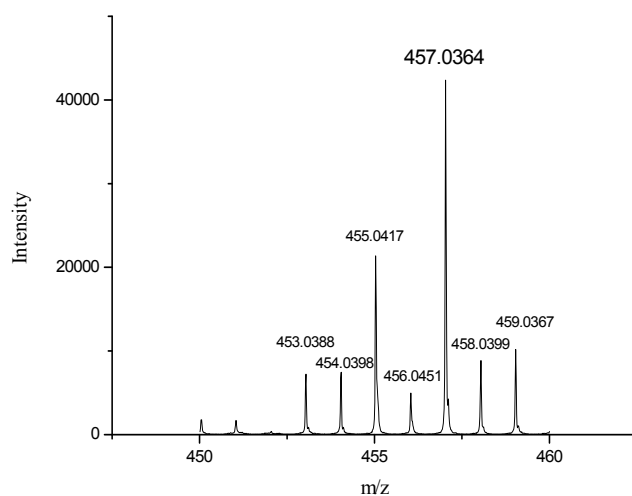
The following solutions were prepared: An OPA stock solution containing 0.512 g OPA in 25 mL methanol; a boric acid solution containing 0.2 M boric acid and 0.2 M potassium chloride; a borate buffer solution containing equal volumes of boric acid solution and 0.2 M sodium hydroxide (pH  $9.9 \pm 0.05$ ) (Kutlan and Molnar-Perl, 2003); the OPA/NAC derivatising reagent (containing equimolar amounts of OPA and NAC) made up of 0.4 g NAC in 20 mL borate buffer mixed with 5 mL OPA stock solution (final pH  $9.3 \pm 0.05$ ). The derivatising agent was prepared at least 90 min before use and stored in a refrigerator at 4°C for no more than 9 days (Molnar-Perl and Vasanits, 1999).

After reaction of the derivatising reagent containing equimolar amounts of OPA/NAC with L-SeMet (1 mL, 0.1 mM) and the solution allowed to stand at room temperature for 7 min (Molnar-Perl and Bozor, 1998; Molnar-Perl and Vasanits, 1999; Kutlan and Molnar-Perl, 2003). The structure of the derivative was investigated by high resolution time-of-flight mass spectrometry (HR MS-TOF). This was carried out on a Bruker micrOTOF-Q instrument controlled by Compass software version 1.3 (Bruker Daltronics, Bremen, Germany). Samples of reaction product were introduced by direct infusion at 3  $\mu\text{L}/\text{min}$  into the electrospray ionization (Kutlan *et al.*) source in the negative ion mode with a

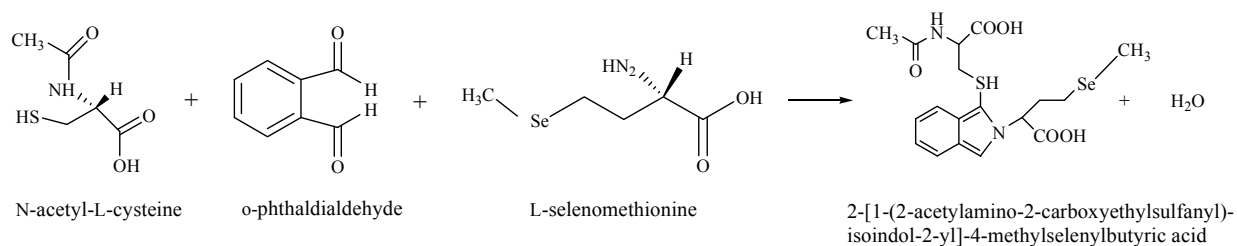
capillary voltage of +4500 V and 5 L/min of a dry gas (99% nitrogen) at 180°C. Sampling was averaged for 2 min over the m/z range 50-1000 amu. External calibration with sodium formate clusters used 15 calibration points from 90 to 1050 amu and a quadratic plus HPC (High Precision Calibration) line fit. SigmaFit™ of isotopic patterns was used to investigate the presence and structure of the L-SeMet derivative (Pelzing *et al.*, 2004).

HR MS-TOF confirmed the formation of a 1:1:1 derivative between OPA, NAC and L-SeMet, namely {2-[1-(2-acetylamino-2-carboxyethylsulfanyl)-isoindol-2-yl]-4-methylselenylbutyric acid}, based on the fact that the monoisotopic peak at m/z 457.0364 was found to be within 10 ppm of the calculated mass for C<sub>18</sub>H<sub>21</sub>N<sub>2</sub>O<sub>5</sub>SSe (m/z 457.0342) (Figure 2.1a). The proposed derivatisation reaction is shown in Figure 2.1b.

a



b



**Figure 2.1.** (a) Isotopic profile of the derivative obtained by HR MS-TOF; the structure was confirmed by the fact that the monoisotopic peak at  $m/z$  457.0364 agreed with the calculated value of  $m/z$  457.0342. (b) Proposed derivatisation reaction between OPA/NAC and L-SeMet.

### 2.1.3 Sample preparation

Sample for analysis incubates of L-SeMet or selenite (initially  $\sim 0.5$  mM) in 10% w/w caecum content suspensions made up in PBS. Samples (100  $\mu$ L) were vortexed with 10  $\mu$ L 30% sulphosalicylic acid to precipitate protein. After

centrifugation at 12000 x g for 2 min, a 20  $\mu$ L aliquot of supernatant was added to 200  $\mu$ L methanol, 200  $\mu$ L 0.2 M boric acid solution and 50  $\mu$ L OPA/NAC derivatising reagent to give a final volume of 470  $\mu$ L. After vortexing, samples were allowed to react for 7 min at room temperature (20 - 25°C). Mixtures were then transferred into autosampler vials and placed in an autosampler tray for injection into the HPLC system.

#### **2.1.4. HPLC**

The isocratic HPLC system consisted of a pump (Shimadzu LC-10AT<sub>VP</sub>), autosampler (Shimadzu SIL-10AD<sub>VP</sub>) and fluorescence detector (Hewlett Packard 1046A) all controlled by Classic-VP 7.4 software. Separation was performed on two analytical Luna C<sub>18</sub> columns (5  $\mu$ m, 100 x 2 mm i.d.) connected in series and preceded by a C<sub>18</sub> guard column (4 x 2 mm i.d.) (Phenomenex, CA, USA). The mobile phase consisted of sodium acetate solution (100 mM) : methanol : acetonitrile (60 : 40 : 0.1 v/v) adjusted to pH 7.2 with acetic acid. The injection volume was 20  $\mu$ L and the flow rate 0.2 mL/min. Fluorescence detection involved excitation at 340 nm and emission at 435 nm and quantitation was based on determination of peak area. The run time of the assay was 25 min.

#### **2.1.5. Assay validation**

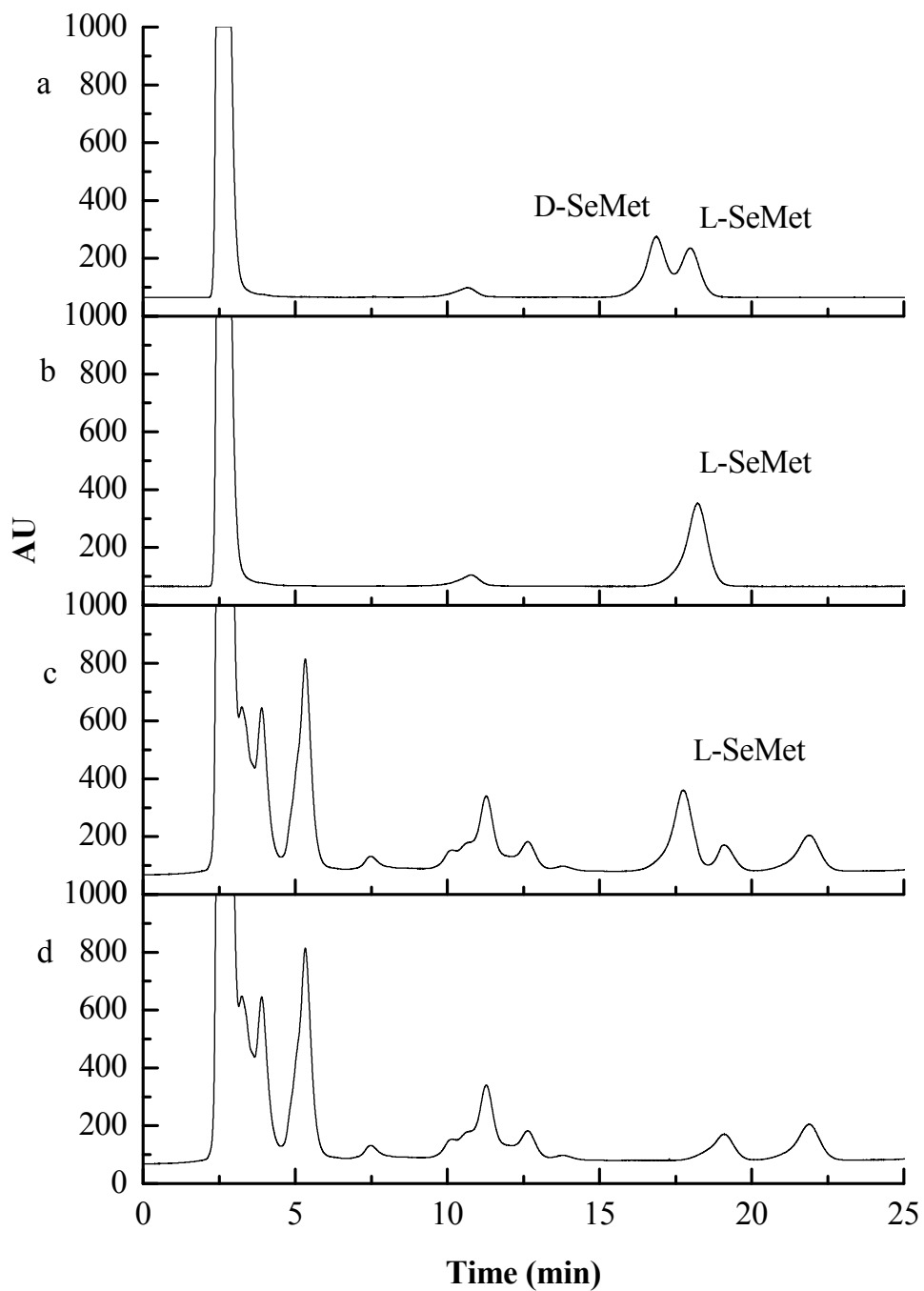
Standard curves were prepared by spiking 10% w/w caecum content suspensions (900  $\mu$ L) with 100  $\mu$ L L-SeMet standard solutions (5, 10, 25, 50, 100, 250, 500, 1000  $\mu$ g/mL) to give calibration standards in the range 0.5 - 100  $\mu$ g/mL. Aliquots (100  $\mu$ L) of calibration standards and QC samples (10, 20, 50 and 100  $\mu$ g/mL) were analysed as described in Section 2.1.3. Recovery was determined by comparison of peak areas in QC samples with those in spiked saline solutions. Accuracy (as percentage of the true value) and precision (as relative standard deviation RSD%) were determined by replicate analyses ( $n = 5$ ) of QC samples on one day and triplicate analyses on five different days. The stability of L-SeMet in



10% caecum content suspensions stored at -20°C was also assessed.

### **2.1.6. Results and Discussion**

In regard to sample preparation, determination of selenium in precipitated protein from samples by graphite furnace atomic absorption spectrometry (GFAAS, detection limit 1 ng/mL) showed negligible amounts of selenium. Chromatograms of DL-SeMet and L-SeMet are shown in Figures 2.2a-d. In analysing DL-SeMet, the D- and L-enantiomers were detected at retention times of 16 and 18 min, respectively, and were free of interference from endogenous peaks in gut content suspensions. Standard curves for L-SeMet were linear in the range 0.5 – 100 µg/mL ( $y = 475208x - 7507$ ;  $r^2 = 0.9992$ ). Accuracy and precision values are shown in Table 2.1. Intra-day and inter-day accuracies were 91.1 – 92.8% and 91.7 – 95.5%, respectively. Intra- and inter-day precisions were < 5%. The limit of detection of L-SeMet (signal-to-noise ratio of 3) was 0.025 µg/mL. L-SeMet was found to be stable in 10% caecum content suspensions stored at -20 °C for over one month.



**Figure 2.2.** Chromatograms of (a) 50  $\mu\text{g/mL}$  DL-SeMet in 0.9% sterile saline solution; (b) 50  $\mu\text{g/mL}$  L-SeMet in 0.9% sterile saline solution; (c) 50  $\mu\text{g/mL}$  L-SeMet in 10% w/w caecum content suspension; (d) blank 10 % w/w caecum content suspension.

**Table 2.1.** Intra-day and inter-day accuracy and precision for the assay of L-SeMet in 10% w/w gut content suspensions. Data are mean  $\pm$  SD based on five replicate analyses on one day and triplicate analyses on five different days.

L-SeMet concentration ( $\mu\text{g/mL}$ )	Intra-day Concentration ( $\mu\text{g/mL}$ )	Precision (RSD%)	Accuracy (%)	Inter-day Concentration ( $\mu\text{g/mL}$ )	Precision (RSD%)	Accuracy (%)
10	$9.1 \pm 0.4$	4.29	$91.1 \pm 3.9$	$9.4 \pm 0.5$	4.91	$93.5 \pm 4.6$
20	$18.5 \pm 0.5$	2.48	$92.3 \pm 2.3$	$19.1 \pm 0.5$	2.56	$95.5 \pm 2.4$
50	$45.8 \pm 1.0$	2.24	$91.7 \pm 2.1$	$45.9 \pm 1.1$	2.45	$91.7 \pm 2.3$
100	$92.8 \pm 4.2$	4.47	$92.8 \pm 4.2$	$94.6 \pm 3.4$	3.55	$94.6 \pm 3.4$

Using the derivatisation conditions described by Hammel *et al.* (1997) (reaction time of 1.5 min), the OPA derivative of L-SeMet was found to be unstable and the method gave non-reproducible results. The OPA/NAC derivatisation of L-SeMet was found to be complete in 7 min and the derivative was stable thereafter. Using OPA/NAC derivatisation followed by reversed phase HPLC, it is possible to separate the enantiomers of most amino acids (Seiler, 1993). NAC is preferable to mercaptoethanol and 3-mercaptopropionic acid due to the stability of its derivative (Medina Hernandez *et al.*, 1991). In the present study, the use of two short narrow bore columns in series resulted in good peak resolution in a reasonable run time (Molnar-Perl and Vasanits, 1999). Analysis of DL-SeMet (Figure 2.2a) revealed that the enantiomers could be resolved indicating this method may be useful to determine the presence of D-SeMet in commercial L-SeMet supplements (Schrauzer, 2000).

HR MS-TOF was used to confirm the identity of the derivative. The derivative was found to have a molecular mass and isotopic profile consistent with the product of a 1:1:1 reaction between L-SeMet, OPA and NAC (Figure 2.1b). Collected fractions of HPLC eluent corresponding to the derivative peaks were also analysed by HR MS-TOF but the reaction product was not detected. This may be due to ionisation interference from components in the collected fraction. In a previous study, it was proposed that OPA-derivatised amino acids decompose during collection of fractions (van Eijk *et al.*, 1999).

This is the first report of an HPLC assay for the quantitation of L-SeMet in a complex matrix such as gut content suspensions. Assay validation shows it is suitable for a study of the metabolism of L-SeMet in gut content suspensions.

## **2.2. Assay for DMSe and DMDS<sub>e</sub>**

GC-MS is commonly used to determine volatile selenium metabolites. The method employed here is based on previous methods to determine DMSe and DMDS<sub>e</sub> in bacterial cultures and selenium in plants (Meija *et al.*, 2002; Ranjard *et al.*, 2002; Ranjard *et al.*, 2003).

### **2.2.1. Materials**

DMSe ( $\geq 99\%$  purity) and DMDS<sub>e</sub> ( $> 96\%$  purity) were obtained from Sigma-Aldrich Inc., NZ. HPLC grade methanol, acetonitrile, hexane and other analytical reagents and solvents were obtained from Sigma-Aldrich and Merck, respectively. An eye shield, face shield, gloves and mask were used during the handling of DMSe and DMDS<sub>e</sub> and all work was carried out in a fume hood. All waste products from the experiments were disposed in biohazard containers.

### **2.2.2. Sample preparation**

Methanol was used as the solvent for DMSe and DMDS<sub>e</sub> (Diplock *et al.*, 1973; Lewis *et al.*, 1974; Gui-bin *et al.*, 1992). To 10% w/w caecum content suspensions (0.5 mL), methanol (1 mL) was added and the contents vortexed and centrifuged at 3500 rpm for 10 min. Supernatants were filtered (0.22  $\mu\text{m}$ ) and maintained at  $-20^{\circ}\text{C}$  until analysis (Meija *et al.*, 2002; Ranjard *et al.*, 2003).

### 2.2.3. GC-MS parameters

The GC-MS system (Agilent Technologies, USA) and parameters are listed in Table 2.2. Chem Station software (Hewlett Packard, Avondale, PA, USA) was used for data analysis. The mass spectrometer was set to scan from  $m/z$  50 to 400 at a rate of 4.1 scans/s using a peak height threshold of 150 counts. An electron multiplier voltage of 1200 V was used to ionise incoming samples. DMSe or DMDS<sub>e</sub> standards were scanned to determine the DMSe or DMDS<sub>e</sub> fragmentation patterns. The identities of DMSe and DMDS<sub>e</sub> were verified with the inbuilt National Institute of Standards and Technology (NIST 98) library in Chem Station software. Based on the DMSe and DMDS<sub>e</sub> fragmentation patterns, the mass spectrometer was set to selective ion mode and scanned for the peaks at  $m/z$  110 and 190 corresponding to the  $[M^+]$  ions of DMSe and DMDS<sub>e</sub>, respectively. MS detection was delayed by 3.5 min to prevent the detector being saturated by solvent front and to preserve the life of the tungsten lamp in the electrical ionisation part of the instrument.

**Table 2.2.** Operating conditions for GC-MS analysis of DMSe and DMDS<sub>e</sub>.

GC-MS	
Model	Agilent 6890N, MS 5973, Injector 7683B series and Autosampler (HP7673)
Column	ZB-5MS capillary column
Column size	60 m x 0.25 mm i.d. x 0.25 $\mu$ m film
Injection volume	1 $\mu$ L
Split ratio	2:1 ( <i>in vitro</i> study) and splitless ( <i>in vivo</i> study)
Carrier gas	H <sub>2</sub> , flow rate 1 mL/min, pressure 6.02 psi
Gas saver	20 mL/min for 2 min
Injector port temperature	210°C
Column temperature	40°C, hold for 5 min and then increase at 5°C/min to 120°C

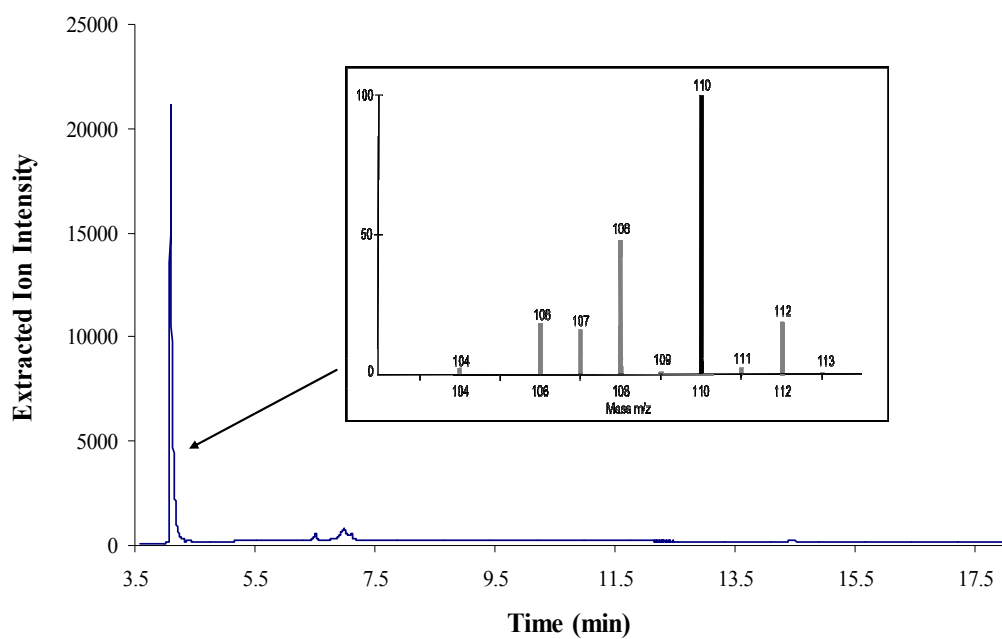
#### 2.2.4. Assay validation

Calibration standards were prepared by spiking 10% w/w caecum content suspensions with methanol solutions of DMSe (63.9 mM) or DMDS<sub>e</sub> (50.7 mM) to give concentrations of 0.001 - 3.19 mM DMSe and 0.001 - 1.01 mM DMDS<sub>e</sub>. QC samples at concentrations of 0.013, 0.128 and 1.28 mM for DMSe and 0.010, 0.051 and 0.507 mM for DMDS<sub>e</sub> were also prepared. Calibration standards and QC samples were analysed as described in 2.2.2. Recovery was determined by comparison of peak areas in QC samples with those in spiked saline solutions. Accuracy (as percentage of the true value) and precision (as RSD%) were determined by replicate analyses ( $n = 3$ ) of QC samples.

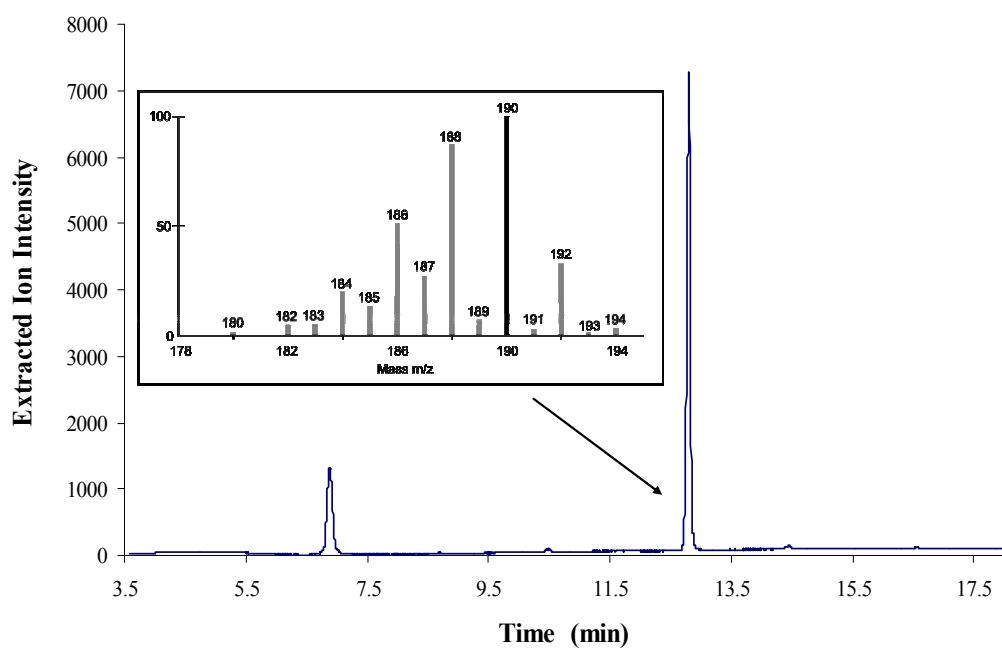
#### 2.2.5. Results and Discussion

The retention times for DMSe and DMDS<sub>e</sub> were 4.1 and 12.8 min, respectively (Figure 2.3). Standard curves were linear in the range 0.001 – 3.19 mM ( $y = 1585x + 4240$ ;  $r^2 = 0.9994$ ) for DMSe and 0.001 – 1.01 mM ( $y = 5009x - 1811$ ;  $r^2 = 0.9955$ ) for DMDS<sub>e</sub>. The limits of detection based on the formulation as described in previous study (Meija *et al.*, 2002) were 1.27  $\mu$ M for DMSe and 1.01  $\mu$ M for DMDS<sub>e</sub>. Precision was < 5% for both compounds with recoveries in the range 90.6 – 93.8%. DMSe and DMDS<sub>e</sub> in gut content suspensions stored at -80°C were found to be stable. DMSe and DMDS<sub>e</sub> in methanol solutions stored at -20°C were also found to be stable for over three months. A GC-MS chromatogram of a sample showing the presence of both DMSe and DMDS<sub>e</sub> is shown in Figure 2.4.

a

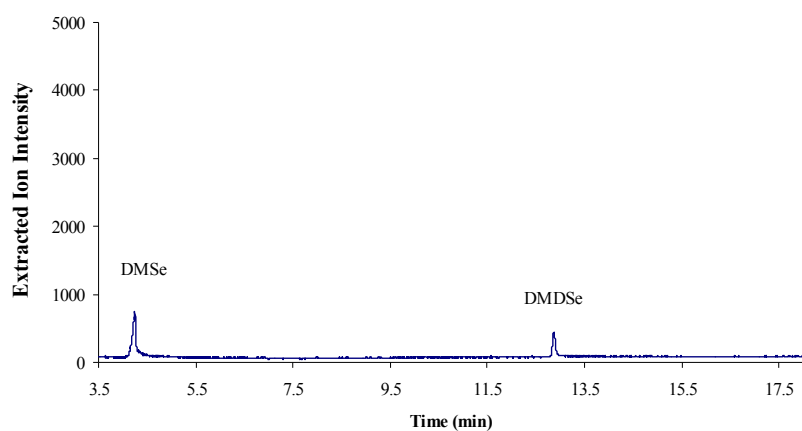


b

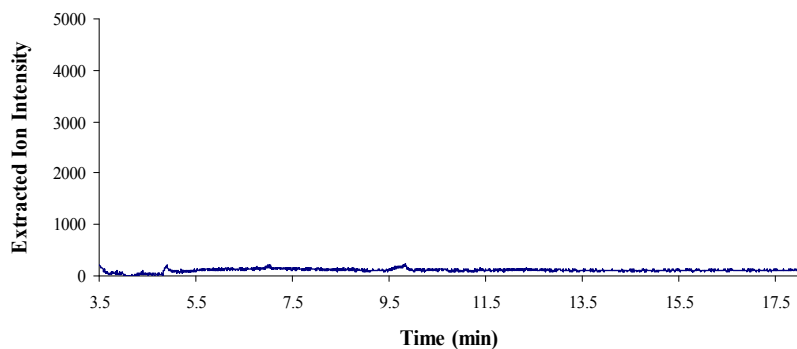


**Figure 2.3.** GC-MS chromatograms of (a) DMSe (3.19 mM) and (b) DMDS (2.54 mM) calibration standards. Inserts above the chromatograms are the mass spectra of DMSe and DMDS.

a



b



**Figure 2.4.** GC-MS chromatograms of (a) DMSe and DMDSe formed after incubation of selenite (0.58 mM) with a 10% w/w caecum content suspension at 37°C over 24 h and (b) a blank 10% w/w caecum content suspension.

### 2.3. Assay for selenite

The assay for selenite was based on the HGAAS methods of Cabon and Erler (1998) and Sherrard *et al.* (2004). The HGAAS system consisted of an AAnalyst 800™ atomic absorption spectrometer (Perkin Elmer, USA) with an MHA-FIAS-400 flow injector and an AS-90 plus autosampler. The system was controlled by WinLab32 software. A mixing chamber in the MHA-FIAS combined three separate flows of acid, sodium borohydride solution and sample. Selenite is the only selenium species susceptible to rapid reduction by sodium borohydride to



form hydrogen selenide. The H<sub>2</sub>Se produced is transferred in a steam of argon to a quartz tube heated to 900°C. The hydride is broken down to gaseous elemental selenium which is then determined using a selenium lamp (196.0 nm resonance line). Selenate is also reduced by sodium borohydride solution but the reaction is too slow to cause interference in the assay of selenite.

### **2.3.1. Materials**

A selenium standard solution containing sodium selenite (12.66 mM) was obtained from BDH Laboratory Supplies, UK. Other analytical reagents and solvents were obtained from Sigma-Aldrich and Merck, respectively. A standard reference material (SRM) of rice flour (SRM 1568a) (4.81 ± 0.51 nmol selenium/g) for use in assay validation was obtained from the National Institute of Standards and Technology, Gaithersburg, MD, USA.

### **2.3.2. Sample preparation**

Aliquots of 10% w/w caecum content suspensions (20 µL) were diluted with 9.8 mL distilled deionised water and mixed in a vortex mixer. A 500 µL aliquot was then diluted with 9.5 mL 20% hydrochloric acid and analysed by HGAAS. Samples were analysed in triplicate.

### **2.3.3. Assay validation**

Selenium standards were prepared by diluting the sodium selenite standard solution in 20% HCl to concentrations of 0.032, 0.063 and 0.127 µM. The SRM 1568a was digested in nitric acid followed by perchloric acid. Replicate samples ( $n = 15$ ) of SRM 1568a were analysed to determine precision and accuracy. Validation was performed using standard procedures employed by the Trace Element Research Laboratory, Department of Human Nutrition, University of Otago (Krittaphol *et al.*, 2006). The SRM validates the method for both accuracy

of the standard curve and the digestion procedure. Accuracy and precision was determined by comparison with the certified value of SRM 1568a.

#### **2.3.4. Results and Discussion**

The extensive dilution of samples reduced matrix effects to zero enabling validation to be performed using standard solutions and the SRM. The standard curve was linear for the three standards ( $y = 0.04166x$ ;  $r^2 = 0.9920$ ). The mean  $\pm$  SD (%RSD) for SRM 1568a was  $4.73 \pm 0.15$  nmol/g (3.2%) compared with the certified value of  $4.81 \pm 0.51$  nmol/g.

#### **2.4. Assay for total selenium**

Total selenium in tissues was determined using a method similar to that for analysis of selenite outlined in Section 2.3. The tissue of interest was digested by heating with nitric acid followed by perchloric acid during which selenium in the tissues is oxidised to selenite as described by Pettersson *et al.* (1988), Tiran *et al.* (1993) and Krittaphol *et al.* (2006). The total selenium was then determined by HGAAS with a detection limit of 25 ng/g (Vijan and Wood, 1976).

Analysis of total selenium in serum was carried out using GFAAS. The system consisted of an AAnalyst 800™ atomic absorption spectrometer (Perkin Elmer, USA) using a selenium lamp, a transversely-heated graphite atomizer (THGA) with Zeeman background correction and a palladium nitrate matrix modifier (Nève *et al.*, 1987). Total serum selenium has been previously analysed by GFAAS with a detection limit of 1 ng/L (Jacobson and Lockitch, 1988; Krittaphol *et al.*, 2006).

### 2.4.1. Materials

A selenium standard solution containing sodium selenite and SRM 1568a were obtained as described in 2.3.1. A tissue SRM of bovine liver (SRM 1577a,  $8.99 \pm 0.89$  nmol selenium/g) was obtained from the National Institute of Standards and Technology, Gaithersburg, MD, USA. Two certified reference materials (CRM) for use in serum analysis were UTAK Reference Plasma (Cat #8634, 1.52 (1.14 - 1.90)  $\mu$ M, UTAK Laboratories Inc, Valencia, CA), and Seronorm (Lot #JL4409, 1.05 (0.97 - 1.13)  $\mu$ M, Seronorm<sup>TM</sup> Trace Element Serum, Laboratories of SERO AS, Billingstad, Norway). Pooled serum was obtained from the Trace Element Research Laboratory, University of Otago. Palladium nitrate was obtained from Sigma-Aldrich Inc., NZ. Other analytical reagents and solvents were obtained from Sigma-Aldrich and Merck, respectively.

### 2.4.2. Sample preparation

#### 2.4.2.1. Tissues

For digestion of samples, 0.3 g (wet weight) gut contents, liver or kidney were digested with 69% nitric acid initially at 50°C after which the temperature was slowly increased to 100°C until the solution was clear. The solution was cooled after which 1 mL perchloric acid was added carefully and then digested until the solution was again clear. The solution was then cooled and diluted with 20% hydrochloric acid and total selenium measured by HGAAS.

#### 2.4.2.2. Serum

Serum samples (20  $\mu$ L) were analysed directly without digestion by GFAAS based on a modification of the method of Jacobson and Lockitch (1988).

### **2.4.3. Assay validation**

#### **2.4.3.1. Tissues**

Selenium standards were prepared by diluting the sodium selenite standard solution in 20% HCl to concentrations of 0.032, 0.063 and 0.127  $\mu\text{M}$ . Replicate samples of previously-digested SRM 1568a and SRM 1577a were analysed ( $n = 15$ ) to determine the precision and accuracy of the method.

#### **2.4.3.2. Serum**

This determination employed a standard addition method. The sodium selenite standard solution was diluted to 3.80  $\mu\text{M}$  with diluents (containing 0.5% Triton X-100 and 6% glycerol in distilled deionised water) and further diluted into a matrix made up of 50% pooled serum to give final concentrations of 0.48, 0.95 and 1.52  $\mu\text{M}$ . A blank consisting of 50% pooled serum in diluents was also prepared. The software of the AA spectrometry automatically adjusts for matrix effects during the determination of the calibration curve. The three point calibration curve was linear  $R^2 > 0.99$  with a zero intercept. Accuracy and precision was assessed after each batch of ten samples using the two CRMs. Multiple aliquots of a pooled serum control were included with each analysis of samples to assess precision.

### **2.4.4. Results and Discussion**

GFAAS is commonly used to analyse small samples of serum ( $\sim 10 \mu\text{L}$ ) for selenium whereas HGAAS is commonly used for analysis after digestion and dilution of larger tissue samples ( $\sim 0.3 \text{ g}$ ). It is convenient to measure serum selenium directly by GFAAS as a digestion step is not needed and because tissue cannot be conveniently and accurately introduced into the graphite furnace.

#### 2.4.4.1. Tissues

The standard curve was linear for the three standards ( $y = 0.04165x$ ;  $r^2 = 0.9960$ ). The mean  $\pm$  SD (%RSD) for SRM 1568a and SRM 1577a were  $4.86 \pm 0.24$  nmol/g (4.9%) and  $9.44 \pm 0.32$  nmol/g (3.4%) compared with the certified values (mean  $\pm$  SD) of  $4.81 \pm 0.51$  nmol/g and  $8.99 \pm 0.89$  nmol/g, respectively.

#### 2.4.4.2. Serum

The standard curve was linear for the three standards ( $y = 0.00127x$ ;  $r^2 = 0.9997$ ). In determination of accuracy and precision, the means  $\pm$  SD (% RSD) were  $1.60 \pm 0.04$   $\mu$ M (2.5%) ( $n = 18$ ) for UTAK and  $1.01 \pm 0.02$   $\mu$ M (2.0%) ( $n = 18$ ) for Seronorm<sup>TM</sup>, compared to the corresponding certified means and expected ranges given by the manufacturers of 1.52 (1.14 – 1.90)  $\mu$ M and 1.05 (0.97 – 1.13)  $\mu$ M, respectively. The mean (SD) (% RSD) of total selenium for 18 aliquots of the pooled serum control ( $n = 18$ ) was  $1.25 \pm 0.06$   $\mu$ M (4.8 %) compared with the reference value of 1.14 - 1.38  $\mu$ M.

### 2.5. Conclusion

All the assays as described in this Chapter provided satisfactory results on validation indicating the assays are suitable for application in the research reported in this thesis. The HPLC and HGAAS assays were used for the quantitation of L-SeMet and selenite, respectively in *in vitro* and *ex vivo* studies. The GC-MS assay was used for the quantitation of volatile selenium metabolites in *in vitro*, *ex vivo* and *in vivo* studies. In the *in vivo* study, GFAAS was used to determine total selenium in serum whereas HGAAS was used for assay of total selenium in tissues.

## CHAPTER 3

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### Metabolism of L-selenomethionine and selenite by rat gut contents\*

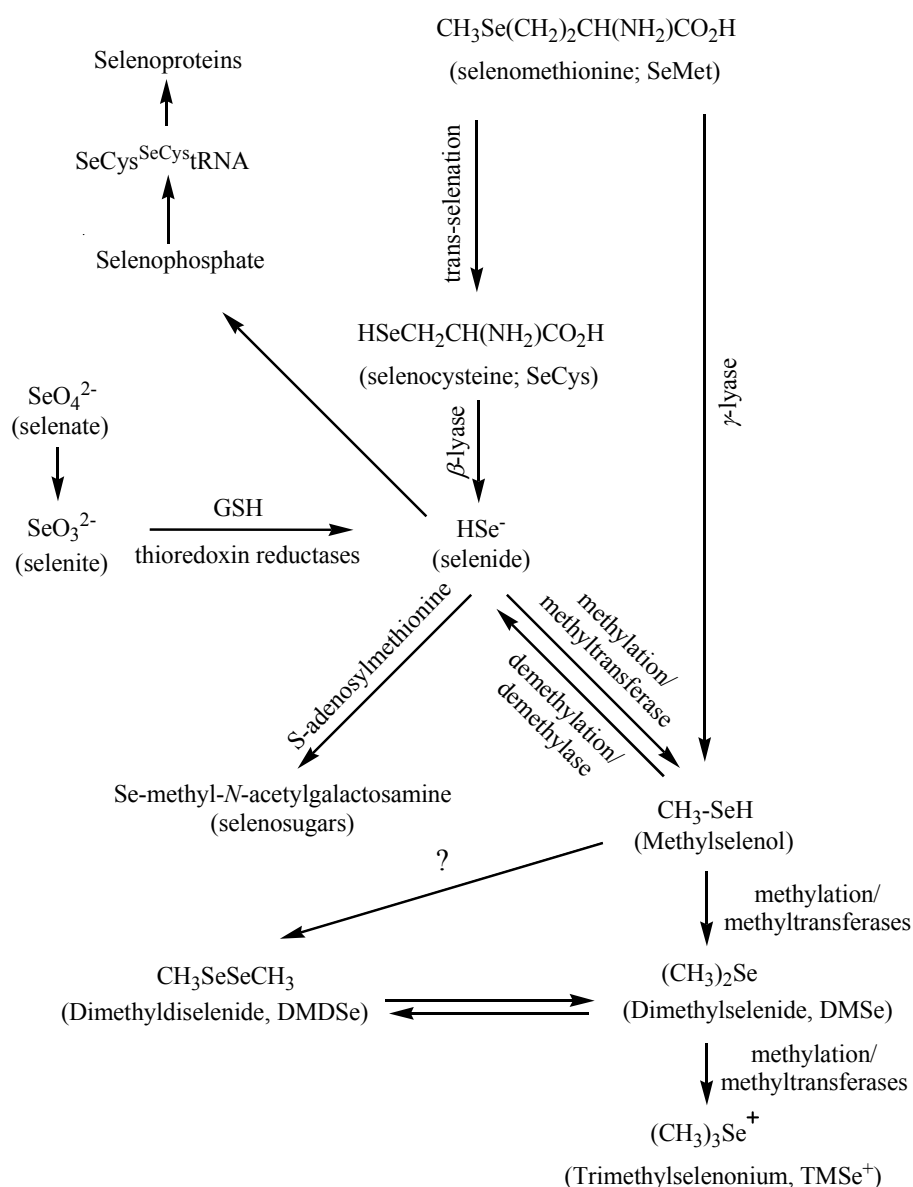
#### 3.1. Introduction

As described in Section 1.4, selenium metabolism in mammalian liver and kidney leads to the incorporation of selenium into Sels (Suzuki, 2005; Suzuki *et al.*, 2007). The main excretory routes for selenium are via the methylated selenium compounds, DMSe and TMS<sup>+</sup>, and selenosugars.

DMSe is the main excretion product in faeces and in the exhaled breath whereas TMS<sup>+</sup> and selenosugars are the main excretion products in urine. It has been shown that in bacteria, both organic and inorganic selenium compounds are converted to methylated derivatives via selenide (Suzuki, 2005) (Figure 3.1). L-SeMet is converted via methylselenol formed by  $\gamma$ -lyase (Suzuki *et al.*, 2007) or via SeCys formed by the trans-selenation pathway and then by  $\beta$ -lyase (Ohta and Suzuki, 2008). In contrast, selenite is transformed to selenide via reduction by glutathione and by TrxRs (Zakharyan *et al.*, 2005; Behne *et al.*, 2009). Selenide is subsequently methylated to methylselenol which undergoes further methylation to DMSe and TMS<sup>+</sup> by methyltransferases (Ohta and Suzuki, 2008).

As described in Section 1.8.1, several studies have shown that bacteria in soil and fresh water are able to degrade L-SeMet and selenite to DMSe and DMDS<sub>2</sub>Se (Dungan and Frankenberger Jr, 2001; Ranjard *et al.*, 2003) (Figure 3.1). Previous studies have also shown that bacteria can demethylate selenium compounds and

release hydrogen selenide (Doran and Alexander, 1977; Dungan and Frankenberger Jr, 2001). Some bacteria reduce selenite to elemental selenium (Oremland *et al.*, 2004) and one organism, *Bacillus selenitireducens*, can transform elemental selenium to hydrogen selenide (Herbel *et al.*, 2003). These various reactions are thought to serve as protective measures against selenium toxicity (Stolz *et al.*, 2006).



**Figure 3.1.** Selenium metabolic pathways in bacteria as indicated by previous studies (Doran and Alexander, 1977; Ranjard *et al.*, 2002; Suzuki, 2005; Suzuki *et al.*, 2007).

The ability of bacteria in the mammalian GIT to metabolise selenium is less well known. A previous study to investigate how selenium status affects Sel biosynthesis in conventional and germ-free mice indicated that bacteria may compete with the host for selenium when selenium status is low (Hrdina *et al.*, 2009). Another study of SeCys derivatives (Se-*p*-methoxybenzylselenocysteine and Se-methylselenocysteine) presented to gastrointestinal bacterial species showed that the derivatives were transformed by  $\beta$ -lyase to selenide and methylated to methylselenol (Schwiertz *et al.*, 2008). Whether methylselenol or other methylated selenium compounds are produced by the gut microbiota subsequent to the ingestion of selenium supplements has not been investigated.

The aim of the study reported in this Chapter was to examine the metabolism of L-SeMet and selenite in rat gut contents *ex vivo*. In particular, the formation of methylated selenium compounds, DMDS<sub>e</sub> and DMSe, as markers of a detoxification process by microorganisms was investigated. Incubations of relatively high concentrations of L-SeMet and selenite (~ 0.5 mM) with rat gut content suspensions were carried out to reflect the situation after ingestion of large doses of these selenium supplements. This concentration is high based on the estimated intestinal concentration produced by an oral dose of 2 mg selenium/kg in Wistar rats (Shiobara *et al.*, 1998). This was estimated based on a rat body weight of 300 g and an estimated weight of the rat gut of 10 g. A dose of 2 mg selenium/kg or 0.6 mg/300 g equates to a concentration of selenium in the gut contents of 60  $\mu$ g/g. The concentration of L-SeMet and selenite used in incubations is 100  $\mu$ g/mL (0.51 mM L-SeMet and 0.58 mM selenite) which contains not more than 60  $\mu$ g selenium. The concentrations of L-SeMet, selenite, DMSe and DMDS<sub>e</sub> were determined as described in Chapter 2.



## 3.2. Methods

### 3.2.1. Animals and gut content suspensions

The study protocol was approved by the University of Otago Committee on Ethics in the Care and Use of Laboratory Animals, Dunedin, New Zealand. Adult male Wistar rats (age 8-10 months, body weight 250-300 g) were sacrificed by asphyxiation with carbon dioxide and a midline incision made to access the gut. The whole intestinal tract was removed and placed in 0.9% ice-cold sterile saline solution. The contents of the jejunum, ileum, caecum and colon were expelled separately and maintained at 4°C during preparation. Gut contents were diluted with PBS to give 10% w/w suspensions, flushed with nitrogen and homogenised. Bacteria in suspensions were counted after serial 10-fold dilutions. Aliquots (1 mL) of  $10^{-2}$  -  $10^{-5}$  dilutions ( $n = 5$ ) were anaerobically incubated at 37°C for 48 h on sheep blood agar. The numbers of colonies on the plates were used to determine the number of colony-forming units per gram (cfu/g). The number of bacteria present in contents of caecum and colon was found to be  $10^{10}$  cfu/g compared to  $10^5$  cfu/g and  $10^9$  cfu/g in contents of jejunum and ileum, respectively (detailed data presented in Chapter 5, Table 5.1). Total protein content of 10% w/w suspensions ( $n = 5$ ) was determined using the Quick Start™ Bradford protein assay (Bio-Rad, California, USA) using bovine serum albumin (Sigma) as standard. Protein contents (g/L, mean  $\pm$  SD) were found to be; caecum  $0.573 \pm 0.050$ , colon  $0.536 \pm 0.087$ , jejunum  $0.230 \pm 0.025$ , and ileum  $0.245 \pm 0.057$ .

### 3.2.2. Incubation with gut content suspensions

L-SeMet (50  $\mu$ L, 5.10 mM) or selenite (50  $\mu$ L, 5.78 mM) was added to suspensions of gut contents (450  $\mu$ L), flushed with nitrogen and incubated anaerobically in sealed 600  $\mu$ L Eppendorf tubes at 37°C for 3 h. Incubations of solutions of L-SeMet (0.51 mM) and selenite (0.58 mM) in PBS served as

controls. Samples were removed at 0, 1, 2 and 3 h and stored at -20°C pending analysis for L-SeMet and selenite. Similar incubations were carried out in 2 mL PTFE/silicone sealed-glass vials and analysed for DMSe and DMDS<sub>e</sub> by GC-MS. To establish metabolic stability of DMSe and DMDS<sub>e</sub> under incubation conditions, DMSe (10 µL) or DMDS<sub>e</sub> (10 µL) were added to 990 µL 10% w/w caecum content suspensions to give concentrations of 129 and 106 mM, respectively and incubated as before.

### 3.2.3. Statistical analyses

Statistical analysis was performed using SPSS 15.0 for Windows. Unpaired two-tailed Student's t-test was used to test differences in protein content in different gut segments. A linear mixed model was used to compare L-SeMet, selenite DMSe and DMDS<sub>e</sub> concentrations over time and between suspensions of the contents of jejunum, ileum, caecum and colon. The components of the linear mixed model were rat (n) x segment (n) x time (n); segment and time are fixed factors and rat is a random factor. To examine relationships between concentrations of L-SeMet and DMDS<sub>e</sub> and selenite and DMSe, linear regression analysis was performed. A *p* value < 0.05 was considered statistically significant. Statistical tests showed that the data produced were normally distributed.

## 3.3. Results

### 3.3.1. L-SeMet incubations

No significant metabolism of L-SeMet was observed during incubation in PBS. Incubations with gut content suspensions over 3 h resulted in 30% loss in caecum, 15% loss in colon and little or no metabolism in jejunum and ileum (Figure 3.2). DMDS<sub>e</sub> but not DMSe was detected. High DMDS<sub>e</sub> concentrations were produced in contents of caecum ( $22.1 \pm 3.3$  µM) and colon ( $19.2 \pm 2.8$  µM) compared to

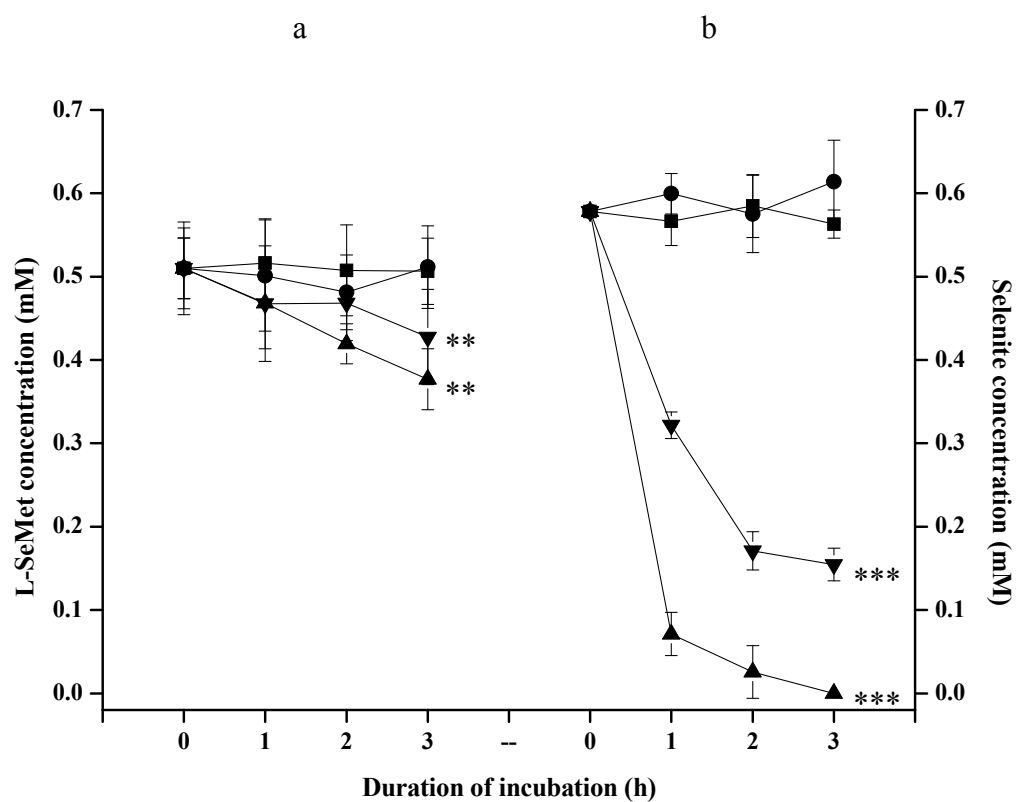
jejunum ( $6.2 \pm 1.3 \mu\text{M}$ ) and ileum ( $4.4 \pm 1.9 \mu\text{M}$ ) (Figure 3.3). The DMDSe concentration in caecum contents at 3 h corresponded to  $8.7 \pm 1.3\%$  of the amount of L-SeMet added (based on the reaction  $2\text{L-SeMet} \rightarrow \text{DMDSe}$ ) and  $28.9\%$  of the amount of L-SeMet reacted. Significant linear relationships were found between L-SeMet concentration and DMDSe concentration in suspensions of caecum ( $R^2 = 0.83$ ,  $p < 0.0001$ ) and colon ( $R^2 = 0.41$ ,  $p < 0.01$ ) contents, but not in those of jejunum and ileum contents. The slopes of the linear relationships in caecum and colon were  $-0.1281$  and  $-0.0903$  consistent with the production of other metabolites in parallel reactions or the conversion of DMDSe to other compounds.

### 3.3.2. Selenite incubations

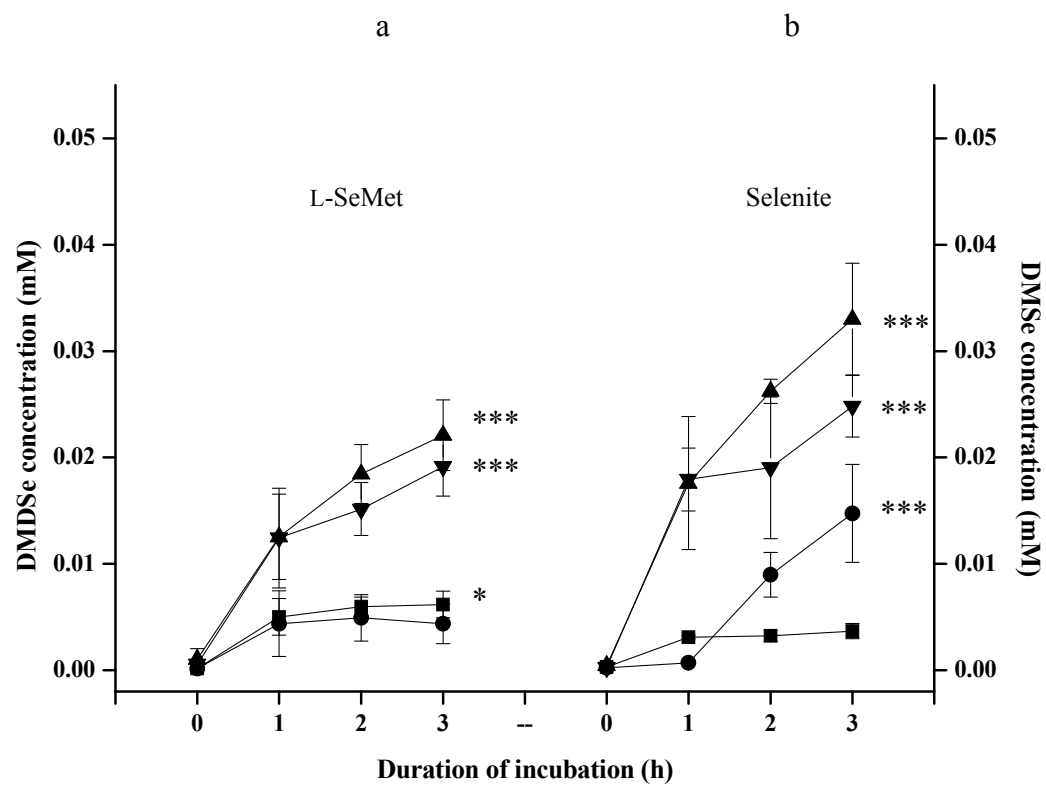
No significant metabolism of selenite was observed during incubation in PBS. Incubations with gut content suspensions over 3 h resulted in 100% loss in caecum, 73% loss in colon and little or no metabolism in jejunum and ileum (Figure 3.2). Metabolism of selenite was accompanied by the formation of a red precipitate previously shown to be amorphous elemental selenium (Oremland *et al.*, 2004), most notably in caecum content suspensions. DMSe was also produced in the order caecum > colon > ileum  $\approx$  jejunum (Figure 3.3) but there were no significant correlations between concentrations of selenite and DMSe in any of the gut content suspensions. The increase in DMSe in suspensions of caecum contents over 3 h corresponded to  $5.7 \pm 0.9\%$  of the amount of selenite added.

### 3.3.3. Incubations of DMSe and DMDSe

Incubation of DMDSe with 10% w/w suspensions of caecum contents produced DMSe (13% of DMDSe added), whereas incubation of DMSe produced unknown products (28% of DMSe added).



**Figure 3.2.** Changes in the concentration of (a) L-selenomethionine (L-SeMet) and (b) selenite during anaerobic incubations of L-SeMet (0.51 mM) and selenite (0.58 mM) at 37°C over 3 h with 10% w/w suspensions of the contents of rat jejunum —■—; ileum —●—; caecum —▲— and colon —▼—. Data are mean  $\pm$  SD, ( $n = 5$ ). \*\*  $p < 0.001$ , \*\*\*  $p < 0.0001$ , 3 h vs 0 h.



**Figure 3.3.** Formation of (a) dimethyldiselenide (DMDSe) and (b) dimethylselenide (DMSe) during anaerobic incubations of L-SeMet (0.51 mM) and selenite (0.58 mM), respectively at 37°C over 3 h with 10% w/w suspensions of the contents of rat jejunum —■—; ileum —●—; caecum —▲— and colon —▼—. Data are mean  $\pm$  SD, ( $n = 5$ ). \*  $p < 0.05$ , \*\*\*  $p < 0.0001$ , 3 h vs 0 h.

### 3.4. Discussion

The present study of the metabolism of L-SeMet and selenite by gut content suspensions from four segments of the rat intestinal tract *ex vivo* shows that both compounds are metabolised to the greatest extent in suspensions of caecum contents. Bacterial counts and protein analysis showed that the number of cells and the protein concentrations in suspensions of caecum and colon contents were similar indicating the microbiota in rat caecum are inherently more metabolically active towards L-SeMet and selenite than the microbiota in colon. Given the much greater relative size of the colon in humans, it is likely that metabolism of selenium compounds in the human gut would occur mainly in the colon.

Several studies have described selenium metabolic pathways *in vivo* (Suzuki, 2005) and in environmental samples such as soil (Doran and Alexander, 1977) and fresh water (Ranjard *et al.*, 2003). These studies show that DMSe and DMDS<sub>e</sub> can both be produced from L-SeMet and selenite via selenide and methylselenol. DMSe is the main product of selenite biotransformation whereas DMDS<sub>e</sub> is the main product of L-SeMet biotransformation (Doran and Alexander, 1977; Ranjard *et al.*, 2002) as found in our study. Interestingly, Dungan and Frankenberger Jr (1999; 2001) showed that the production of DMDS<sub>e</sub> or DMSe from selenite by bacterial sewage sludge is dependent on the concentration of selenite, a high concentration (5.78 mM) producing DMDS<sub>e</sub> and a low concentration (0.58 mM) producing DMSe as the predominant product. The low concentration used by Dungan and Frankenberger Jr was similar to that used here and indicates our results are consistent for selenite.

It is generally considered that all nutritional selenium sources are transformed via selenide and methylselenol to DMSe and TMSe<sup>+</sup> (Ohta and Suzuki, 2008). L-SeMet is known to produce DMDS<sub>e</sub> in a hydride generation system using sodium borohydride and hydrochloric acid where it was suggested to form via rapid oxidative dimerisation of methylselenol (Chatterjee *et al.*, 2001). Presumably the

same dimerisation of methylselenol occurs in bacteria after its production from selenide via the action of  $\beta$ -lyase on SeCys or via the direct action of  $\gamma$ -lyase on L-SeMet. The former activity is commonly found in bacteria in the human intestinal tract (Schwiertz *et al.*, 2008) and the latter seems to occur at high concentrations of L-SeMet (Okuno *et al.*, 2001; Okuno *et al.*, 2005). Whatever the case, there is ample evidence that bacteria (Doran and Alexander, 1977; Dungan and Frankenberger Jr, 2001; Ranjard *et al.*, 2002; Ranjard *et al.*, 2003; Stolz *et al.*, 2006) and yeast (Dietz *et al.*, 2004) can convert L-SeMet to DMDS<sub>e</sub> and our study provides the first evidence that DMDS<sub>e</sub> is formed by gut microbiota in the intestinal tract.

In the present study, incubation of DMDS<sub>e</sub> with suspensions of caecum contents produced some DMSe, whereas incubation of DMSe produced unknown products. The fact that DMSe was not produced from DMDS<sub>e</sub> during L-SeMet incubations is presumably due to the low concentration of DMDS<sub>e</sub> produced over 3 h. In the case of selenite, the production of DMSe was low due to its subsequent conversion to other products including insoluble elemental selenium. The fact that no elemental selenium was observed during incubation of DMDS<sub>e</sub> with gut content suspensions is surprising given that the reaction would be expected to involve  $\text{DMDS}_e \rightarrow \text{DMSe} + \text{Se}^\circ$ .

In conclusion, the results of this study show that metabolism of L-SeMet and selenite in rat gut contents occurs in a manner similar to that observed in various microorganisms *in vitro*. The formation of volatile methylated selenium compounds and elemental selenium in the intestinal tract suggests that the gut microbiota play an important role in protecting the host from toxicity due to high doses of selenium supplements.

## CHAPTER 4

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### Metabolism of L-selenomethionine and selenite by probiotic bacteria and rat gut contents \*

#### 4.1. Introduction

In Chapter 3 it was found that the metabolism of L-SeMet and selenite in rat gut contents was similar to that in microbial cultures (Dungan and Frankenberger Jr, 2001; Ranjard *et al.*, 2003). Products of L-SeMet and selenite metabolism included the volatile compounds, DMSe and DMDS<sub>e</sub> and, in the case of selenite, elemental selenium as found in previous reports (Ranjard *et al.*, 2002; Ranjard *et al.*, 2003; Oremland *et al.*, 2004). Based on the findings reported in Chapter 3, it appears that gut microorganisms may play an important role in protecting the host from toxicity after high intake of selenium supplements. Probiotics can potentially alter selenium metabolism in the gut but little information is available to corroborate this. In this Chapter, the ability of probiotics to metabolise L-SeMet and selenite *in vitro* and their effect on the metabolism of L-SeMet and selenite by rat gut contents *ex vivo* was investigated.

As stated in Section 1.9, probiotics are microorganisms that can confer a health benefit on the host (Dunne *et al.*, 1999; Maldonado Galdeano and Perdigon, 2006; Jia *et al.*, 2008). The present study focuses on probiotic bacteria of the genera *Lactobacillus*, *Bifidobacterium* and *Streptococcus*. The specific strains selected have been previously used in laboratory animal and human studies to investigate (a) the formation of short chain fatty acids in faeces, (b) the determinants

\*Krittaphol *et al.* (2011) *Biological Trace Element Research* DOI 10.1007/s12011-011-9057-2  
(Epub ahead of print)

\*Krittaphol *et al.* (2009) *Annals of Nutrition and Metabolism* 55 (suppl 1): 127 (abstract)

\*Krittaphol *et al.* (2008) 13<sup>th</sup> TEMA conference, Puñon, Chile



of oral malodour and (c) the effect of different levels of probiotic dosing on the intestinal microbiota. Little information is available relating to the effect of probiotics on selenium metabolism (Johansson *et al.*, 1998; Burton *et al.*, 2006a; Ahmed *et al.*, 2007).

Lactobacilli are gram-positive, non-spore forming rods that are catalase negative, usually non-motile and unable to reduce nitrate. *Lactobacillus* species are dominant in the gut and are commonly found in human (Wold, 1999; Fooks and Gibson, 2002). The potential health benefits of ingesting lactobacilli include the maintenance and restoration of normal intestinal balance and improvement of milk product digestibility (Fotiadis *et al.*, 2008). Lactobacilli are also involved in immunomodulation (enhancing the immune response to pathogens), reducing serum cholesterol, enhancing the absorption of calcium and producing B vitamins (Collins and Gibson, 1999; Schrezenmeir and de Vrese, 2001).

Bifidobacteria are gram-positive, non-spore forming rods which are major inhabitants of the human intestinal tract (Moore and Moore, 1995; Gibson and Collins, 1999). Bifidobacteria are commonly used as probiotics in dairy products (i.e. yoghurt and cheese) and are important in the maintenance of intestinal homeostasis as well as having the potential to enhance the immune system in both animals and humans (Prasad *et al.*, 1998; Arunachalam *et al.*, 2000; Gill *et al.*, 2001).

*Streptococcus salivarius*, a gram-positive, chain-forming coccus, is an inhabitant of the human upper respiratory tract. When used as a probiotic, this species can help displace more harmful bacteria and thereby improve the health of the oral cavity (Burton *et al.*, 2006b). In addition, *Streptococcus salivarius* K12 has been found to inhibit inflammatory responses in human bronchial epithelial cells (Cosseau *et al.*, 2008). *S. salivarius* K12 was used in this study because of its availability as a commercial probiotic product in New Zealand.

Both single strains and combinations of strains have been used in probiotic studies (Johansson *et al.*, 1998; Yesovitch *et al.*, 2004; Burton *et al.*, 2006a; Ahmed *et al.*, 2007; Larkin *et al.*, 2007). From these studies, it was found that treatment with a combination of probiotics provides improved health benefits compared to single probiotics (Matsumoto and Benno, 2004; Mohan *et al.*, 2006; De Preter *et al.*, 2007).

Individually, selenium and probiotics play a role in immune function and prevent some forms of cancer (Ryan-Harshman and Aldoori, 2005; Fotiadis *et al.*, 2008). The combination of these two supplements may involve a synergistic enhancement of immune response. Selenium is the first trace element to be studied in conjunction with probiotics (Mazo *et al.*, 2007). Probiotic bacteria with intracellular accumulated selenium have been used as a delivery system to improve selenium status. This selenium can then be transformed to selenoenzymes containing SeCys and/or L-SeMet, which increase the overall selenium content of the bacterial cell proteins (Calomme *et al.*, 1995a; 1995b).

A few studies have used selenium-enriched probiotics to improve health benefits. Krizkova *et al.* (2002) demonstrated by the use of the *Euglena* assay (an antimutagenic test) that the increased antimutagenic activity of *Enterococcus faecium* M-74 in the presence of a selenium-based medium provided protection against the genotoxicity of ofloxacin and acridine orange. In a rat study, Rovenský *et al.* (2002) showed that organic selenium-enriched *E. faecium* M-74 in combination with methotrexate reduced the symptoms in a rat model of rheumatoid arthritis. The results of this study suggest selenium-enriched probiotics may be a useful adjunct in the treatment of rheumatoid arthritis.

Even though details of the mechanisms of probiotic action remain unclear, it is possible that a combination of probiotics and a selenium supplement produces synergistic health benefits. This is based on the fact that probiotics and selenium have roles in immune function and may act as anticarcinogens. The aims of the

research reported in this Chapter were to investigate the ability of some probiotic strains of human origin to metabolise L-SeMet and selenite and the influence of these strains on the metabolism of L-SeMet and selenite in rat gut contents *ex vivo*. A selection of two strains of each of four species was first investigated after which one strain of each species was selected for incorporation into a mixture for further studies. Antibiotic-resistant strains were compared with wild type strains to ensure that their behaviour was similar to the wild type strains. These antibiotic-resistant probiotics were subsequently used in an *in vivo* study (Chapter 5) in which the viability of probiotics in the GIT after oral dosing was assessed.

## **4.2. Materials and methods**

### **4.2.1. Materials**

Streptomycin, spectinomycin and rifamicin were obtained from Sigma-Aldrich, Inc., New Zealand. De Man Rogosa Sharpe (MRS) agar and culture medium, Difco Columbia Agar Base (CAB) agar and Rogosa SL agar were from BD Biosciences, UK. Other analytical solvents and reagents were mainly from Merck and Sigma-Aldrich, respectively. Eight probiotic strains, two strains of each of four species, were provided by BLIS Technologies Ltd, New Zealand: *Streptococcus salivarius* strains K12 and Mia, *Lactobacillus rhamnosus* strains 67B and L2H, *Lactobacillus acidophilus* strains L10 and La742 and *Bifidobacterium lactis* strains LAFTI<sup>®</sup> B94 and Danisco<sup>®</sup> HN019.

### **4.2.2. Animals and gut contents**

The study protocol is described in Chapter 3. The contents of the jejunum, ileum, caecum and colon were expelled separately and maintained at 4°C during preparation. Gut contents were diluted with PBS, flushed with nitrogen gas and homogenised to give 10% w/w suspensions.

### 4.2.3. Incubation with individual probiotic strains

Probiotic *S. salivarius* strains K12 and Mia, *L. rhamnosus* strains 67B and L2H, *L. acidophilus* strains L10 and La742 and *B. lactis* strains LAFTI<sup>®</sup> B94 and Danisco<sup>®</sup> HN019 grown in MRS cultures (500 mL) were harvested by centrifugation (3100 x g) at 4°C for 15 min and resuspended in PBS [(1-5)x10<sup>10</sup> cfu/mL]. After counting, each suspension of the eight probiotic strains were incubated with L-SeMet and selenite in the same way as described in Section 3.2.2. L-SeMet, selenite, DMSe and DMDSe were analysed as described in Chapter 2.

### 4.2.4. Preparation of antibiotic-resistant probiotic strains

Antibiotic-resistant probiotic strains were prepared by serial subculture of the parental strains on antibiotic-containing agar as follows: *S. salivarius* K12 on MRS agar supplemented with 500 µg/mL streptomycin and 500 µg/mL spectinomycin; *L. rhamnosus* 67B on Rogosa SL agar supplemented with 1000 µg/mL spectinomycin; *L. acidophilus* L10 on Rogosa SL agar supplemented with 250 µg/mL rifampicin and *B. lactis* LAFTI<sup>®</sup> B94 on MRS agar supplemented with 125 µg/mL rifampicin. Colonies of antibiotic-resistant *S. salivarius* K12, *L. rhamnosus* 67B and *L. acidophilus* L10 were then grown in 100 mL MRS broth for 18 h at 37°C under 5% carbon dioxide in air. *Bifidobacterium lactis* LAFTI<sup>®</sup> B94 was grown for 2 days at 37°C under an anaerobic atmosphere. The MRS cultures (100 mL) were used to inoculate second batches of MRS broth to produce the final cultures. These MRS cultures were used in incubations described in Sections 4.2.5, 4.2.6 and 5.2.2. This method was used to ensure there were sufficient inoculums of cells and that the cultures reached the stationary phase of the cell cycle before harvesting. A bacterial count of the fresh cultures was performed by diluting 1 mL aliquots of suspensions serially (10-fold) in PBS and plating samples of the 10<sup>-2</sup>-10<sup>-5</sup> dilutions on appropriate antibiotic-supplemented

agar (as described above). The number of colonies in the probiotic suspensions was generally in the range  $10^9$ - $10^{10}$  cfu/mL.

#### **4.2.5. Incubation with antibiotic-resistant probiotic strains**

Antibiotic-resistant probiotic *S. salivarius* strain K12, *L. rhamnosus* strain 67B, *L. acidophilus* strain L10 and *B. lactis* strain LAFTI<sup>®</sup> B94 grown in MRS cultures (500 mL) were harvested and resuspended as described in Section 4.2.3. After counting, each suspension of the four probiotic strains was incubated with L-SeMet and selenite in the same way as described in Section 3.2.2 except the duration of incubation was extended to 24 h. Parallel incubations were carried out with wild type probiotic strains. Control incubations were carried out in PBS. L-SeMet, selenite, DMSe and DMDS<sub>2</sub>Se were analysed as described in Chapter 2.

#### **4.2.6. Incubation with mixtures of probiotics and gut contents**

Probiotic cells grown in MRS cultures (500 mL) were harvested and resuspended in PBS as described in Section 4.2.3. After counting, suspensions of the four probiotic strains were mixed in appropriate volumes to give 10 mL of a suspension (ca.  $3 \times 10^{10}$  cfu/mL) containing equal numbers of each strain. Rat gut contents (1 g wet weight) were added to probiotic suspensions to give final 10% w/w suspensions of gut contents containing probiotics (ca.  $10^{10}$  cfu/mL). Samples were flushed with nitrogen gas and homogenized immediately prior to incubation.

Incubations of L-SeMet and selenite with gut contents suspensions or mixtures of suspensions and probiotic cells were carried out in the same way as described in Section 3.2.2 except the duration of the incubation was extended to 24 h. All experiments were carried out in triplicate or quintuplicate. Controls were performed by incubating either L-SeMet, selenite, DMSe or DMDS<sub>2</sub>Se in PBS under the same conditions. L-SeMet, selenite and volatile selenium metabolites were analysed as described in Chapter 2.

#### 4.2.7. Statistical analysis

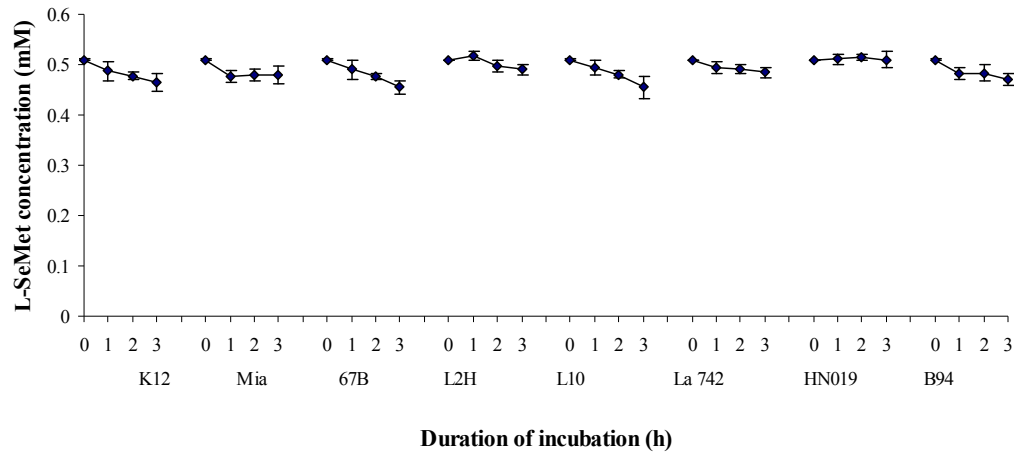
Statistical analysis of experimental data was performed using SPSS 15.0 for Windows. A linear mixed model was used to compare L-SeMet, selenite, DMSe or DMDSe concentrations in the presence and absence of probiotics over time, between probiotic strains and between gut content suspensions prepared from different gut segments. The Student's two-tailed t-test for unpaired data was used to test differences in concentration at a given time. Statistical significance was set at  $p < 0.05$ .

### 4.3. Results

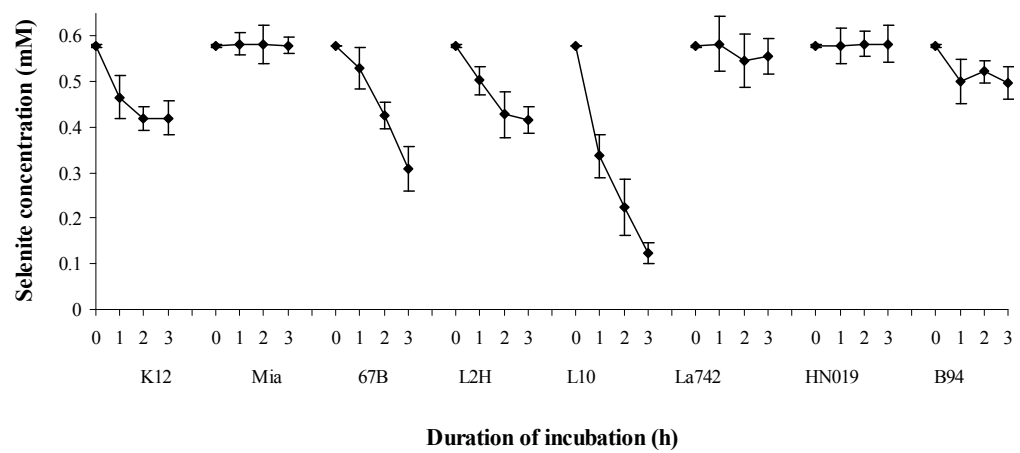
#### 4.3.1. Incubation with individual probiotic strains

After incubation of *S. salivarius* strains K12 and Mia, *L. rhamnosus* strains 67B and L2H, *L. acidophilus* strains L10 and La742 and *B. lactis* strains LAFTI<sup>®</sup> B94 and Danisco<sup>®</sup> HN019 with either L-SeMet (0.51 mM) or selenite (0.58 mM) at 37°C for 3 h, the metabolism of selenite was found to be greater than that of L-SeMet for all strains except *S. salivarius* Mia and *B. lactis* Danisco<sup>®</sup> HN019 (Figure 4.1). Comparison between the two strains of each species showed that the two selenium compounds were most effectively metabolised by *L. acidophilus* L10, *L. rhamnosus* 67B, *S. salivarius* K12 and *B. lactis* LAFTI<sup>®</sup> B94. These four strains were selected for further investigation.

a



b



**Figure 4.1.** Changes in concentration of (a) L-SeMet (initially 0.51 mM) and (b) selenite (initially 0.58 mM) in anaerobic incubations with *S. salivarius* K12 (K12) and Mia (Mia), *L. rhamnosus* 67B (67B) and L2H (L2H), *L. acidophilus* L10 (L10) and La742 (La742) and *B. lactis* Danisco® HN019 (HN019) and LAFTI® B94 (B94) over 3 h at 37°C. Data are mean  $\pm$  SD, ( $n = 3$ ).

### 4.3.2. Incubation with antibiotic-resistant probiotic strains

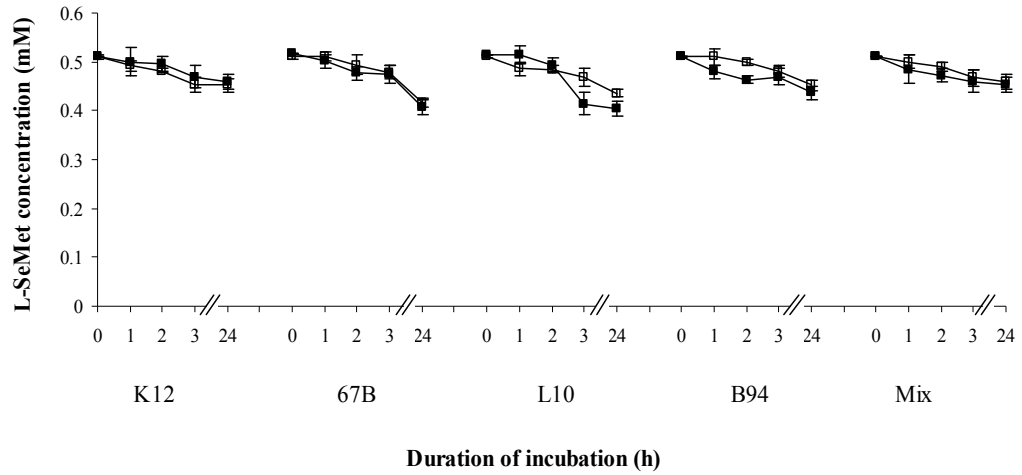
In control incubations of L-SeMet or selenite in PBS at 37°C for 24 h, concentrations were found to be stable (data not shown). The antibiotic-resistant strains were found to metabolise L-SeMet and selenite to a similar extent as the corresponding wild type strains (Figure 4.2). In incubations with the individual antibiotic-resistant strains, L-SeMet was metabolised more by *L. rhamnosus* 67B (18%) and *L. acidophilus* L10 (15%) and less by *S. salivarius* K12 and *B. lactis* LAFTI® B94 (~11%) (Figure 4.2a). Selenite metabolism by the lactobacilli strains was complete (*L. acidophilus* L10) or almost complete (*L. rhamnosus* 67B) but only 26% and 47% of selenite was metabolised by *S. salivarius* K12 and *B. lactis* LAFTI® B94, respectively (Figure 4.2b). Metabolism by the mixture corresponded to an average of the extent of metabolism by the individual strains. The mixture of probiotics metabolised 10% of L-SeMet (Figure 4.2a) and 100% of selenite (Figure 4.2b) at 24 h.

In control incubations of DMDSe or DMSe in PBS at 37°C for 24 h, concentrations were unchanged (data not shown). The concentrations of DMDSe and DMSe formed from L-SeMet and selenite in incubations with the individual antibiotic-resistant strains are shown in Figure 4.3. DMDSe was formed exclusively from L-SeMet for the first 3 h of incubation after which some DMSe was also formed by each of the probiotic strains except *B. lactis* LAFTI® B94 (Figure 4.3a). The formation of DMDSe from L-SeMet at 24 h by individual probiotic strains corresponded to 18 - 46% of the L-SeMet lost. The total concentrations of DMDSe and DMSe produced from L-SeMet by *S. salivarius* K12, *L. rhamnosus* 67B, *L. acidophilus* L10 and *B. lactis* LAFTI® B94 and the mixture of the four strains at 24 h corresponded to approximately 80, 44, 47, 46 and 36% of the L-SeMet lost, respectively. In incubations of L-SeMet with the mixture of probiotics, only DMDSe was detected (Figure 4.3a).

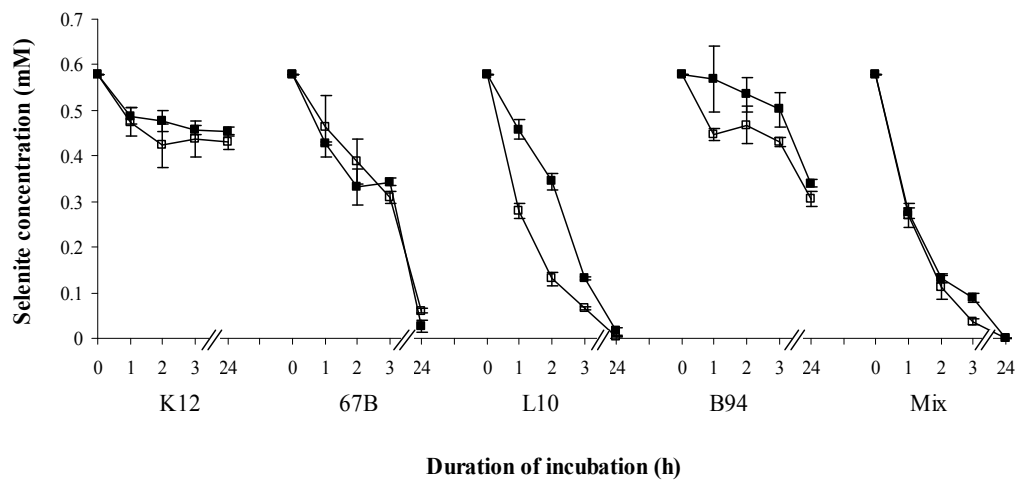


The metabolism of selenite by the probiotic strains produced mainly DMSe (0-4.8% of selenite lost at 24 h) except in the case of *B. lactis* LAFTI<sup>®</sup> B94 where only DMDSe was detected (Figure 4.3b). Some DMDSe was also detected at times up to 3 h for *L. acidophilus* L10 and *L. rhamnosus* 67B (Figure 4.3b). The formation of both DMDSe and DMSe was irregular and not consistent with the relative amounts of selenite metabolised (Figure 4.2b). It was accompanied by the formation of a red precipitate of elemental selenium, most notably in the case of the two lactobacilli strains. In incubations of selenite with the mixture of probiotics, only DMSe was produced with the level at 24 h accounting for 1% of selenite.

a

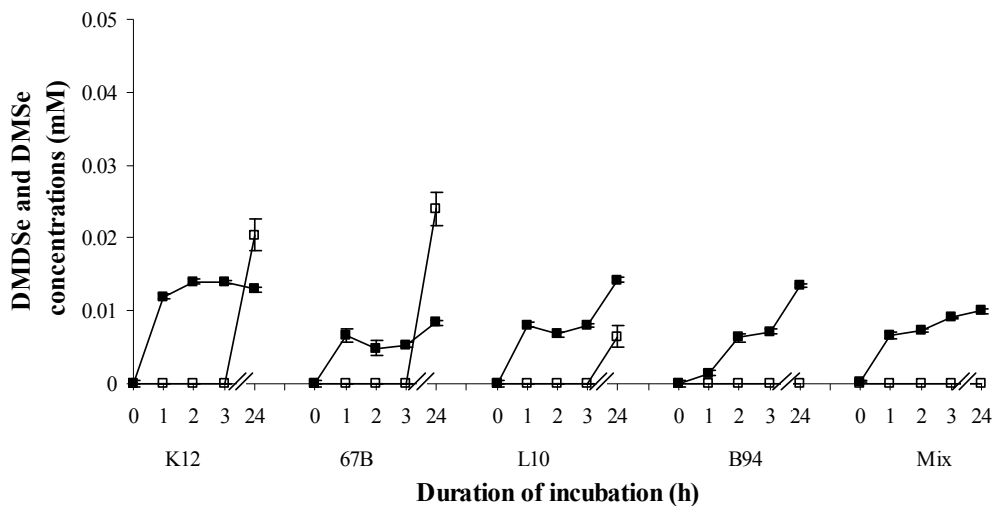


b

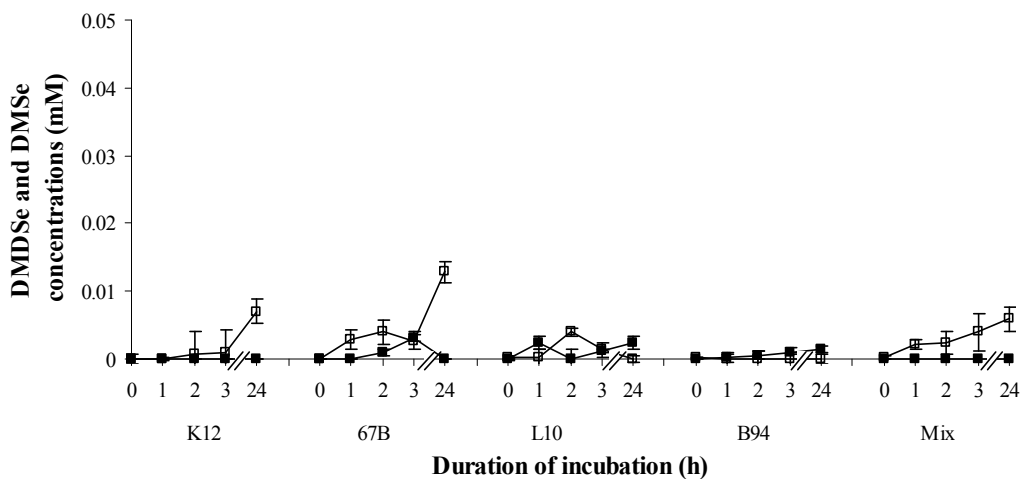


**Figure 4.2.** Changes in concentration of (a) L-SeMet (initially 0.51 mM) and (b) selenite (initially 0.58 mM) in anaerobic incubations at 37°C for 24 h with wild type —■— or antibiotic-resistant strains —□— of *S. salivarius* K12 (K12), *L. rhamnosus* 67B (67B), *L. acidophilus* L10 (L10), *B. lactis* LAFTI® B94 (B94) ( $n = 3$ ) and a mixture (Mix) ( $n = 5$ ) of the four probiotic strains. Data are mean  $\pm$  SD.

a



b



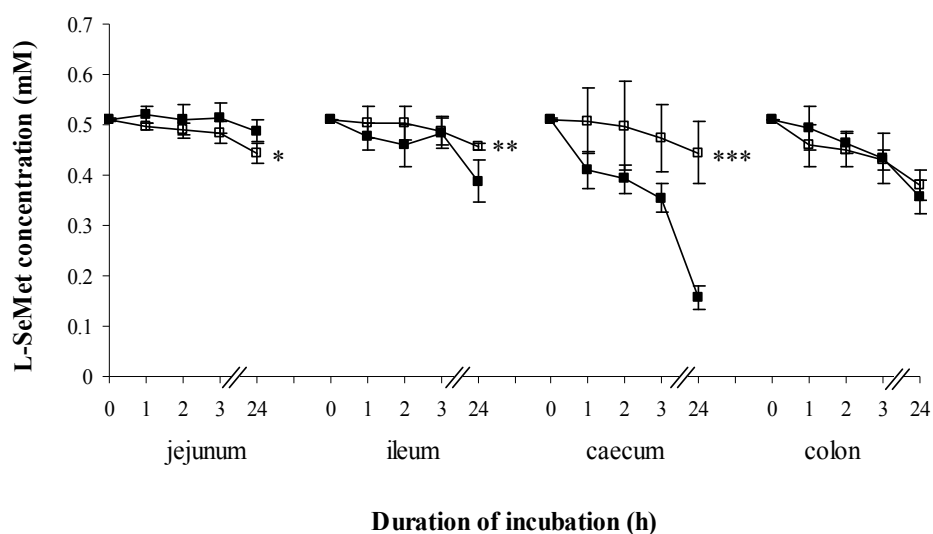
**Figure 4.3.** Formation of DMDSe —■— and DMSe —□— during anaerobic incubations at 37°C over 24 h of (a) L-SeMet (0.51 mM) and (b) selenite (0.58 mM) with individual antibiotic-resistant strains of *S. salivarius* K12 (K12), *L. rhamnosus* 67B (67B), *L. acidophilus* L10 (L10), *B. lactis* LAFTI® B94 (B94) ( $n = 3$ ) and a mixture (Mix) ( $n = 5$ ) of the four probiotic strains. Data are mean  $\pm$  SD.

### 4.3.3. Incubation with gut content suspensions and probiotic strains

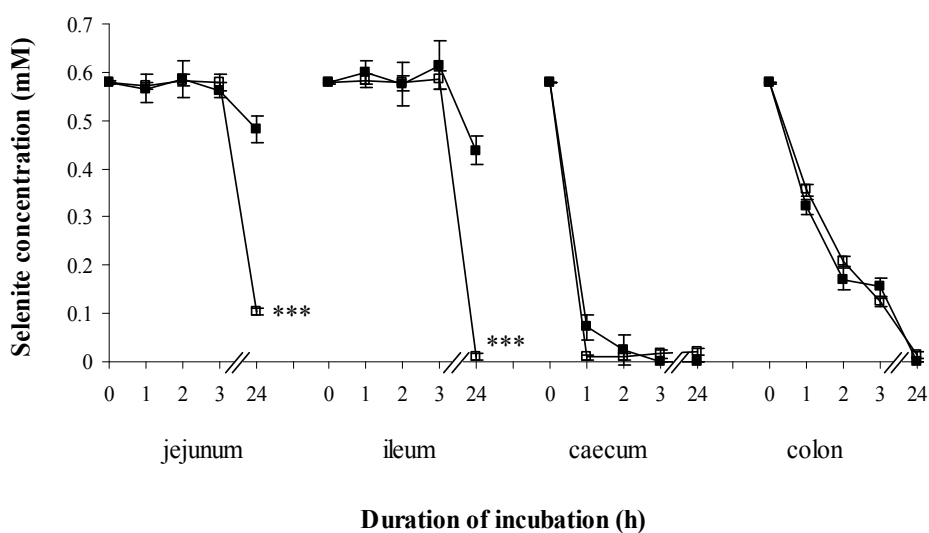
When incubated with gut content suspensions for 24 h, L-SeMet was metabolised to the greatest extent by caecum contents (70%) (Figure 4.4) as found previously over 3 h (Figure 3.2). Selenite was metabolised 100% by both caecum and colon contents at 24 h (Figure 4.5). This is consistent with the results for caecum contents at 3 h but more extensive in colon contents than predicted by the 3 h data (Figure 3.2).

The metabolism of L-SeMet in incubations with a combination of probiotics and gut content suspensions from the different gut segments appeared to be less for ileum and caecum contents than in the corresponding gut contents alone (Figure 4.4). In contrast, the metabolism of selenite in incubations with the combination of probiotics and gut contents at 24 h was significantly enhanced for jejunum (84% vs. 16%;  $p < 0.0001$ ) and ileum (100% vs. 24%;  $p < 0.0001$ ) (Figure 4.5). Selenite metabolism in the mixture of probiotics and gut contents was again accompanied by the formation of a red precipitate of elemental selenium particularly in incubations with caecum and colon contents.

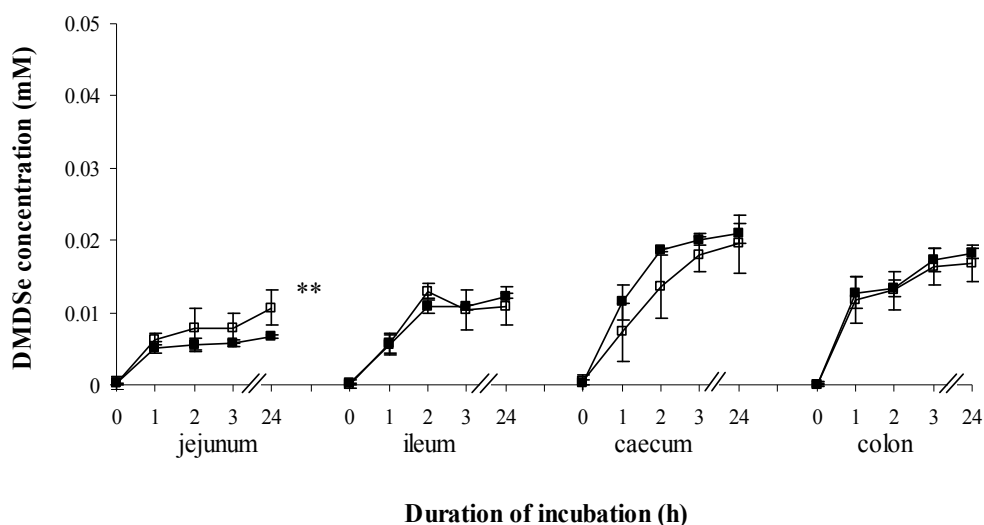
In regard to the formation of volatile selenium metabolites, the combination of probiotics with jejunum contents produced significantly more DMDS<sub>2</sub> from L-SeMet at 24 h than in the corresponding gut contents alone (Figure 4.6) consistent with the significantly greater loss of L-SeMet in the combination (Figure 4.4). The greater formation of DMDS<sub>2</sub> in caecum contents alone (Figure 4.6), although not significantly different, is also consistent with the significantly higher loss of L-SeMet in caecum contents alone (Figure 4.4). No DMSe was detected following incubation of L-SeMet with gut content suspensions either in the presence or absence of probiotics.



**Figure 4.4.** Changes in concentration of L-SeMet (initially 0.51 mM) in anaerobic incubations at 37°C over 24 h with 10% w/w suspensions of the contents of rat jejunum, ileum, caecum and colon in the presence —□— or absence —■— of probiotics. Data are mean  $\pm$  SD, ( $n = 5$ ). \*( $p < 0.05$ ), \*\*( $p < 0.01$ ), \*\*\*( $p < 0.001$ ) for differences in L-SeMet concentration between gut contents in the presence and absence of probiotics at 24 h.

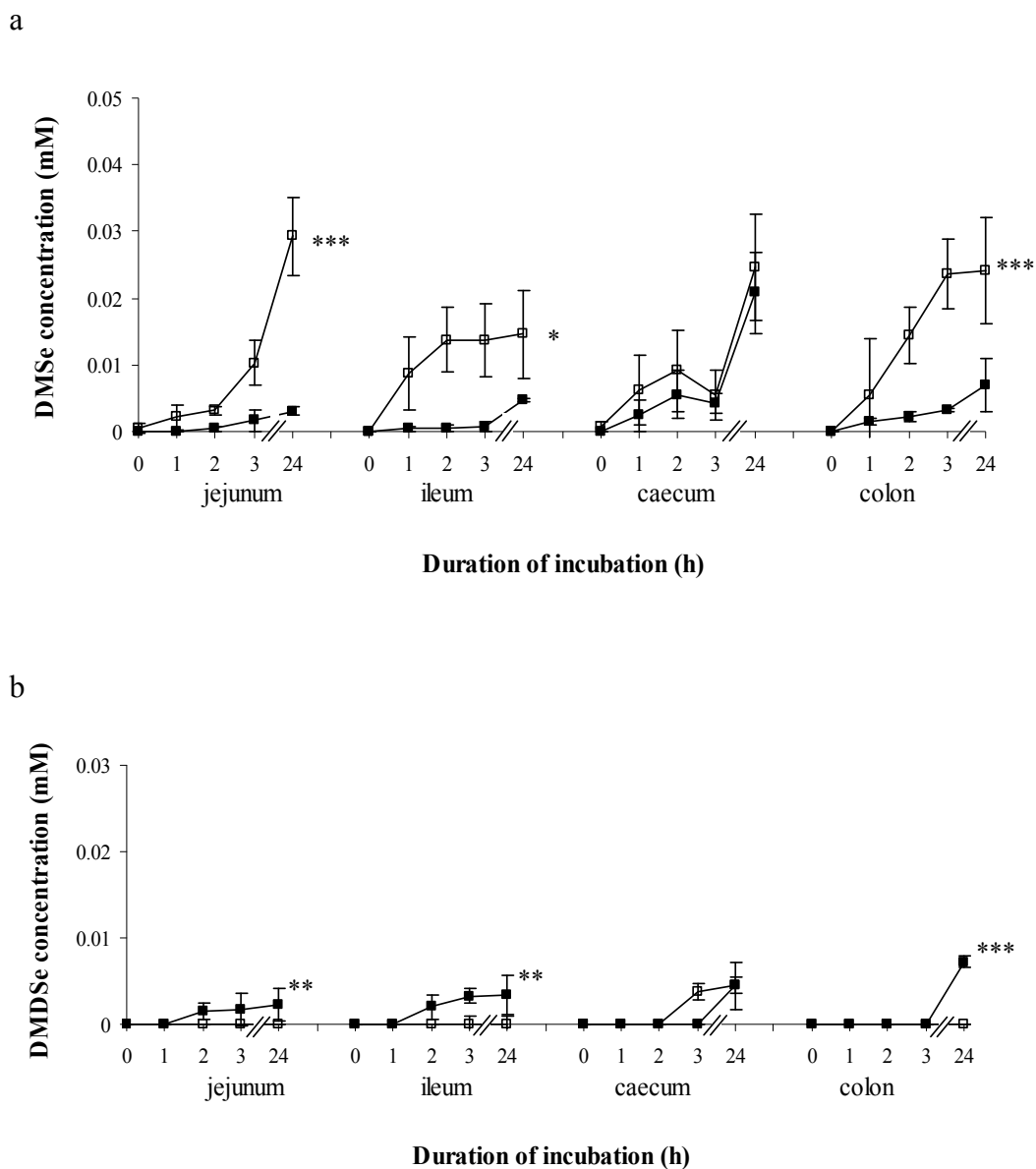


**Figure 4.5.** Changes in concentration of selenite (initially 0.58 mM) in anaerobic incubations at 37°C over 24 h with 10% w/w suspensions of the contents of rat jejunum, ileum, caecum and colon in the presence —□— or absence —■— of probiotics. Data are mean  $\pm$  SD, ( $n = 5$ ). \*\*\*( $p < 0.0001$ ) for differences in selenite concentration between gut contents in the presence and absence of probiotics at 24 h.



**Figure 4.6.** DMDSe formed during anaerobic incubations at 37°C over 24 h of L-SeMet (0.51 mM) with 10% w/w suspensions of the contents of rat jejunum, ileum, caecum and colon in the presence —□— or absence —■— of a mixture of probiotics. Data are mean  $\pm$  SD, ( $n = 5$ ). \*\*( $p < 0.01$ ) for differences in DMDSe concentration between gut contents in the presence and absence of probiotics at 24 h.

For all gut segments with probiotics, the DMS<sub>2</sub>Se level formed from selenite at 24 h was higher than with gut contents alone (Figure 4.7a). The formation of DMS<sub>2</sub>Se was significantly higher in jejunum and ileum contents consistent with the significantly greater loss of selenite in jejunum and ileum contents with probiotics (Figure 4.5). DMDSe was also formed after incubations of selenite for 24 h with each of the gut contents alone (Figure 4.7b). In gut contents with probiotics, selenite conversion to DMDSe was only detectable in caecum contents after 3 h (Figure 4.7b).



**Figure 4.7.** Formation of (a) DMSe and (b) DMDSa during anaerobic incubations at 37°C over 24 h of selenite (0.58 mM) with 10% w/w suspensions of the contents of rat jejunum, ileum, caecum and colon in the presence —□— or absence —■— of probiotics. Data are mean  $\pm$  SD, ( $n = 5$ ). \*( $p < 0.05$ ), \*\*( $p < 0.01$ ); \*\*\*( $p < 0.001$ ) for differences in (a) DMSe or (b) DMDSa concentration between gut contents in the presence and absence of probiotics at 24 h.

#### 4.4. Discussion

The results of the present study show that both L-SeMet and selenite can be metabolised by the four selected probiotic strains and especially by the two strains of lactobacilli. Lactobacilli are commonly used to produce yoghurt or fermented dairy products where the increased production of organic acids leads to a decrease in pH (Salazar *et al.*, 2009). When compared to bifidobacteria and streptococci, the production of organic acids by lactobacilli was found to be higher indicating that this genera has a higher metabolic rate (Salazar *et al.*, 2009). In the present study, the two strains of lactobacilli were particular efficient at metabolising selenite (Figure 4.2).

The present study shows that formation of DMDSe from L-SeMet at 24 h by individual probiotic strains corresponds to 18 - 46% of the L-SeMet lost. The upper limit of this range is consistent with the results of a previous study using suspensions of *Pseudomonas stutzeri* showing 53% of L-SeMet was converted to DMDSe over 12 h (Doran and Alexander, 1977). In addition, the probiotic bacteria except *B. lactis* LAFTI<sup>®</sup> B94 metabolised L-SeMet to both DMDSe and DMSe (Figure 4.3a) consistent with previous studies in bacteria from the genera *Pseudomonas* and *Corynebacterium* (Doran and Alexander, 1977; Ranjard *et al.*, 2003). Surprisingly, the formation of DMSe was not detected in incubations of L-SeMet with the mixture of probiotics as found in incubations with rat gut content suspensions (Chapter 3). This suggests a mixture of different species of bacteria can display a metabolic profile different from that expended on the basis of metabolism by the individual species alone.

Based on the metabolic pathway of L-SeMet and selenite outlined in Sections 1.4 and 3.1, it is known that methylselenol is converted to DMSe by a methylation process. The other volatile selenium compound, DMDSe, is produced by the oxidation of methylselenol (Figure 3.1) (Freney, 1967; Doran and Alexander, 1977). For this oxidation to occur under anaerobic conditions requires an



alternative electron sink to oxygen. The nature of this electron sink is currently unknown.

A previous study showed that thiopurine methyltransferase plays a role in methylation of organic and inorganic selenium to form DMSe and DMDSe (Ranjard *et al.*, 2002). DMDSe may also be demethylated leading to an increase in the level of DMSe. This corresponds to the results of a study by Doran and Alexander (1977) who reported that DMSe was formed when DMDSe was incubated with strains of *Xanthomonas* and *Corynebacterium*.

Previous studies have shown that selenium compounds are metabolised in the liver and kidney (Swanson *et al.*, 1991; Suzuki, 2005) but little was previously known about the role of the gut microbiota in selenium metabolism (Schwiertz *et al.*, 2008; Hrdina *et al.*, 2009). The results of the present study show that probiotics can influence selenium metabolism when combined with rat gut contents *ex vivo* and increase or decrease the formation of methylated selenium metabolites and, in the case of selenite, elemental selenium. A previous study suggested that probiotic bacteria can interact with indigenous gut microbiota and change their metabolic activity (Collado *et al.*, 2009). Based on the results presented here, it appears that this interaction may also affect selenium metabolism in the gut.

Supplemental probiotics may provide a useful means of reducing the toxicity of selenium supplements by affecting their biotransformation to methylated selenium metabolites and elemental selenium. Whether probiotic treatment can alter the disposition of selenium after oral doses of L-SeMet and selenite is the subject of Chapter 5.

## CHAPTER 5

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### **The influence of probiotic treatment on the disposition of selenium after oral administration of L-selenomethionine and selenite to rats\***

#### **5.1. Introduction**

The results in Chapter 3 show that metabolism of L-SeMet and selenite in rat gut contents from normal rats is similar to that mediated by microbial cultures *in vitro* (Dungan and Frankenberger Jr, 2001; Ranjard *et al.*, 2002). The results of *in vitro* and *ex vivo* studies reported in Chapter 4 show that probiotics metabolise L-SeMet and selenite to volatile selenium compounds and elemental selenium and can modulate the metabolism of L-SeMet and selenite by rat gut contents.

Details of the basis for beneficial probiotic activities are still incomplete although three types of activity have been suggested; (a) competition with pathogens for adhesion receptors thereby reducing the ability of pathogens to colonise the intestine (Collado *et al.*, 2007), (b) production of various antimicrobial substances such as acids, bacteriocins and antibiotic-like substances (Rolfe, 2000; Chen *et al.*, 2003; Dierksen *et al.*, 2007) and (c) immunomodulation (Cosseau *et al.*, 2008) by enhancing the levels of secretory antibody (IgA) to bring about changes in cell-mediated immunity (Park *et al.*, 2002). These effects of probiotics are strain and dose-dependent.

The potential advantages of treatment with a combination of a selenium supplement and probiotic bacteria include enhanced probiotic activity and clinical improvement of chronic disorders such as cancer, chronic pancreatitis, febrile

neutropenia, and arthritis (Križková *et al.*, 2002; Önning *et al.*, 2003; Chen *et al.*, 2005; Mego *et al.*, 2005; Rovenský *et al.*, 2005; Mego *et al.*, 2006; Mössner, 2006). Improvements in probiotic induced antioxidant defence and redox control of enzymes and proteins may enhance selenium control of cancer and immune function (Ryan-Harshman and Aldoori, 2005). Clearly, there is a need for a greater understanding of the relationship between probiotics and the host's response to selenium supplements.

Probiotic treatment can improve gastrointestinal function (Maldonado Galdeano and Perdigon, 2006; Collado *et al.*, 2009) and bring about an improvement in selenium status (Önning *et al.*, 2003; Mazo *et al.*, 2007; Zhang *et al.*, 2009). Whether this is because probiotic cells remain viable and thereby directly contribute to selenium metabolism during their transit through the GIT or because probiotic treatment modulates selenium metabolism by the indigenous microbiota is presently unknown (Ranjard *et al.*, 2002). The aim of the *in vivo* study reported in this Chapter was to investigate the effects of probiotic treatment on the disposition of selenium after oral administration of L-SeMet and selenite to rats. The mixture of four antibiotic-resistant probiotic strains was formulated in a mixture of lyoprotectants to help maintain cell viability. A lyoprotectant control group was included in the study to investigate whether lyoprotectants alone modulate selenium disposition.

## **5.2. Materials and methods**

### **5.2.1. Materials**

All materials except lyoprotectants were obtained as described in previous Chapters. Lyoprotectant is a substance included in a formulated product to maintain cell viability. The mixture of lyoprotectants containing trehalose,

maltodextrin and lactitol in a proprietary formulation was obtained from BLIS Technologies Ltd.

### **5.2.2. Preparation of the probiotic mixture**

The preparation of the four antibiotic-resistant probiotic strains is described in Chapter 4. Probiotic cells grown in MRS culture (2 L) were harvested by centrifugation (9800 x *g*) at 4°C for 10 min and resuspended in 50 mL of the corresponding culture supernatant, mixed with 50 mL of the lyoprotectant mixture and adjusted to pH 7.1 - 7.4 using 0.1 M NaOH. Aliquots (1.5 mL) of the suspension were frozen at -80°C for subsequent use. Serial 10-fold dilutions of 1 mL aliquots of each probiotic suspension were prepared in PBS and counts obtained following plating and incubation on the appropriate antibiotic-containing agar medium (as described in Chapter 4). These counts were typically in the range  $1 \times 10^9$  -  $1 \times 10^{10}$  cfu/g. To prepare the probiotic mixture, probiotic suspensions of each of the four probiotic strains were thawed at room temperature and combined in appropriate volumes to give a mixture containing equal numbers of each strain (total cell count ca.  $1 \times 10^{10}$  cfu/g) immediately prior to administration to rats. The lyoprotectant mixture diluted 1:1 with sterile distilled deionised water served as the vehicle.

### **5.2.3. Animals and protocol**

Rats were maintained on a rat chow formulation (Specialty Feeds, Western Australia) containing 0.3 mg selenium/kg. All nutritional parameters of this diet met or exceeded the National Research Council guidelines for rats (New South Wales Department of Primary Industries: Animal Research Review Panel, 2007). The calculated total selenium provided by rat chow was 0.03 mg/kg/day. This is estimated on a daily intake of 30 g rat chow containing 0.3 mg/kg and an average rat weight of 300 g (Rubino *et al.*, 2005), but below the LD<sub>50</sub> for both forms of selenium (Klug *et al.*, 1950; Hassoun *et al.*, 1995).

A control experiment was performed in which three groups of rats ( $n = 3/\text{group}$ ) were given saline (0.1 mL/100 g body weight) or an oral dose of 2 mg selenium/kg as L-SeMet or selenite by gavage. These three groups are hereafter described as untreated rats. Two other groups of rats ( $n = 6/\text{group}$ ) were given 3 mL aliquots of the probiotic mixture twice a day for three days and, at the time of the last probiotic dose, also given an oral dose of L-SeMet or selenite (2 mg selenium/kg). Two other groups were treated in a similar manner with the probiotic vehicle. These four groups are hereafter described as treated rats. Blood was collected from the tail vein of all untreated rats and of five rats in each treatment group before and at 2, 5 and 10 h after selenium administration and by cardiac puncture at 24 h, immediately after rats were asphyxiated with carbon dioxide. Serum was prepared by centrifugation at 15000 x g for 10 - 15 min and stored at  $-80^{\circ}\text{C}$  for subsequent analysis of selenium.

A midline incision was made to access the gut. The contents of the jejunum, ileum, caecum and colon were expelled separately and maintained on ice at  $4^{\circ}\text{C}$  during separation into two portions. One portion was retained for selenium analysis and the other was diluted with PBS to give 10% w/w suspensions. Aliquots (500  $\mu\text{l}$ ) of the gut content suspensions were frozen in Eppendorf tubes at  $-80^{\circ}\text{C}$  for subsequent analysis of L-SeMet, selenite and in sealed-top glass vials for subsequent analysis of DMDS<sub>2</sub>Se and DMS<sub>2</sub>Se. Livers and kidneys were also collected and stored at  $-80^{\circ}\text{C}$  for subsequent analysis of selenium. Samples were analysed as described in Chapter 2.

The sixth rat in each treatment group was also sacrificed at 24 h and the jejunum, ileum, caecum and colon removed for determination of the counts of total bacteria and of each of the antibiotic-resistant probiotic strains. Suspensions of gut contents (10% w/w) were diluted serially (10-fold) in PBS and samples of the  $10^{-2}$  -  $10^{-5}$  dilutions plated on blood agar (Difco CAB supplemented with 5% human blood and 0.1%  $\text{CaCO}_3$ ) and on the appropriate antibiotic-supplemented agar (as described in Chapter 4). All plates were anaerobically incubated at  $37^{\circ}\text{C}$  for four

days. The number of colonies on plates was used to determine the number of colony-forming units per gram (cfu/g gut contents) of the gut contents.

#### **5.2.4. Statistical analysis**

Statistical analysis of the experimental data was performed using SPSS 15.0 for Windows. A linear mixed model was used to compare L-SeMet, selenite or selenium concentrations in different groups and in different tissues. The Student's two-tailed t-test for unpaired data was used to test differences in area under the curve of serum selenium concentration-time profiles and the serum and tissue levels of selenium at a given time. Statistical significance was set at  $p < 0.05$ .

### **5.3. Results**

#### **5.3.1. Bacterial counts of gut content suspensions**

Corresponding total bacterial counts in the contents of jejunum, ileum, caecum and colon in probiotic and vehicle treated rats given L-SeMet and selenite were similar (Table 5.1b and c). Total bacterial counts were also similar to levels in normal rats (determined as described in Section 3.2.1) except in jejunum where counts were  $10^3 - 10^4$  cfu/g higher than in normal rats (Table 5.1a). Of the four antibiotic-resistant probiotic strains, only *L. acidophilus* L10 and *L. rhamnosus* 67B were detected in all four segments of the gut of probiotic treated rats in approximately equal numbers which were  $10^2 - 10^4$  cfu/g less than the corresponding total bacterial counts. The counts of the two strains of lactobacilli in the four segments of the intestine were within  $10^2$  cfu/g of each other in rats receiving L-SeMet or selenite.

**Table 5.1.** Bacterial counts ( $\text{Log}_{10}$  cfu/g gut contents) of contents of jejunum, ileum, caecum and colon from (a) normal rats ( $n = 5$ ) and rats given a single oral dose (2 mg selenium/kg) of (b) L-SeMet ( $n = 1$ ) or (c) selenite ( $n = 1$ ) after treatment with either probiotics (P) or vehicle (V).

## a) Normal rats†

	Jejunum	Ileum	Caecum	Colon
Total bacterial counts	5.1 ± 0.05	9.2 ± 0.07	10.3 ± 0.15	10.3 ± 0.04

† Data are mean ± SD from Chapter 3.

## b) L-SeMet

		Jejunum	Ileum	Caecum	Colon
Total bacterial counts	(P)	9	9	8	9
	(V)	9	9	9	9
<i>L. acidophilus</i> L10	(P)	7	6	6	6
<i>L. rhamnosus</i> 67B	(P)	7	5	7	7
<i>S. salivarius</i> K12	(P)	ND	ND	ND	ND
<i>B. lactis</i> LAFTI®B94	(P)	ND	ND	ND	ND

ND = not detected

## c) Selenite

		Jejunum	Ileum	Caecum	Colon
Total bacterial counts	(P)	8	8	9	9
	(V)	8	9	9	10
<i>L. acidophilus</i> L10	(P)	6	6	8	8
<i>L. rhamnosus</i> 67B	(P)	6	5	8	8
<i>S. salivarius</i> K12	(P)	ND	ND	ND	ND
<i>B. lactis</i> LAFTI®B94	(P)	ND	ND	ND	ND

ND = not detected

### 5.3.2. L-SeMet, selenite and volatile metabolites in gut contents

In rats given L-SeMet after probiotic or vehicle treatment, L-SeMet was found in the jejunum (4.0, 4.9  $\mu\text{M}$ ) and ileum (1.5, 1.1  $\mu\text{M}$ ) (Table 5.2). In vehicle treated rats, it was also found in the caecum (1.4  $\mu\text{M}$ ) and at a very low level in colon. In untreated rats given L-SeMet, L-SeMet was found (2.9-3.8  $\mu\text{M}$ ) in all segments of the gut. The levels were significantly higher in ileum, caecum and colon than in corresponding sections of treated rats. DMDSe and DMSe were also present in gut contents of treated rats given L-SeMet at levels up to  $\sim 20 \mu\text{M}$ . DMDSe was higher in probiotic than vehicle treated rats except in colon and DMSe was also higher in probiotic than vehicle treated rats in jejunum and ileum. In untreated rats given L-SeMet, only DMDSe was detected and only in jejunum.

Levels of selenite were very low ( $< 0.037 \mu\text{M}$ ) in all segments of the gut in both treated and untreated rats. DMDSe and DMSe were present in treated rats but there were no discernable trends. In untreated rats given selenite, neither DMSe nor DMDSe were detected.



**Table 5.2.** Concentrations ( $\mu\text{M}$ ) of L-SeMet, selenite, DMDSe and DMSe in gut contents of (a) untreated rats and rats given a single oral dose (2 mg selenium/kg) of (b) L-SeMet or (c) selenite after treatment with either probiotics or vehicle (Data are mean  $\pm$  SD,  $n = 5$ ).

## a) Untreated rats

	L-SeMet	DMDSe	DMSe	Selenite	DMDSe	DMSe
Jejunum	3.8 $\pm$ 0.2	17.6 $\pm$ 5.4	ND	0.036 $\pm$ 0.025	ND	ND
Ileum	3.2 $\pm$ 0.2	ND	ND	0.037 $\pm$ 0.027	ND	ND
Caecum	3.7 $\pm$ 0.3	ND	ND	ND	ND	ND
Colon	2.9 $\pm$ 0.4	ND	ND	ND	ND	ND

ND = not detected

## b) L-SeMet

	Probiotics			Vehicle		
	L-SeMet	DMDSe	DMSe	L-SeMet	DMDSe	DMSe
Jejunum	4.0 $\pm$ 0.1	15.1 $\pm$ 15.4	19.2 $\pm$ 18.3	4.9 $\pm$ 0.61	7.4 $\pm$ 8.0	1.6 $\pm$ 3.5
Ileum	1.5 $\pm$ 1.2	8.2 $\pm$ 13.3	12.1 $\pm$ 18.1	1.1 $\pm$ 1.1	1.2 $\pm$ 2.8	1.2 $\pm$ 2.2
Caecum	ND	8.5 $\pm$ 11.7	0.15 $\pm$ 0.34	1.4 $\pm$ 1.0	5.3 $\pm$ 12.0	1.8 $\pm$ 2.5
Colon	ND	2.2 $\pm$ 5.0	0.98 $\pm$ 1.34	0.09 $\pm$ 0.06	15.4 $\pm$ 21.2	2.2 $\pm$ 3.3

ND = not detected

## c) Selenite

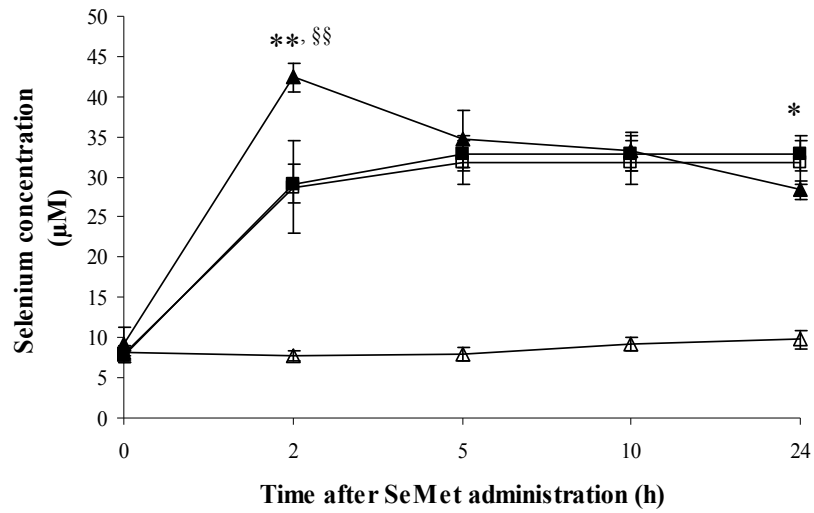
	Probiotics			Vehicle		
	Selenite	DMDSe	DMSe	Selenite	DMDSe	DMSe
Jejunum	0.016 $\pm$ 0.002	13.7 $\pm$ 18.9	1.9 $\pm$ 1.95	0.010 $\pm$ 0.001	1.5 $\pm$ 3.3	10.2 $\pm$ 12.3
Ileum	0.016 $\pm$ 0.003	ND	0.85 $\pm$ 0.82	0.016 $\pm$ 0.002	14.8 $\pm$ 20.9	3.7 $\pm$ 7.1
Caecum	0.003 $\pm$ 0.001	4.1 $\pm$ 9.2	1.4 $\pm$ 1.3	0.001 $\pm$ 0.000	5.6 $\pm$ 8.9	2.8 $\pm$ 5.8
Colon	0.003 $\pm$ 0.001	10.7 $\pm$ 24.0	11.1 $\pm$ 18.8	0.001 $\pm$ 0.000	3.9 $\pm$ 8.6	2.6 $\pm$ 5.8

ND = not detected

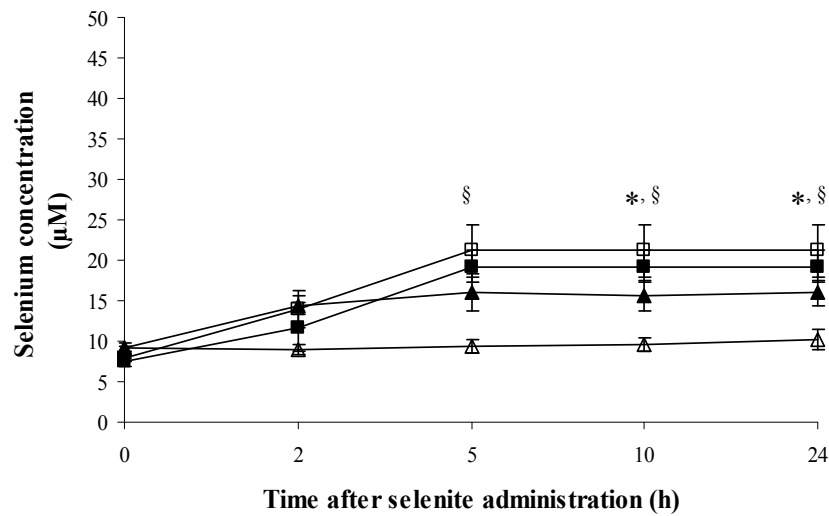
### 5.3.3. Serum selenium levels

Serum selenium levels were higher following L-SeMet administration than after selenite administration (Figure 5.1). For both L-SeMet and selenite, no significant differences were found in the area under the curve (AUC<sub>0-24 h</sub>) of corresponding serum selenium concentration-time profiles between rats treated with probiotics and those treated with vehicle. Serum selenium levels in untreated rats given saline were  $8.6 \pm 0.9 \mu\text{M}$ , respectively. In rats given L-SeMet, the AUC<sub>0-24 h</sub> in untreated rats was not significantly different from that in treated rats but the serum selenium at 2 h was significantly higher and the level at 24 h significantly lower (Figure 5.1a). In rats given selenite, the AUC<sub>0-24 h</sub> in untreated rats was significantly lower ( $p < 0.01$ ) than that in treated rats and serum selenium levels at 5, 10 and 24 h were also significantly lower (Figure 5.1b).

a



b



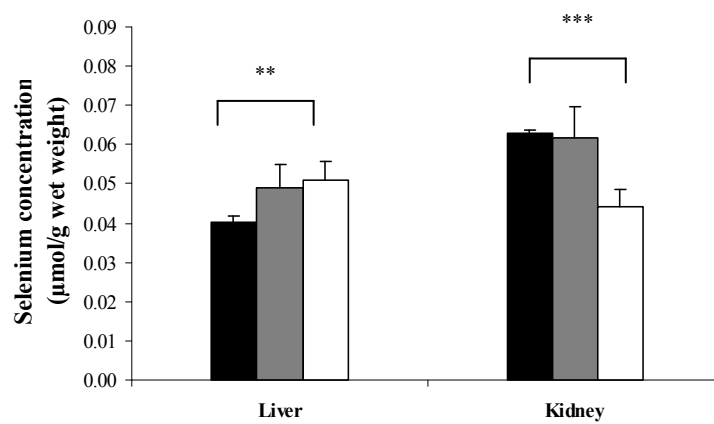
**Figure 5.1.** Serum selenium concentration ( $\mu\text{M}$ ) in rats given saline ( $n = 3$ )  $\text{---}\Delta\text{---}$ , and those given a single oral dose (2 mg selenium/kg) of either (a) L-SeMet or (b) selenite alone ( $n = 3$ , untreated rats)  $\text{---}\blacktriangle\text{---}$ , or after treatment with either probiotics ( $n = 5$ )  $\text{---}\blacksquare\text{---}$  or vehicle ( $n = 5$ )  $\text{---}\square\text{---}$  (treated rats). Data are mean  $\pm$  SD.

\* ( $p < 0.05$ ) and \*\* ( $p < 0.01$ ) for differences in serum selenium concentration between rats treated with probiotics and untreated rats; § ( $p < 0.05$ ) and §§ ( $p < 0.01$ ) for differences in serum selenium concentration between rats treated with vehicle and untreated rats.

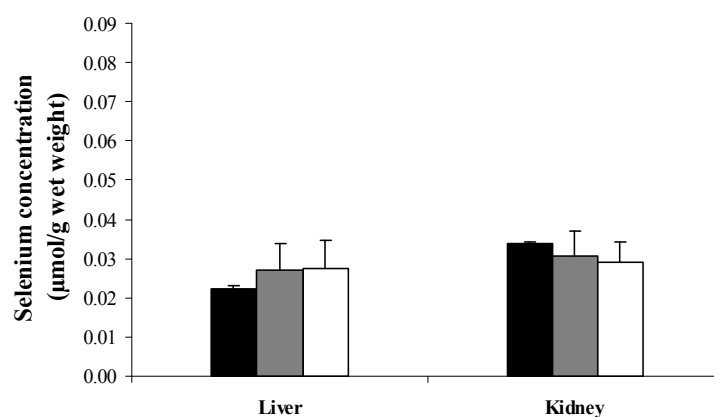
### 5.3.4. Selenium in liver and kidney

In rats treated with probiotics and vehicle given either L-SeMet or selenite, there were no significant differences in selenium levels in liver or kidney tissue (Figure 5.2). However, the level in probiotic treated rats given L-SeMet was significantly higher in the liver and significantly lower in the kidney than in untreated rats given L-SeMet alone (Figure 5.2a). A similar trend was observed in rats given selenite, but none of the differences was significant (Figure 5.2b).

a



b



**Figure 5.2.** Selenium concentration ( $\mu\text{mol/g}$  wet weight) in the liver and kidney 24 h after single oral doses (2 mg selenium/kg) of either (a) L- SeMet or (b) selenite either alone (untreated rats) ( $n = 3$ ) (black bars) or after treatment with either vehicle ( $n = 5$ ) (gray bars) or probiotics ( $n = 5$ ) (white bars) (treated rats). Data are mean  $\pm$  SD. \*\*( $p < 0.01$ ), \*\*\*( $p < 0.001$ ) for the indicated differences in selenium concentration.

## 5.4. Discussion

In considering the total bacterial counts (Table 5.1), the larger populations of microorganisms in the jejunum of rats treated with either probiotics or vehicle is presumably the result of stimulation of microbial growth by one or more components of the lyoprotectant mixture. While trehalose is known to assist survival of bacterial cells (Higashiyama, 2002; Crowe, 2007) and maltodextrin to enhance the ability of intestinal bacteria to ferment carbohydrates (Olano-Martin *et al.*, 2000), lactitol is an established prebiotic (Kummel and Brokx, 2001) known to promote the growth of probiotic lactobacilli and bifidobacteria (Cummings and Macfarlane, 2002; Finney *et al.*, 2007). In the present study, two strains of lactobacilli were found in all segments of the gut of probiotic treated rats but the numbers were not sufficient to increase the total bacterial counts in ileum, caecum and colon nor to entirely explain the increase in counts in the jejunum.

The ability of lactobacilli but not *S. salivarius* K12 to remain viable in the rat gut 24 h after the last dose of probiotics is consistent with the results of a previous study by Lee *et al.* (2009) involving a similar treatment of rats with antibiotic-resistant probiotic strains of the same species. However, the absence of viable bifidobacteria in the gut found here is in sharp contrast to the results of Lee *et al.* (2009) who found viable *B. lactis* HN019 in ileum and colon contents for up to 7 days after the last dose of probiotics. In general, survival rates of bifidobacteria strains are strain-specific. As previously reported, bifidobacteria of human origin are vulnerable to low pH and bile salts but apparently have an intrinsic ability to survive the presence of pancreatin (Charteris *et al.*, 1998). Of the strains tested, representatives of *Bifidobacteria animalis* ssp. *lactis* appeared to be the most capable of surviving gastric transit, which is probably due to their enhanced acid tolerance compared to other *Bifidobacterium* species (Matsumoto *et al.*, 2004; Mättö *et al.*, 2004; Ventura *et al.*, 2004; Sánchez *et al.*, 2006). Although some strains of *B. lactis* appear to be more capable of surviving gastric transit due to their enhanced acid tolerance (Ding and Shah, 2007; Masco *et al.*, 2007), the

results of the present study suggest that, even among the various *B. lactis* strains, there can be considerable differences in ability to survive gastrointestinal transit.

For the *in vivo* study, HPLC was used to determine L-SeMet in serum. Although the C<sub>max</sub> of total serum selenium was equivalent to 8.2 µg/mL L-SeMet, a concentration that would be detectable by HPLC, no L-SeMet was detected. The result suggested that ingested L-SeMet was transformed to other selenium compounds that were present in serum. Possibly a minimal amount of L-SeMet may be bound to albumin (Agli *et al.*, 1998).

In regard to serum selenium levels, the selenium concentration in untreated rats given saline (Figure 5.1) is similar to that found in a previous study (~ 8 µM) of rats fed a selenium adequate diet (0.2 mg selenium/kg/day) (Shiobara *et al.*, 1998). The observation that serum selenium levels were higher following L-SeMet administration than after selenite administration is also consistent with the findings of previous studies (Thomson and Stewart, 1973; Han *et al.*, 2009). As L-SeMet is absorbed in all segments of the gut (> 90% of an orally administered dose) (Vendeland *et al.*, 1992a), a high serum selenium concentration is to be expected. Selenite is absorbed mainly in the ileum and most of the absorbed selenite is efficiently excreted by the kidneys (Swanson *et al.*, 1991; Thomson, 2007).

The fact that rats treated with either probiotics or vehicle showed serum selenium levels that were more sustained after L-SeMet administration and significantly higher after selenite administration than in untreated rats indicates that one or more components of the lyoprotectant mixture either increase metabolism of the two selenium compounds or increase their absorption and/or that of their metabolites. The lower levels of L-SeMet and higher levels of DMDS<sub>2</sub>Se and DMSe found in the ileum, caecum and colon of treated rats as compared to untreated rats (Table 5.2) indicates that increased metabolism in the gut lumen is the most likely explanation.

Greater absorption of selenium from the intestinal tract should result in higher selenium levels in the liver and kidney. SeMet is known to concentrate in liver and kidney where it replaces Met in proteins (Beilstein and Whanger, 1988; Schrauzer, 2003). In the present study, selenium levels in both the liver and kidney of untreated rats given L-SeMet were higher than corresponding levels in untreated rats given selenite. This is consistent with a previous study in rats given either selenium yeast (organic selenium) or selenite (Han *et al.*, 2009). The fact that probiotic treatment modulated liver and kidney levels of selenium over and above the effects of the vehicle shows that probiotics do influence selenium disposition. Why the level of selenium increased in liver and decreased in kidney is currently unknown and awaits further research.

The results of the present study suggest that the combination of probiotics and lyoprotectants acts as a synbiotic as found in previous studies (Peuranen *et al.*, 2004; Furrie *et al.*, 2005; De Vrese and Schrezenmeir, 2008; Fotiadis *et al.*, 2008; Kalliomäki, 2009). A prebiotic is defined as “a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one of a limited number of bacteria in the colon...” (Gibson and Roberfroid, 1995). Recently, the definition was expanded to “a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microbiota that confers benefits upon the host in well-being and health” (Gibson *et al.*, 2004; Roberfroid, 2007). Lactitol meets the criteria of a prebiotic in that it is a poor substrate for mammalian enzymes, is not absorbed from the GIT (Soontornchai *et al.*, 1998) but can be fermented by microbiota in the large intestine and stimulate the growth of lactobacillus and bifidobacteria (Peuranen *et al.*, 2004). Although several compounds such as polydextrose, lactosucrose, lactose and pectic oligosaccharides may be prebiotics, there remains insufficient evidence to justify their classification as prebiotics (Soontornchai *et al.*, 1998; Peuranen *et al.*, 2004; Roberfroid, 2007). The findings in the present study may result from the prebiotic activity of lactitol but further study is required

to investigate whether any of the other components of the lyoprotectant mixture also act as prebiotics.

In conclusion, treatment with the probiotic preparation modulates selenium uptake from selenium supplements compared with untreated rats. Follow-up studies of the nature of this effect particularly as mediated by the lyoprotectant components are warranted.



# CHAPTER 6

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## Summary, limitations, future studies

### 6.1. Summary

Prior to this study, few investigations of selenium metabolism by commensal microorganisms in mammals have been carried out (Schwiertz *et al.*, 2008; Hrdina *et al.*, 2009). Previous studies have investigated the ability of probiotics containing selenium to improve gut function and selenium status (Křižková *et al.*, 2002; Mego *et al.*, 2005; Yang *et al.*, 2009) but this thesis is the first to describe the metabolism of L-SeMet and selenite in probiotic bacteria and rat gut contents as well as the effect of probiotic treatment on the disposition of selenium after the oral administration of L-SeMet and selenite to rats. The results suggest probiotics and the gut microbiota can metabolise selenium supplements and that probiotic treatment can modulate gut microbiota-mediated metabolism.

*Ex vivo* studies of metabolism in different segments of the rat intestinal tract showed that L-SeMet and selenite metabolism is greatest in caecum as found in previous studies (Hall *et al.*, 1992; Kurosawa *et al.*, 2005). The higher rate is attributed to the larger population of metabolically active microorganisms in the caecum (Hall *et al.*, 1992; Kurosawa *et al.*, 2005). The biotransformation of both L-SeMet and selenite by rat gut contents was found to be similar to that mediated by microbial cultures *in vitro* (Reamer and Zoller, 1980; Ranjard *et al.*, 2003). In fact, the present study showed a significant linear relationship between L-SeMet concentration in caecum content suspensions and DMDSe concentration, this volatile metabolite accounting for 8.7% of the amount of L-SeMet added and

28.9% of the L-SeMet lost. For selenite, metabolism in caecum and colon contents was more extensive than that of L-SeMet producing DMSe accompanied by elemental selenium. Although metabolism of selenium supplements was known to occur in the liver and kidney, this study shows that the gut microbiota also plays a role in their biotransformation.

Information on metabolism of L-SeMet and selenite by probiotics is limited. *In vitro* studies indicate that different probiotic strains and species have different inherent capacities to metabolise L-SeMet and selenite. The impact of probiotics on selenium metabolism in different segments of the gut *ex vivo* is also different. This study shows that the two lactobacilli metabolised selenite more efficiently than L-SeMet. This result may explain the increase in selenite metabolism in the jejunum and ileum contents when probiotics were present. It is unknown why the metabolism of selenite by lactobacilli is greater than that by the other two probiotic strains tested. One possibility is that lactobacilli produce organic acids which decrease pH and create an environment that helps improve fermentation and bacterial enzymatic activity towards selenite (Alzate *et al.*, 2008). However, the PBS pH 7.4 used to prepare 10% gut content and probiotic suspensions should hold the pH at 7.4 and not interfere with the metabolism of selenite and/or alter the enzymatic activity of microorganisms during incubation. In the case of L-SeMet, why metabolism of L-SeMet decreased when probiotics were present is currently unknown. It is speculated that probiotics may trigger the gut microbiota to increase or decrease their enzymatic activity to metabolise selenite or L-SeMet. However, the mechanism by which probiotic bacteria/ gut microbiota convert selenite to elemental selenium under the anaerobic conditions in the present study is unknown.

In the *in vivo* study, probiotic treatment increased the number of bacteria in the jejunum but not in other segments of the gut. However, the number of viable probiotic cells detected in gut contents after treatment was much less than the number of indigenous gut bacteria indicating only a small number of probiotic

cells survives transit through the intestinal tract. This may be why treatment with the lyoprotectant vehicle was found to alter selenium levels to almost the same extent as treatment with the probiotic suspension although selenium levels in liver and kidney of probiotic treated rats were significantly different compared to corresponding levels in untreated rats. It is possible that lyoprotectants containing lactitol may have a prebiotic effect to promote the growth of gut microbiota. Thus, it appears that probiotic treatment can affect selenium disposition *in vivo* but the magnitude of the effect is modest in relation to the growth stimulant effect of the lyoprotectants.

## 6.2. Limitations

There are a number of limitations associated with this study. First, a rat model was used to investigate the metabolism of L-SeMet and selenite rather than a clinical study in human. Since the rat caecum is larger and the colon is shorter than in humans, the results of the *ex vivo* and *in vivo* studies can only be extrapolated to humans with caution. However, the difficulty of obtaining human gut contents and tissues makes a similar study in humans impossible.

L-SeMet (organic form) and selenite (inorganic form) were studied in this thesis because these compounds are commonly used as selenium supplements. Since it L-SeMet and selenite labelled with a specific selenium isotope were unavailable, it was difficult to establish the total disposition of selenium after oral doses of these compounds. Because selenium undergoes a complex series of biochemical reactions and incorporation into many proteins, radiolabelled compounds are the best means of studying their metabolic fate. To compensate for the lack of radiolabelled compounds, metabolism was investigated using high selenium concentrations (detailed in Chapter 3) and relatively high doses.

Hydrogen selenide (or HSe<sup>-</sup>) is highly reactive and is converted to methylselenol. Because of methodological difficulties, no attempt was made to entrap any hydrogen selenide and methylselenol produced. Instead, the present study focused on the volatile metabolites, DMDS<sub>e</sub> and DMSe, which are more readily detected and quantified. Determination of elemental selenium was considered but the methodology is difficult due to gut matrix interference. A previous study by Oremland *et al.* (2004) showed that it is possible but the isolation of elemental selenium requires a large volume of growth medium (500 mL), a complicated multi-step extraction procedure and ultracentrifugation (10,000 g). Because of the small volumes and complex matrix involved in the present study, the analysis was considered too difficult to attempt.

### **6.3. Future studies**

There are several factors to be considered in relation to future studies. The first concerns the variation in metabolic activity of probiotic strains or species. In addition, the number of probiotics that reach the intestinal tract in a viable state is strain and species dependent (Sánchez *et al.*, 2006; Masco *et al.*, 2007; Lee *et al.*, 2009). Research into optimising probiotic treatment to increase the proportion of administered probiotics surviving in the intestinal tract is needed.

Another issue relates to the effect of lyoprotectants. Lyoprotectants are used to maintain cell viability and, in particular, to protect probiotic cells during storage at low temperatures (Higashiyama, 2002; Zayed and Roos, 2004; Crowe, 2007). Any or all of the three components of the lyoprotectant mixture used in the *in vivo* study could enhance probiotic enzyme activity and/or the metabolic activity of the indigenous gut microbiota (Ammann *et al.*, 1988; Soontornchai *et al.*, 1998; Kummel and Brokx, 2001; Mineo *et al.*, 2002; Finney *et al.*, 2007). In particular, lactitol is a sugar alcohol (Soontornchai *et al.*, 1998) known to act as a prebiotic (Kummel and Brokx, 2001) which can be fermented by some bacteria of the large

intestine (Finney *et al.*, 2007) to form short chain fatty acids which promote the growth of lactobacilli and bifidobacteria (Cummings and Macfarlane, 2002; Peuranen *et al.*, 2004). A future study to investigate the effect of different lyoprotectants on selenium metabolism by the gut microbiota is warranted.

Other future studies are required to better define the effect of probiotics on selenium disposition in both normal and selenium-deficient rats. The latter study would provide data of immediate relevance to human and animal health since conditions arising from selenium deficiency have a particular importance. Further research should also aim to (1) investigate other selenium supplements such as Se-methylselenocysteine, a compound from which *in vivo* higher concentrations of the putative anticarcinogenic agent, methylselenol, are produced, (2) determine whether probiotic treatment can increase the production of elemental selenium from selenite (Zhang *et al.*, 2004; Wang *et al.*, 2007; Zhang *et al.*, 2008) and (3) investigate the nature of the elemental selenium produced by the gut microbiota and whether it is subsequently bioavailable.

### **6.3. Conclusions**

The results of the research reported in this thesis emphasise the importance of the gut microbiota in the metabolism of selenium supplements and suggest potential benefits of adjunctive probiotic treatment in maintaining adequate selenium status.

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